Received: 14 March 2021 | Revised: 10 July 2021 | Accepted: 12 July 2021

DOI: 10.1096/fba.2021-00037

RESEARCH ARTICLE

FASEB BioAdvances WILEY

ROCK inhibitors modulate the physical properties and adipogenesis of 3D spheroids of human orbital fibroblasts in different manners

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Abstract

To elucidate the pharmacological effects of Rho-associated coiled-coil containing protein kinase inhibitors (ROCK-is), ripasudil (Rip), Y27632, and KD025, on human orbital fatty tissue, the human orbital fibroblasts (HOFs) were three-dimensional (3D) cultured for 12 days. The effects of ROCK-is on the physical properties of the 3D-cultured HOF spheroids, including their sizes and physical stiffness, their adipogenesis by lipid staining, and the mRNA expression of adipogenesis-related genes, *PPARγ* and *AP2*, and extracellular matrix (ECM) including collagen (COL) 1, 4, and 6, and fibronectin were analyzed. A significant increase in the sizes, physical stiffness, lipid staining, and mRNA expression of adipogenesis-related genes, *COL4* and *COL6*, and a decrease in *COL1* expression were observed with adipogenesis (DIF+). In the presence of ROCK-is, such DIF+-induced effects were differently modulated as follows: (1) the sizes were not affected or significantly enhanced by Rip, Y27632, or KD025, (2) the physical stiffness was significantly decreased in Rip and Y27632, but was substantially increased in KD025, (3) the lipid staining was further enhanced or significantly suppressed by Rip, Y27632, or KD025, and both *PPARγ* and *AP2* expression were significantly downregulated or upregulated by KD025 or Rip, and (4) Rip upregulated the expression of *COL4*, Y27632 upregulated the expression of *COL1*, *COL4*, and *COL6*, and KD025 upregulated the expression of *COL1* and *COL4*. This study indicates that ROCK-is significantly and differently modulate physical properties of the 3D HOF spheroids as well as their adipogenesis.

KEYWORDS

human orbital fibroblasts (HOFs), Rho kinase, ROCK, ROCK inhibitor, three-dimensional (3D) tissue culture

1 | INTRODUCTION

The only evidence-based therapy for glaucomatous optic neuropathy, the most frequent disease leading to irreversible blindness,[1–3] is to suitably lower the intraocular pressure (IOP) by antiglaucoma medications and/or glaucoma surgeries.[4] Among antiglaucoma medications, prostaglandin analogues (PGs) are recognized as the first-line medication due to their great hypotensive efficacy in addition to very few systemic side effects.[5] Recently, PGs induced periocular side effects, including deepening of the upper eyelid sulcus (DUES) and other manifestations have been identified among their long-term users.[6] PG-induced atrophy of orbital adipose tissues was suspected as a possible mechanism for causing DUES,[7] Since then, antiglaucoma medication-induced periocular side effects have begun to attract great attentions. To study this periocular effects especially toward orbital fatty tissue, we successfully obtained an in vivo model replicating DUES pathogenesis by using three-dimensional (3D) cell cultures of 3T3-L1 cells[8] as well as human orbital fibroblasts (HOFs).[9] Thus, we suggested that our 3D spheroid culture methods will rationally be applicable to study the periocular effects of several antiglaucoma drugs especially on orbital fatty tissues.

Rho-associated coiled-coil containing protein kinases (ROCKs), belonging to the serine–threonine protein kinase family, are well known to be involved in the regulation of actin cytoskeleton remodeling.[10–14] Two types of ROCKs, ROCK1 (ROKβ) and ROCK2 (ROKα) share their amino acid compositions of the carboxyl termini, the catalytic kinase domain and the Rho-binding domain except their coiled-coil region.[15,16] Functionally, ROCK1 and ROCK2 play important roles in the regulation of actin cytoskeleton organization, cytokinesis, differentiation, apoptosis, glucose metabolism, cell adhesion/motility, and inflammation.[17–19] The expression of ROCKs are also recognized within the ocular and periocular tissues, including the trabecular meshwork, ciliary muscles, and the retina,[15,16] and therefore, these are involved in the ocular pathophysiology in several ocular diseases such as cataracts, retinopathy, and corneal dysfunction.[10,11,20–23] These observations, in turn, strongly suggest that ROCKs may alternatively become therapeutic targets for these ocular diseases. In fact, it was revealed that ROCK inhibitors (ROCK-is) could reduce IOP in several animal models,[24,25] and one of the ROCK-is, ripasudil hydrochloride hydrate (Rip), a nonselective ROCK-i, is already available as a new type of medications for the treatment of glaucoma and ocular hypertension.[26,27] Since it has been revealed that conjunctival hyperemia as one of periocular side effects of Rip, Rip and other ROCK-is may cause unknown other periocular manifestations.[26,27] In addition, since, as described above, ROCKs, ROCK1 and ROCK2, multiply contribute pathophysiology in general, it is of great interest to elucidate periocular effects of ROCK-is including Rip, especially toward orbital fatty tissues. In fact, our recent pilot study using a 3D spheroid culture of the 3T3-L1 cells, which is known as the most popular preadipocyte used for adipogenesis-related study, suggested that ROCK-is significantly affect physical properties, adipogenesis, and extracellular matrix (ECM) expression of the 3T3-L1 3D spheroids.[28] Taken together, these observations rationally allowed us to study further the effects of ROCK-is toward HOFs.

Therefore, in the present study, we studied the effects of several ROCK-is, including nonselective ROCK-is, Y27632 and Rip, and a selective ROCK2 inhibitor, KD025, on lipid metabolism, especially on adipocyte volume, and their ECM expression through adipogenesis on our 3D-cultured HOFs.

2 | MATERIALS AND METHODS

This study, which was performed at the Sapporo Medical University Hospital, Japan, was approved by the institutional review board (approved number, 312-3190) and according to the tenets of the Declaration of Helsinki as well as national laws for the protection of personal data. Informed consent was obtained from all participants in this study.

2.1 | Isolation of HOFs and 3D cultures of HOFs

Human orbital fibroblasts were isolated surgically as described previously using orbital fat explants from four patients with orbital fat herniation. Thereafter, 3D cultures of HOFs and induction of their adipogenic differentiation were processed for 12 days as described recently.[29,30] For evaluating the efficacy of ROCK-is, 1 or 10 μM ripasudil (Rip), Y27632, or KD025 were added from Day 1 through Day 12.

2.2 | Lipid staining of 3D-cultured HOFs by BODIPY

BODIPY lipid staining of 3D-cultured HOF spheroids was performed as described previously.[29,31,32] Briefly, 4% paraformaldehyde-fixed 3D spheroids were incubated in a mixture of 0.1% BODIPY (#D3922; Thermo Fisher Scientific), 0.1% DAPI (#D523; Doujin), and 0.1% phalloidin (#20553; Funakoshi) in PBS containing 3% bovine serum albumin for 3 h. The fluorescence intensity of the BODIPY was detected using a Nikon A1 confocal microscope (Tokyo, Japan) and quantified using the Image J software version 2.0.0 (NIH).

2.3 | Quantitative PCR

Using total RNA extraction by a RNeasy mini kit (Qiagen), and the reverse transcription by the SuperScript IV kit

(Invitrogen) were processed to make cDNA according to the manufacturer's instructions. The real-time PCR with the Universal Taqman Master mix was performed using a StepOnePlus machine (Applied Biosystems/Thermo Fisher Scientific). cDNA levels expressed as fold-change relative to the expression of a housekeeping 36B4 (*Rplp0*) gene was calculated. Sequences of the primers and Taqman probes used are given below:

Human RPLP0

Probe: 5′-/56-FAM/CCCTGTCTT/ZEN/CCCTGGGCATCAC/3IABkFQ/-3′; forward: 5′-TCGTCTTTAAACCCTGCGTG-3′; reverse: 5′-TGTCTGCTCCCACAATGAAAC-3′.

Human COL1A1

Probe: 5′-/56-FAM/TCCAGGGCC/ZEN/AAGACGAAGACATC/3IABkFQ/-3′, forward: 5′-GACATGTTCAGCTTTGTGGAC-3′; reverse: 5′-TTCTGTACGCAGGTGATTGG-3′.

Human COL4A1

Probe: 5′-/56-FAM/TCATACAGA/ZEN/CTTGGCAGCGGCT/3IABkFQ/-3′, forward: 5′-AGAGAGGAGCGAGATGTTCA-3′;reverse:5′-TGAGTCAGGCTTCATTATGTTCT-3′.

Human COL6A1

Forward: 5′-CCTCGTGGACAAAGTCAAGT-3′; reverse: 5′-GTGAGGCCTTGGATGATCTC-3′.

Human FN1

Forward: 5′-CGTCCTAAAGACTCCATGATCTG-3′; reverse: 5′-ACCAATCTTGTAGGACTGACC-3′.

2.4 | Microindentation force measurement

icroindentation force of the spheroids was measured using a microsqueezer (CellScale) as described previously.[30] Briefly, a single spheroid placed on a 3 mm × 3 mm plate was compressed to achieve 50% deformation in 20 s using a microcamera. The required strain (μN) was measured and force/displacement (μN/μm) was calculated.

2.5 | Statistical analysis

All statistical analyses were performed using Graph Pad Prism 8 (GraphPad Software). To analyze the difference between groups, a group analysis with two-way ANOVA

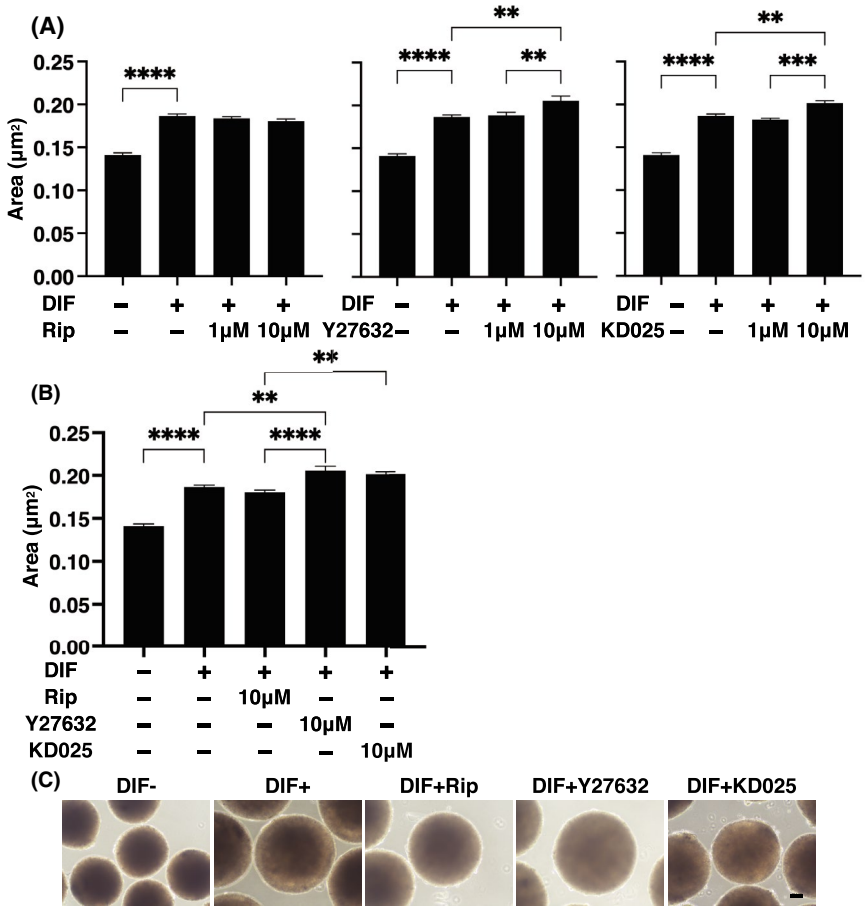

FIGURE 1 Effects of ROCK-is on the area sizes of the 3D human orbital fibroblast (HOF) spheroids during adipogenesis. At Day 12, the mean area sizes (μm^2) of the 3D spheroids of HOFs preadipocytes (DIF−) and their adipogenic differentiation (DIF+) without or with 1 or 10 μM ripasudil (Rip), Y27632, or KD025 are plotted in panel (A). Among the different ROCK-is forms, to compare their effects toward HOFs adipogenesis, the mean area sizes (μm^2) of 10 μM of each ROCK-is were replotted in panel (B), and their representative phase contrast microscopic images are shown in panel (C). All experiments were performed in triplicate using fresh preparations, each of which consisted of 16 spheroids. Data are presented as arithmetic means ±standard error of the mean (SEM). $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ (ANOVA followed by Tukey's multiple comparison test). ROCK, Rho-associated coiled-coil containing protein kinase

followed by Tukey's multiple comparison test were performed. Data are presented as arithmetic means ± SEM.

3 | RESULTS

To study the pharmacological effects of several ROCK-is on orbital fatty tissues, physical properties such as sizes and stiffness of 3D HOFs spheroids were investigated in the presence of 1 or 10 μM ripasudil (Rip), Y27632, or KD025. The 3D cultures of HOFs at Day 12 and sizes of each 3D spheroid among the experimental groups were plotted as shown in Figure 1A,B. As described in our previous studies,[29,31] uniform round-shape spheroidal 3D spheroids from 20,000 HOFs cells were obtained (Figure 1C), and upon adipogenic induction (DIF+), these sizes were significantly enlarged. This DIF+-induced enlargements of 3D HOF spheroids were substantially enhanced in the presence of Y27632 or KD025 and this enhancement was concentration dependent, although such enlargement effects were not observed in the presence of Rip (Figure 1A,B). Similarly, microindentation analysis indicated that the physical stiffness of 3D HOF spheroids were significantly increased by DIF+, and the additive effects of ROCK-is to DIF+ were also concentration dependent. However, the efficacies among the ROCK-is forms were significantly different, that is, pan-ROCK-is, Rip and Y27632, or ROCK2-i, KD025, induced significant suppressive or enhancing effects (Figure 2).

To elucidate on the underlying mechanisms of ROCK-is-induced effects on the physical properties of 3D HOF spheroid, lipid staining by BODIPY and mRNA expression of adipogenesis-related genes, *PPARγ* and *AP2*, and ECMs including collagen 1 (*COL1*), *COL4*, *COL6*, and fibronectin (FN) were studied in the presence of 10 μM ROCK-is, which caused more effects on the physical properties as above. As shown in Figure 3, upon DIF+, their staining intensities and gene expression of *PPARγ* and *AP2* were significantly increased as observed in our previous studies.[29,31,32] In the presence of Rip or Y27632, such DIF+ induced increase in BODIPY staining was further enhanced; although the gene expression of *PPARγ* was not altered, the gene expression of *AP2* was significantly increased by Rip. Surprisingly, KD025 induced significant downregulation of *PPARγ* and *AP2* expression, which is more evident in the latter, and almost no staining by BODIPY. In terms of mRNA expression of ECMs, *COL4* and *COL6* were downregulated or upregulated upon DIF+ as similarly observed in our previous studies.[29,31,32] In the presence of ROCK-is, such DIF+-induced changes of *COL4* were all enhanced. While in contrast, DIF+-induced expression of *COL1* and *COL6* were significantly enhanced by Y27632 and KD025, and Y27632, respectively. These diversity in the efficacies among ROCK-is may be attributed to differences in their preference for ROCK1 or ROCK2 inhibitory activities (Rip; ROCK 1<ROCK 2, Y-27632; ROCK 1>ROCK 2)[33,34] in addition to the paradoxical inhibitory effects of KD025 as described by Diep et al.[35]

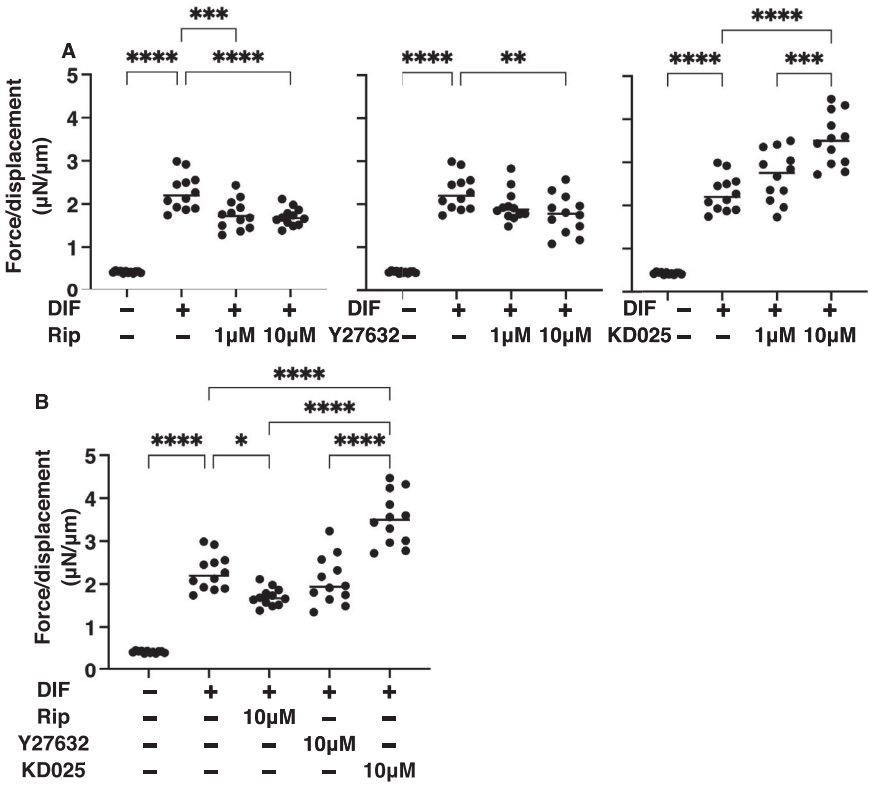

FIGURE 2 Effects of ROCK-is on physical stiffness of the 3T3-L1 3D spheroids. At Day 12, the 3D human orbital fibroblast (HOF) spheroids of preadipocytes (DIF−) and their adipogenic differentiation (DIF+) without or with 1 or 10 μM ripasudil (Rip), Y27632, or KD025 were subjected to a physical solidity analysis using a microsqueezer. The force required to induce deformation until half diameter was reached (μN/μm force/displacement) were measured and the data are potted in panel A. Among the different ROCK-is forms, to compare their effects toward HOFs adipogenesis, the physical stiffness of 10 μM of each ROCK-is were replotted in panel B. All experiments were performed using freshly prepared 12–20 spheroids. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ (ANOVA followed by Tukey's multiple comparison test). ROCK, Rho-associated coiled-coil containing protein kinase

4 | DISCUSSION

ROCKs are known to negatively regulate adipocyte differentiation.[36] In the 3T3-L1 cells, ROCKs are known to inhibit adipogenesis, and in turn, their inhibitors, Y-27632 and fasudil are identified to stimulate adipocyte differentiation.[36] In the present study, pan-ROCK inhibitors, Rip and Y27632, also significantly enhanced adipogenesis of the 3D HOF spheroids. As it was shown that ROCK2 but not ROCK1 is responsible for the suppressive effects toward their adipogenesis,[36] we expected higher enhancement effects of adipogenesis by a specific inhibitor of ROCK2, KD025. However, in contrast, KD025 inhibited mRNA expression of PPARγ slightly and AP2 substantially in the 3D HOF spheroids, in which almost no BODIPY lipid staining was observed. Such paradoxical anti-adipogenesis effects by KD025 in the 3T3-L1 cells were also reported.[35] Furthermore, this paradoxical phenomenon by KD025 was also observed in the stiffness analysis of the 3D HOF spheroids (Figure 2). In their study, key genes such as *PPARγ* and *C/EBPα* were significantly blocked by KD025, whereas the expression of early adipogenic genes was not affected. In addition, the end stage of adipogenesis, KD025 did not affect lipid accumulation, and mitotic clonal expansion. Based on these findings, they suggested that KD025 regulates its targets during the intermediate stage of adipogenesis. To support this suggestion, in our current study, suppressive effects of mRNA expression toward *AP2* were much more evident than those of *PPARγ*.

In terms of the expression of major ECMs in adipocytes or adipose tissues, it has been reported that main types of adipose ECM are the main fibril-forming COL1 and microfibrillar COL4 and COL6. COL1 is the most abundant ECM, which serve to impart stiffness to tissues and organs.[37] COL4 and six foam network of COLs and are involved in a variety of cellular functions including the regulation of fibril assemblies and organization, integrating cells, and matrix structures and/or the integration of different matrix structures such as basement membranes.[38,39] In agreement with these network-forming ECMs have the capacity to organize the 3D tissue architecture.[40,41] ROCK-is-induced upregulation of COL1, COL4, or COL6 was observed within the 3D HOF spheroids, in which their sizes were increased in the present study. FN forms a weak molecular conformation and can be changed by the binding of allosteric partners or strain resulting from cell contractile forces.[42] In the present study, FN expression were not significantly affected by ROCK-is. Therefore, unbalance between unchanged FN and significant upregulation of the network-forming COLs, COL4, and COL6 by ROCK-is, may presumably cause substantial decrease in the physical stiffness of the 3D HOF spheroids. This speculation is rationally supported by the fact that the increase in 3D spheroid size was induced in the presence of 10 μM ROCK-is, but not in the presence of 1 μM ROCK-is as shown in Figure 1, and in the presence of 10 μM concentrations of ROCK-is, the gene expression of ECM were modified as shown in Figure 4. Similar to this, the inhibition of ROCKs is also known to reduce the extent of mechanical tension and stiffness in cells, and decrease ECM synthesis and rigidity in various cell types.[43,44]

Concerning the periocular effects of ROCK-is especially on orbital fatty tissues, only limited information is currently available. However, in our previous studies,

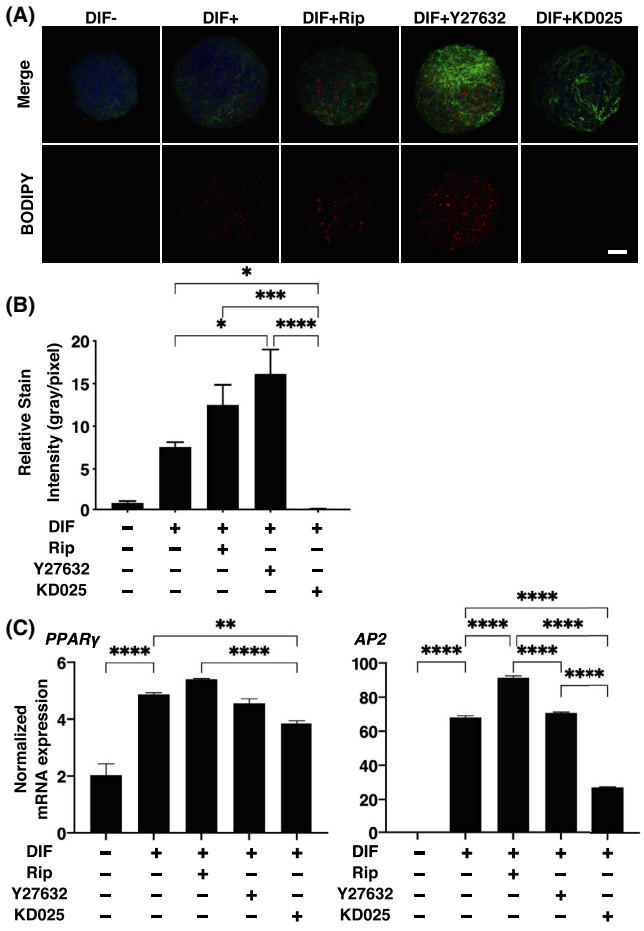

FIGURE 3 Effects of ROCK-is on adipogenesis of 3D human orbital fibroblast (HOF) spheroids. The 3D HOF spheroids from Day 12 was prepared under several conditions: preadipocytes (DIF−) and their adipogenic differentiation (DIF+) without or with 10 μM ripasudil (Rip), Y27632, or KD025. These samples were immunostained with DAPI (blue), phalloidin (green), and BODIPY (red) (panel A, scale bar: 100 μm) and their staining intensities were plotted (panel B). The mRNA expressions of adipogenesis-related genes including *Pparγ* and *Ap2* under above conditions were plotted in panel ©. All experiments were performed in duplicate using fresh preparations, each consisting of 16 spheroids. Data are presented as arithmetic means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ (ANOVA followed by Tukey's multiple comparison test). ROCK, Rho-associated coiled-coil containing protein kinase

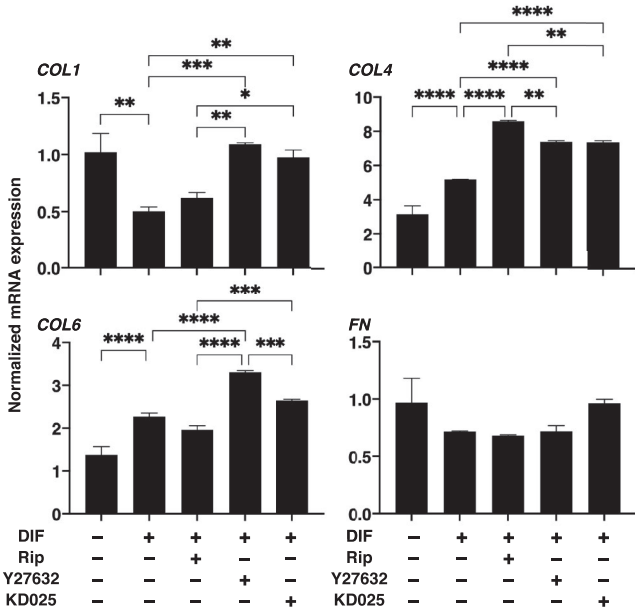

FIGURE 4 Effects of ROCK-is on mRNA expressions of mRNA expression of ECMs in 3D human orbital fibroblast (HOF) spheroids. At Day 12, the 3D HOF spheroids of preadipocytes (DIF−) and their adipogenic differentiation (DIF+) without or with 10 μM ripasudil (Rip), Y27632, or KD025 were subjected to a quantitive PCR analysis to estimate the mRNA expression of ECMs. All experiments were performed in duplicate using fresh preparations, each of which consisted of 16 spheroids. Data are presented as arithmetic means ± SEM. COL1, collagen 1; COL4, collagen 4; COL6, collagen 6; ECM, extracellular matrix; FN, fibronectin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$ (ANOVA followed by Tukey's multiple comparison test). ROCK, Rho-associated coiled-coil containing protein kinase

we demonstrated that pan-ROCK-is, Rip and Y27632, induced the formation of significantly larger sized 3D spheroids from DIF+ 3T3-L1 cells[28] or DIF-HOFs obtained from patients with Grave's orbitopathy.[45] However, a paradoxical phenomenon of physical stiffness of the 3D[28] spheroid was observed upon adipogenesis, that is, an increase (HOF)[30,46] but a decrease (3T3-L1)[32] was observed in addition to the paradoxical inhibition of the ROCK2 inhibitor, KD025 toward adipogenesis[35] were also observed in our current study. The underlying mechanisms responsible for causing these paradoxical phenomena remain unknown because of study limitations and need to be elucidated.

Our current observations indicated that ROCK-is significantly altered the physical properties as well as adipogenesis of 3D HOF spheroids. However, in a clinical situation, adipocytes are already differentiated when they are exposed to glaucoma medications, and further study on the effects of ROCK-is toward undifferentiated preadipocytes in addition to investigations related to the study limitations as above will be required.

AUTHOR CONTRIBUTIONS

Y.I. designed and performed experiments, analyzed data, and wrote the paper. H.I, M.W., and U.A. analyzed the data. H.O. analyzed the data and provided conceptual advice. F.H. designed the experiments, analyzed the data, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no conflicts of interest associated with this manuscript.

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How to cite this article: Hikage F, Ichioka H, Watanabe M, Umetsu A, Ohguro H, Ida Y. ROCK inhibitors modulate the physical properties and adipogenesis of 3D spheroids of human orbital fibroblasts in different manners. *FASEB BioAdvances*. 2021;3:866–872. https://doi.org/10.1096/fba.2021-00037