Role of Caveolin-1 for Blocking the Epithelial-Mesenchymal Transition in Proliferative Vitreoretinopathy

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Preliminary studies have shown that Caveolin-1 plays a key role in many important processes. Caveolin-1 is a 21- to 24-kDa integral membrane protein and has been adequately investigated by a number of biochemical studies. Caveolin-1 was found at the endoplasmic reticulum and Golgi complex and predominantly at the plasma membrane. It has been adequately investigated by a number of biochemical studies. Caveolin-1 was found to be involved in the pathogenesis of PVR by blocking the epithelial-mesenchymal transition (EMT). EMT is one of the main concepts in the pathogenesis of fibrovascular membranes (FVMs) in PVR. In this study, we examined the expression of Caveolin-1 in human FVMs from patients with PVR. We also examined the role of Caveolin-1 in the pathogenesis of PVR. Western blotting, real-time PCR, and immunohistochemistry were performed with human FVMs and mouse eyes with PVR. Cell migration assays were performed to evaluate the involvement of Caveolin-1 in EMT using primary human and mouse RPE cells. Results. Caveolin-1 was expressed in human FVMs and upregulated in the mouse eyes with PVR. The alpha-smooth muscle actin (αSMA) expression and migration ability were increased in RPE cells with knockout or knockdown of Caveolin-1. Immunohistochemistry showed reduced morphology and expression of ZO-1. In addition, migration assays showed that Caveolin-1 reduction increased RPE cell migration abilities.

Conclusions. These results indicated that Caveolin-1 in RPE cells prevents PVR by blocking EMT.

Keywords: caveolin-1, proliferative vitreoretinopathy, epithelial-mesenchymal transition, retinal detachment, retinal pigment epithelium

Retinal detachment (RD) and its advanced status, proliferative vitreoretinopathy (PVR), are leading causes of visual impairment in humans. Although the robust improvement in surgical instruments has enabled a very high rate of structural attachment in RD, RDs with severe complications, including retinal breaks, multiple retinal tears, and vitreous hemorrhage, often develop into PVR. Proliferative vitreoretinopathy is characterized by fibrotic changes in the detached retina combined with fibrovascular membranes (FVMs) and subretinal bands (SRBs). The reason why certain cases of RD develop into PVR has not been completely clarified. Nevertheless, previous studies have shown the strong involvement of the epithelial-mesenchymal transition (EMT) in FVMs with PVR: RPE cells spread into the vitreous cavity through retinal breaks in the detached retina, transform into fibroblasts, and migrate both on the surface and beneath the retina, resulting in the onset of PVR. Transforming growth factor-beta (TGF-β), which is much higher in eyes with PVR than in uncomplicated eyes with RD, is the major driver for the induction of EMT in many cells, and presumably in RPE cells as well. Oral steroid intakes that supposedly suppress TGF-β activity did not robustly contribute to the prevention of PVR in surgical RD cases. Therefore, a better understanding of the precise mechanism and the discovery of a novel therapeutic target for PVR is necessary.

Caveolin-1 is a 21- to 24-kDa integral membrane protein and has been adequately investigated by a number of biochemical studies. Caveolin-1 was found at the endoplasmic reticulum and Golgi complex and predominantly at the plasma membrane. It has also been found in many cells, such as adipocytes, endothelia, and epithelial cells. Recently, many studies have shown that Caveolin-1 plays a key role in many important...
biological events in cancer research, including tumor metastasis and angiogenesis.\textsuperscript{14–16} Caveolin-1 is of particular interest because it also plays an important role in the EMT of cancer biology and of tissue fibrosis.\textsuperscript{14,16–18} Therefore, we hypothesized that Caveolin-1 has a pivotal role in the pathogenesis of PVR. To confirm our hypothesis, we examined the expression of Caveolin-1 in the human FVMs from eyes with PVR and in mouse tissues with PVR. In addition, we studied the role of Caveolin-1 in PVR using mice that lacked the Cav-1 gene and using primary human and mouse RPE cell lines both in vivo and in vitro.

**Materials and Methods**

**Patients and Sample Collection**

All tissues were collected during surgeries except the corneal and retinal tissues that were used for the Caveolin-1 Western blot and quantitative RT-PCR. Corneal and retinal tissues were obtained from the normal control donor eye from the Minnesota Lions Eye Bank (Minneapolis, MN, USA) and San Diego Eye Bank (San Diego, CA, USA). Tissues were stored at \(-80^\circ\text{C}\) immediately after extraction until further use. We excluded the patients with severe systemic diseases, such as autoimmune diseases or cancers. The study followed the guidelines of the Declaration of Helsinki and was approved by the Nagoya University Hospital Ethics Review Board. We obtained a written informed consent from each patient.

**Proliferative Vitreoretinopathy Induction in Mice**

Wild-type C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan), and Caveolin-1 knockout (Cav-1\(^{-/-}\)) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The surgical method that was used to generate the mouse PVR model has been precisely described by Saika et al.\textsuperscript{19} A linear surgical method was used to generate the mouse PVR purchased from Jackson Laboratory (Bar Harbor, ME, USA). The obtained a written informed consent from each patient.

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**Cell Culture and Primary Cell Preparation**

Primary human RPE (hRPE) cells were purchased from Lonza (Walkersville, MD, USA) and used for in vitro assays. The cells were grown in the Dulbecco's modified Eagle's medium premixed with Ham's F-12 nutrient mixture (1:1 ratio; Sigma-Aldrich Corp., St. Louis, MO, USA) and supplemented with 10% fetal bovine serum (FBS) and streptomycin/penicillin G antibiotics (Sigma-Aldrich Corp.). The primary hRPE cells were transfected with Stealth small interfering RNA (siRNA; Invitrogen, Carlsbad, CA, USA) targeting CAVELONIN-1 (HS1414466) and the negative control (siRNA_Ctrl). The primary mouse RPE (mRPE) cells were collected from the wild-type mice and the Cav-1\(^{-/-}\) mice as previously described.\textsuperscript{20,21}

**Immunohistochemistry and Immunocytochemistry**

The immunohistochemistry for human ocular tissues has been previously described.\textsuperscript{22} In brief, the tissues were fixed with 10% neutral buffered formalin. The immunohistochemical staining was performed using the rabbit antibody against human Caveolin-1 (1:200; Cell Signaling Technology, Beverly, MA, USA), and the staining without primary antibodies (Negative Ctrl) were performed to assess the specificity of staining. The bound antibody was detected with a Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, USA), and the enzyme complex was visualized with both horseradish peroxidase (HRP) and an alkaline phosphatase blue substrate kit (Vector Laboratories). Levamisole (Vector Laboratories) was used to block any endogenous alkaline phosphatase activity. Mouse eyes induced with PVR were fixed and cryoprotected and 10-μm sections were obtained as previously described.\textsuperscript{23} The sections were stained with zsMA antibody (1:200; Sigma-Aldrich Corp.) and visualized with Alexa 488 (1:1000; Invitrogen) conjugated antibody. For cultured hRPE cells after siRNA transfected, the cells were maintained in medium with 1% FBS for 48 hours and then fixed with 100% methanol, stained with rabbit antibodies against zonula occludens-1 (ZO-1, 1:100; Invitrogen), zsMA antibody (1:200; Sigma-Aldrich Corp.), and visualized with Alexa 488 or 594 (1:1000, Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were obtained using a scanning laser confocal microscope (A1-Rsi; Nikon, Tokyo, Japan). Relative fluorescence intensities of zsMA signals were analyzed as previously described.\textsuperscript{24}

**Protein and RNA Isolation**

In the mouse PVR model, retinal samples including FVM were carefully isolated from the eyes at 7 days after inducing PVR. In this model, abundant pigmented cells, presumably PPE cells, adhered to the retina samples (retina/RPE). For protein collection, the retina/RPE complex and cultured human and mouse cells were lysed in radioimmunoassay buffer (Sigma-Aldrich Corp.) with a protease inhibitor cocktail

**Table. Characteristics of the Patients/Subjects for Immunohistochemistry, Western Blotting, and Real-Time PCR**

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DME, diabetic macular edema; ILM, internal limiting membrane; VMTS, vitreo-macular traction syndrome.

* Donor eyes from Minnesota Lions Eye Bank/San Diego Eye Bank.
The lysate was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was collected. The protein concentrations were determined using a Bradford assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. For the RT-PCR analyses, the total RNA was purified using a Qiagen RNeasy Mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol; the RNA concentration and quality were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Protein and RNA from human samples were prepared following the same method.

Quantitative RT-PCR
The total RNA was reverse transcribed using a Transcriptor Universal cDNA Master Kit (Roche Diagnostics) starting with 2 μg total RNA from each sample. Reverse transcription PCR was performed using the Thunderbird Probe qPCR Mix (Toyobo Life Science, Osaka, Japan) and a Gene Expression Assay containing primers and a FAM dye-labeled TaqMan probe for detecting human CAVEOLIN-1 (Hs00971716_m1; Applied Biosystems, Foster City, CA, USA). The PCR cycles consisted of a pre-denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturing steps at 95°C for 15 seconds and annealing and extending steps at 60°C for 60 seconds. The relative expressions of the target genes were determined by the 2^{-ΔΔCt} method.

Western Blotting
Protein (30–70 μg) samples from the human and mouse tissues or culture cells were run on SDS precast gels (Wako, Osaka, Japan) and transferred to polyvinylidene difluoride membranes. Because mouse ocular tissues are very small, the tissues from four to five eyes were mixed together and used as

**Figure 1.** Existence of Caveolin-1 in the human FVMs and SRBs from the patients with PVR. Immunohistochemistry showed Caveolin-1-positive cells stained by both HRP (brown) and AP (blue) in FVMs and SRBs from three independent patients with PVR. The specificity of Caveolin-1 staining was confirmed by the absence of reaction production. Scale bar: 50 μm.
The transferred membranes were washed in TBS-T (0.05M Tris, 0.138M NaCl, and 0.0027M KCl, pH = 8.0, 0.05% Tween 20; Sigma-Aldrich Corp.) and then blocked in 5% nonfat dry milk/TBS-T at room temperature (RT) for 2 hours. The membranes were then incubated with the rabbit antibody against alpha-smooth muscle actin (αSMA, 1:1000; Cell Signaling Technology), Smad2/3 (1:1000; Cell Signaling Technology), and phospho-Smad2/Smad3 (1:1000; Cell Signaling Technology) at 4°C overnight. Protein loading was assessed by immunoblotting using an anti-α/β-tubulin (1:2000; Cell Signaling Technology) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000; Cell Signaling Technology). The HRP-linked secondary antibody was used (1:3,000; Invitrogen) at RT for 1 hour. The signal was visualized with enhanced chemiluminescence (ECL plus; GE Healthcare, Piscataway, NJ, USA) and captured with ImageQuant LAS-4000 Imager (GE Healthcare). Immunoreactive band (caveolin-1) was quantified by using densitometry (ImageJ Software v1.48; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Specific protein expression levels were normalized to the GAPDH protein signal on the same membrane.

Cell Viability Assay

Cell viabilities from hRPE cells and mRPE cells were evaluated using the WST-1 colorimetric assay (Roche Diagnostics) following manufacturer’s instructions. In brief, the plates were analyzed by measuring absorbance at 450 nm (reference at 700 nm) using a plate reader (Bio-Rad, Richmond, CA, USA). Duplicate evaluations were performed for each sample.

Migration Assays

To evaluate migration ability, two different methods, Transwell migration assay and scratch assay, were applied. For Transwell migration assay, from Cav-1−/− or wild-type mice, mRPE cells were replated on the 8-μm pore-size culture inserts (Transwell; Costar, Corning, NY, USA). Transwell membrane separates the upper and the lower chambers; 10% FBS-containing medium was added in the lower chamber, and serum-free medium was added in the upper chamber. After 24 hours, the cells that had migrated through the pores were stained, and the number of migrating cells counted from five vision fields were randomly counted under the microscope (BZ-9000; Keyence, Osaka, Japan) and averaged as n = 1. For scratch assay, mRPE cell from Cav-1−/− or wild-type mice were replated and stimulated by TGF-β2 (10 ng/mL) for 24 hours: this was followed by inflicting a single scratch wound with a p200 pipette tip. The number of cells that migrated into the wound space was assessed by light microscope (FSX100; Olympus, Tokyo, Japan). The migrating cell numbers were counted by ImageJ and averaged. All experiments were performed at least three times.

Statistics

The results were expressed using scatter plot with the horizontal bar representing the median (n = number of
samples). All data were statistically analyzed using the Mann-Whitney U test (unpaired samples). Differences were considered to be statistically significant at $P < 0.05$.

**RESULTS**

Caveolin-1 Expression in Human and Mouse PVR Tissues

We first examined whether the human tissues in the eyes with PVR strongly expressed Caveolin-1. We collected FVMs and SRBs from patients who had undergone vitrectomy surgeries for the treatment of PVR. The characteristics of the patients and the information from the tissues in this study are summarized in the Table. We performed the immunohistochemistry using three independent patients with PVR. We confirmed Caveolin-1 expression in all samples from each patient and double-checked the Caveolin-1 staining using two different substrates, HRP and alkaline phosphatase blue (AP_blue) (Fig. 1). We also performed immunohistochemistry using the same FVMs/SRBs tissues without anti–Caveolin-1 antibody (Negative Ctrl). Immunohistochemistry without anti–Caveolin-1 antibody did not show any specific staining, and the results enhanced the anti–Caveolin-1 staining specificity. We also performed immunohistochemistry with the same tissues as shown in Figure 1 using antibodies against $\alpha$-SMA, CD31, and glial fibrillary acidic protein (GFAP). Supplementary Figure S1 shows that all three samples showed positivity of $\alpha$-SMA and GFAP, and CD31-positive cells were abundantly observed only in the specimens from FVM. We also examined the abundance of Caveolin-1 expression using a Western blot and quantitative RT-PCR (qRT-PCR) (Fig. 2). Western blot was performed using FVMs and SRBs from four independent patients with PVR (Table). Protein lysates of the anterior lens epithelium from the patient with a cataract (negative control) and of the cornea from the normal donor eye (positive control) were used and run together. Western blot showed Caveolin-1 abundance in all FVMs and SRBs from four patients with PVR (Fig. 2a). We also performed qRT-PCR using FVMs and SRBs from three independent patients with PVR and compared the relative expression in the internal limiting membranes (ILMs) from the control patients with epiretinal membranes (ERM), diabetic macular edema, or vitreomacular traction syndrome (Table). CAVOLEIN-1 mRNA levels in FVMs and SRBs were abundantly expressed (268.7, 143.4–1302.5 [median, Q1–Q3], $n = 3$) compared with those in ILMs from the control patients (3.48, 2.24–8.66 [median, Q1–Q3], $n = 3$, Fig. 2b). Similarly, CAVOLEIN-1 mRNA expression in both a rat and mouse eye showed significant changes compared with controls. **$P < 0.01$. N.S., no significant difference.

The Role of Caveolin-1 in PVR

Because Caveolin-1 is ubiquitously expressed in many cells, the discovery that all FVMs/SRBs expressed Caveolin-1 was not surprising. Therefore, we induced PVR in the mouse eyes and examined whether Caveolin-1 expression had been upregu-
those from wild-type (Cav-1) in vivo. To reduce the Caveolin-1 expression in hRPE cells, we used siRNA-Cav-1 obtained from mouse eye with PVR compared with that from the control eyes (Fig. 3a). Interestingly, Cav-1–/– mice showed extremely severe PVR with enhanced αSMA expression in the RPE layer. Of the multiple markers, αSMA and ZO-1 were applied to evaluate EMT, similar to other studies. To reduce the Caveolin-1 expression in hRPE cells, we used siRNA-Cav-1; its knockdown efficacy and cell viability were confirmed (Fig. 4): Caveolin-1 mRNA expression was significantly reduced in hRPE with siRNA-Cav-1 (0.14, 0.12–0.20 [median, Q1–Q3], n = 6, P = 0.004) compared with that with control siRNA (siRNA_Ctrl; 0.80, 0.58–1.00 [median, Q1–Q3], n = 6, Fig. 4a) and Caveolin-1 protein expression (densitometry) was significantly reduced in hRPE with siRNA-Cav-1 (0.26, 0.23–0.34 [median, Q1–Q3], n = 6, P = 0.016) compared with that with siRNA_Ctrl (0.46, 0.39–0.47 [median, Q1–Q3], n = 6, Figs. 4b, 4c), whereas hRPE cell viability with siRNA-Cav-1 (0.89, 0.77–1.00 [median, Q1–Q3], n = 12) did not show significant changes compared with those with control siRNA (1.00, 0.87–1.13 [median, Q1–Q3], n = 12, P = 0.112; Fig. 4d). Small interfering RNA-Cav-1 transfection resulted in ZO-1 decrease with collapsed hexagonal hRPE morphology (Fig. 5a) and αSMA increase (Figs. 5b–d). Relative strength of αSMA fluorescence signal significantly increased by siRNA-Cav-1 (1.79, 1.74–1.85 [median, Q1–Q3], n = 6, P = 0.037) compared with control siRNA (0.99, 0.84–1.08 [median, Q1–Q3], n = 6; Fig. 5c). Alpha-SMA changes were also examined in the mouse ocular tissues before and after inducing PVR in vivo. In the intact status, the αSMA levels did not show obvious differences between wild-type and Cav-1–/– mice. However, consistent with the in vitro results from hRPE cells, the αSMA level was strongly enhanced in the retina/RPE complex in Cav-1–/– mice after inducing PVR. In addition, Cav-1–/– mouse retina/RPE enhanced Smad2/3 phosphorylation after PVR induction (Fig. 5e). Corroborating these results indicated that reduced Caveolin-1 in RPE enhanced EMT.

### Caveolin-1 Knockout RPE Cells Increased Migration

We also examined whether Cav-1–/– mRPE cell migration ability was enhanced. Transwell assay showed significantly larger number of migrating Cav-1–/– mRPE cells (2.10, 1.62–2.25 [median, Q1–Q3], n = 15, P = 0.0003) compared with that of wild-type mRPE cells (1.00, 0.68–1.18 [median, Q1–Q3], n = 15; Figs. 6a–c). Consistently, Cav-1–/– mRPE cells showed a significantly higher number of migrating cells in scratch assay (1.69, 1.00–1.92 [median, Q1–Q3], n = 25, P = 0.014) than wild-type RPE cells (1.00, 0.61–1.46, [median, Q1–Q3], n = 25; Figs. 6d–g). Cell viability of mRPE cells from Cav-1–/– (0.91, 0.83–0.98 [median, Q1–Q3], n = 11) and wild-type (0.91, 0.75–0.95 [median, Q1–Q3], n = 11, P = 0.7, Fig. 4e) was not significantly different.

### DISCUSSION

Epithelial-mesenchymal transition is one of the very important biological events in many organs including ocular tissues. For...
instance, Caveolin-1 is not expressed in the normal lens epithelium (Fig. 2a), but it is upregulated once EMT has been triggered. In the pathogenesis of PVR, both EMT-triggered RPE and transformed glial cells play pivotal roles. Our study suggests that some of the Caveolin-1–positive cells in the FVM or SRB were glial cells rather than myofibroblast or vascular endothelial cells (Supplementary Fig. S1). Although recent studies showed the involvement of glial cell migration in the pathogenesis of PVR,38,29 RPE is believed to be a main player in the induction of EMT in PVR. Indeed, FVMs and SRBs possess a certain number of pigmented cells that can grow ectopically (Fig. 1). In actual clinical situations, ophthalmic physicians often find floating pigmented cells in the vitreous fluid of eyes with RD. It is believed that those floating pigmented cells, presumably RPE cells, attach to the surface of the retina, then initiate EMT and migrate as fibrotic cells. As for the pigmented cells, there are several points that should be considered when handling them in ocular research. For instance, because RPE is pigmented, it is sometimes very difficult to distinguish RPE-oriented pigmentation or diaminobenzidine (DAB)-based colormetric changes in the section. Therefore, in immunohistochemistry, we used two different substrates for the assessment of Caveolin-1 expression. AP_blue staining, which was not hindered by RPE pigmentation, showed the specific localization of the Caveolin-1 in FVMs and SRBs. Combined with the images of DAB staining, we showed the redundancy of Caveolin-1 more confidently in FVMs and SRBs.

**Figure 6.** Migration abilities of primary mRPE cells. (a) The number of migrating mRPE cells through the Transwell membrane was significantly higher in Cav-1−/− mice than those in wild-type (Cav-1+/+/). (b, c) Representative images of mRPE cells from Cav-1+/+ (b) and Cav-1−/− (c) mice. (d) The number of migrating mRPE cells were significantly increased in Cav-1−/− mice compared with that in wild-type mice. (e) An image of mRPE cells immediately following scratch formation. (f, g) Representative images of the migrating mRPE cells in Cav-1+/+ (f) and Cav-1−/− mouse (g). Scale bars: 100 μm. *P < 0.05.
In this study, we confirmed the existence of Caveolin-1 expression in FVMs and SRBs from the eyes with PVR and the increased expression of Caveolin-1 in PVR using the mouse PVR model. We first hypothesized that increased Caveolin-1 promoted EMT in PVR. Nevertheless, Western blotting, immunostaining, and migration assay results showed contrary results to what was expected: Caveolin-1 knockdown and knockout revealed enhanced EMT in both the hRPE and mRPE. Interestingly, Caveolin-1 has been reported to both promote and suppress tumor growth.30–32 These discrepancies revealed various Caveolin-1 functions that were dependent on the cell types and situations. Nevertheless, in most of the tissues related to systemic sclerosis and fibrotic diseases, Caveolin-1 tended to block EMT.18,33–34 The limitation of this study was that we did not show the precise pathway of Caveolin-1-dependent inactivation of Smad2/3. Previous studies revealed that Caveolin-1 blocked extracellular signal-regulated kinases (ERK) 1/2 and c-jun N-terminal kinase (JNK).18,35–37 Phospho-ERK1/2 induces Smad2/3 phosphorylation, which is the main pathway of TGFβ-dependent EMT.38 Indeed, ERK activation leads to increased collagen expression in lung fibroblasts.39 Corroborating these studies suggest that dysregulated Caveolin-1 might fail to block ERK phosphorylation, which results in Smad2/3 activation.39 On the other hand, previous studies showed that Caveolin-1 was upregulated in EMT via the activation of a focal adhesion kinase.14 Epidermal growth factor downregulated Caveolin-1.15 Finding the precise mechanism of the main player that suppresses Caveolin-1 expression in eyes with RD could lead us to new therapeutic approaches in the prevention of PVR.

In conclusion, enhanced expression of Caveolin-1 in FVMs and SRBs blocked EMT. Maintaining Caveolin-1 expression in the ocular tissues could produce novel therapeutic concepts that we do not currently possess.

Acknowledgments

The authors thank Shizuya Saika, Tadasu Sugita, and Norie Nonobe for important clinical and scientific suggestions, and Reona Kimoto, Chisato Ishizuka, and Kazuko Matsuba for technical assistance. Supported by a Grant-in-Aid for Scientific Research B (15H04994; HK) from the Japan Society for the Promotion of Science, Takeda Medical Research Foundation (HK), Takeda Science Foundation (HK), Mishima Kaiichi Memorial Ophthalmic Research Foundation (HK), and Ito chube’e Foundation (HK).

Disclosure: Y. Nagasaka, None; H. Kaneko, None; F. Ye, None; S. Kachi, None; T. Asami, None; S. Kato, None; K. Takayama, None; S.-J. Hwang, None; K. Kataoka, None; H. Shimizu, None; T. Iwase, None; Y. Funahashi, None; A. Higuchi, None; T. Senga, None; H. Terasaki, None

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