

Omidenepag, a Selective, Prostanoid EP2 Agonist, Does Not Suppress Adipogenesis in 3D Organoids of Human Orbital Fibroblasts

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Purpose: The purpose of this study was to present the effects of the prostanoid EP2 agonist, omidenepag (OMD) on human orbital fibroblasts (HOFs) using a three-dimension (3D) cell culture.

Methods: During adipogenesis of 3D HOFs organoids, changes in size, lipids staining, mRNA expression of adipogenesis related genes, *PPAR γ* , *AP2*, and *ADIPOQ*, and extracellular matrix, collagen 1 (COL 1), COL 4, COL 6, and fibronectin (FN), and stiffness by a micro-squeezer were examined in the presence and absence of either 100 nM bimatoprost acid (BIM-A) or 10, 100, or 10,000 nM OMD.

Results: The size of the 3D organoids increased dramatically during adipogenesis, and these were further enhanced in the presence of OMD in contrast to the BIM-A induced suppression effect. The intensity of lipid staining and the mRNA expression of *PPAR γ* were significantly increased upon adipogenesis, and both or latter was markedly inhibited in the presence of OMD or BIM-A, respectively. *AP2* expression was also upregulated by adipogenesis, and was further enhanced by BIM-A. The adipogenesis-induced downregulation of COL 1 and FN, or the upregulation of the expression of COL 4 and COL 6 were all suppressed in the presence of BIM-A. In contrast, OMD caused similar effects on COL 4, COL 6, or FN expression, but caused a significant increase in COL 1 expression. Stiffness was significantly increased upon adipogenesis, and was further increased or substantially decreased by BIM-A or OMD, respectively.

Conclusions: The present study indicates that the FP2 agonist, OMD, had different effects on 3D HOFs organoids, as compared to BIM-A.

Translational Relevance: The current study suggests that OMD may not induce deepening of upper eyelid sulcus (DUES).

Introduction

A selective, non-prostaglandin, prostanoid EP2 agonist prodrug, omidenepag isopropyl (OMDI), has quite recently been available in the treatment of patients with ocular hypertension (OH) and glaucoma.^{1,2} OMDI is converted into the active form, omidenepag (OMD) during its penetration into the eye, where it exerts a hypotensive effect toward elevated intraocular pressure (IOP).^{1,2} It should be noted that OMD is also categorized as a member of the prostanoid receptor family of agonists (i.e. FP

receptor agonists [FP-Rag],³ which are used clinically as first-line drugs because of their great efficacy in decreasing elevated IOPs with minimal systemic side effects.⁴⁻⁶ However, recently, periocular side effects, referred to as “prostaglandin-associated periorbitopathy (PAP)” have been recognized among non-negligible numbers of long-term users of FP-Rags.⁶⁻⁹ Among these, deepening of upper eyelid sulcus (DUES),⁶⁻¹⁰ is cosmetically the most challenging side effect. In terms of a possible molecular pathology of DUES, atrophy of the orbital adipose tissues may primarily be involved in the development of FP-Rags based upon a magnetic resonance (MR) imaging study^{10,11} as well as an in vitro

study using conventional two-dimensional (2D) tissue culture of adipose cell line.^{11,12}

Because the human orbital adipocytes spread within the three-dimensional (3D) conic space, thus, a 3D cell culture system would be desirable in terms of studying the molecular pathology of orbital fat diseases rather than a conventional 2D cell cultures. In fact, Li et al. established a novel in vitro model to evaluate the pathology of Graves' orbitopathy (GO). The model involved the use of a 3D collagen matrix using fibroblasts obtained from patients with GO and normal healthy individuals.¹² Furthermore, our group developed a 3D drop culture method without the need to use a matrix, and found that hypoxia-inducible factor 2 α (HIF2A) is mainly involved in facilitating extracellular matrix (ECM) deposition by inducing the production of lysyl oxidase (LOX) as a possible etiology of GO.¹³ Quite recently, using this 3D tissue drop culture technique, we also observed that FP-Rags significantly altered ECM expression in addition to the adipogenesis in 3T3-L1 cells¹⁴ as well as human orbital fibroblasts (HOFs).¹⁵ Alternatively, 3D culture methods have been established as a glaucoma model. Torrejon et al. found that the overexpression of ECM resulted in an impaired HTM cell phagocytic activity and an increased outflow resistance in a steroid induced glaucoma model using 3D cultures of human trabecular meshwork (HTM) cells using scaffolds.¹⁶ Similarly, a Matrigel assisted 3D culture of the HTM was used to evaluate the degree of chronic oxidative stress to the HTM.¹⁷ Therefore, these collective findings suggest that this 3D drop culture technique would be a suitable method for studies related to the clinicopathology of orbital fat diseases as well as other ocular diseases.

In order to elucidate the effects of OMD on orbital fatty tissue from a clinical perspective, examining whether OMDI could or could not cause DUES, similar to FP-Rags, would be of interest. Yamamoto et al. recently studied the effects of OMD on adipogenesis in 2D cultures using 3T3-L1 cells, and reported that OMD had no effect on adipogenesis.² The 3T3-L1 cells, a mouse preadipocyte cell line, is frequently used as a model system in adipocyte related research.^{2,11,14} Furthermore, our recent pilot study indicated that OMD induced different effects toward 3T3-L1 cells as compared to BIM-A.¹⁸ This prompted us to perform a similar study based on 3D organoid cultures using HOFs rather than 3T3-L1 cells to determine the effects of OMD on human orbital fatty tissues.

Therefore, in the current study, using a 3D drop culture, we analyzed and compared the effects of an FP-Rag, bimatoprost acid (BIM-A) and an EP receptor agonist, OMD, on adipogenesis, ECM expression, and the sizes and physical properties of the 3D HOFs organoids.

Materials and Methods

The current study at Sapporo Medical University Hospital, Japan, was approved by the institutional review board and according to the tenets of the Declaration of Helsinki and national laws for the protection of personal data. Informed consent was obtained from all participants in this study.

3D Organoid Cultures of Human Orbital Fibroblasts

Surgically obtained orbital fat specimens from 4 patients with orbital fat herniation were used for the HOFs and subsequent 3D HOFs organoid cultures and adipogenic differentiation was performed as described previously.^{14,15} To study drug efficacies, 100 nM bimatoprost acid (BIM-A, #16810; Funakoshi, Tokyo, Japan) or 10, 100, or 10,000 nM OMD (a generous gift from Santen Pharmaceutical Co., Ltd., Osaka, Japan) were added. At day 10, the 3D HOFs organoids were collected and used in several analyses, as described below.

Measurement of Sizes and Lipid Staining by BODIPY of 3D HOFs Organoid

Measurement of 3D organoid size by phase contrast bright-field images and lipid staining by BODIPY were performed, as described previously.^{14,15} Both organoid sizes and the BODIPY fluorescence intensity were quantified using the Image J software version 2.0.0 (National Institutes of Health [NIH], Bethesda, MD).

Gene Expression Analysis

The reverse transcription and subsequent quantitative polymerase chain reaction (qPCR) of total RNA extracted from 16 organoids were performed, as described previously,^{14,15} using the following primers and TaqMan probes;

human RPLP0:

Probe, 5'-/56-FAM/CCCTGTCTT/ZEN/CCCTGG GCATCAC/ 3IABkFQ/-3' Forward, 5'-TCGTCTTT AAACCCTGCGTG-3'

Reverse, 5'-TGTCTGCTCCACAATGAAAC-3'

human COL 1A1:

Probe, 5'-/56-FAM/TCCAGGGCC/ZEN/AAGAC GAAGACATC/ 3IABkFQ/-3' Forward, 5'-GACAT GTTCAGCTTTGTGGAC-3'

Reverse, 5'-TTCTGTACGCAGGTGATTGG-3'

human COL 4A1:

Probe, 5'-/56-FAM/TCATACAGA/ZEN/CTTGG CAGCGGCT/31ABkFQ/-3' Forward, 5'-AGAGAGG AGCGAGATGTTCA-3'

Reverse, 5'-TGAGTCAGGCTTCATTATGTTCT-3'

human COL 6A1:

Forward, 5'-CCTCGTGGACAAAGTCAAGT-3'

Reverse, 5'-GTGAGGCCTTGGATGATCTC-3'

human FN1:

Forward, 5'-CGTCCTAAAGACTCCATGATCTG-3'

Reverse, 5'-ACCAATCTTGTAGGACTGACC-3'

human PPAR γ

Probe, 5'-/56-FAM/CTCAT AATG/ZEN/CCATCA GGTGGGGCG/31ABkFQ/-3' Forward, 5'-GTTTC AGAAATGCCTTGCAGT-3'

Reverse, 5'-GGATTCAGCTGGTCGATATCA C-3'

Human AP2:

Probe, 5'-/56-FAM/CAGGAAAGT/ZEN/GGCTG GCATGGC/31ABkFQ/-3' Forward, 5'- ATCACATC CCCATTCACT-3'

Reverse, 5'- ACTTGCTCCAGTGAAAACCTT G-3'

Human ADIPOQ:

Forward, 5'- AGCATCCTGAGCCCTGAT-3'

Reverse, 5'- CCTCACTTCCATTCTGACTGC-3'

human PTGER2:

Probe, 5'-/56-FAM/CCGTCTGCT/ZEN/CCTTGC CTTTAC/31ABkFQ/-3'

Forward, 5'-CTCATTCTCCTGGCT ATCATGA C-3'

Reverse, 5'-CCCATTTTTCTTTTCGGGAAG-3'

Human PTGFR:

Forward, 5'- ACTCAAGTCATCTCCCTCTCA-3'

Reverse, 5'- CTCCGTCTTCTGCTCCTCA-3'

Measurement of the Micro-Indentation Force of the 3D HOFs Organoids

The micro-indentation force analysis of 3D HOFs organoids was performed as reported in a recent study.¹³ The physical stiffness of a single 3D HOFs organoid needed to produce a 50% deformity during 20 seconds is expressed as force/displacement ($\mu\text{N}/\mu\text{m}$).

Statistical Analysis

To analyze the difference among matched multiple group comparisons, 1-way ANOVA was used, followed by a Tukey's multiple comparison test. Data are presented as arithmetic means \pm SEM using GraphPad Prism version 7 (GraphPad Software, San Diego, CA).

Results

Effects of FP-Rag, BIM-A, and EP2 Agonist, OMD Toward Sizes of the 3DHOFs Organoid During 10-Days Culture

To compare effects of FP-Rag, BIM-A, and EP2 agonist on physical property of the 3D HOFs organoid, changes of their sizes were evaluated. Similar to the 3T3-L1 cells,^{14,18} uniform round-shaped 3D organoids were obtained from 20,000 HOFs cells (Fig. 1A). These 3D organoids gradually grew smaller during day 10 differentiation (DIF-) and their sizes significantly increased upon adipogenic differentiation (DIF+; see Figs. 1B, 1C). In the presence of 100 nM BIM-A, the sizes of DIF+ organoids were marked decreased, whereas the DIF induced increase in organoid sizes was further enhanced by the presence of OMD in a concentration dependent manner (see Figs. 1B, 1C). Based upon this result, further investigations in terms of OMD, as detailed below, were performed by using relatively lower concentrations at 10 or 100 nM.

Effects of FP-Rag, BIM-A, and EP2 Agonist, OMD Toward Adipogenesis of the 3DHOFs Organoid

To elucidate additional difference in the effects of FP-Rag, BIM-A, and EP2 agonist toward the 3D HOFs organoid, their adipogenesis was evaluated by lipid staining by BODIPY and the mRNA expression of adipogenesis related genes, including *PPAR γ* , *AP2*, and *ADIPOQ* were determined. As shown in Figure 2, upon adipogenic differentiation (DIF+), the intensity of BODIPY staining was significantly increased, and this increase was substantially inhibited by 100 nM OMD. The mRNA expression of *PPAR γ* or *AP2* was significantly increased upon DIF+. These DIF+ induced enhancement effects of *PPAR γ* were relatively or substantially suppressed in the presence of BIM-A or OMD, respectively, and those of *AP2* were substantially increase by BIM-A.

Effects of FP-Rag, BIM-A, and EP2 Agonist, OMD Toward Major ECM Expressions of the 3DHOFs Organoid

To elucidate underlying mechanisms causing above difference of drug effects between BIM-A and OMD as above, we compared the effects by BIM-A or OMD on the mRNA expression of ECMs, including COL

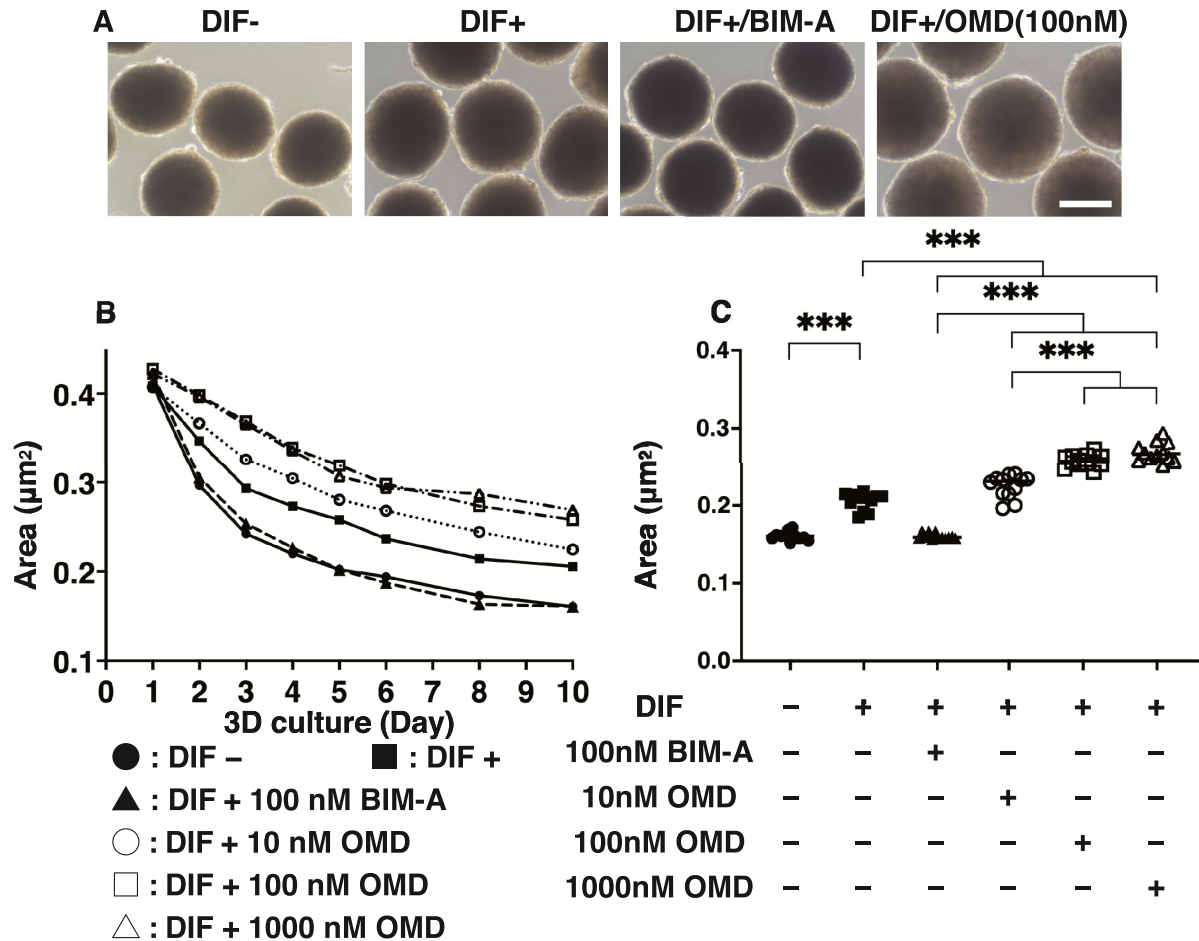


Figure 1. Effects of bimatoprost acid (BIM-A), and omidenepag (OMD) on adipogenesis in 3D cultured human orbital fibroblasts (HOFs). The mean sizes of 3D organoids of HOFs preadipocytes (DIF-, closed circles) and their adipogenic differentiation (DIF+, closed squares) in the presence and absence of 100 nM BIM-A (closed triangles), or OMD (10 nM: open circles, 100 nM: open squares, or 10,000 nM: open triangles) were measured. Representative phase contrast images of each organoid at day 10 under several conditions as above are shown in (A) (scale bar: 100 μ m). Fluctuations in organoid size during a 10-day culture were plotted (B) and those at day 10 were compared among the experimental groups (C). All experiments were performed in triplicate using fresh preparations consisting of 16 organoids each. Data are presented as arithmetic means \pm the standard error of the mean (SEM). *** $P < 0.005$ (ANOVA followed by a Tukey's multiple comparison test).

1, COL 4, COL 6, and FN (Fig. 3), and the physical properties of the 3D HOFs organoids (Fig. 4). Upon DIF+, the mRNA expression of COL 1 and FN, or COL 4 and COL 6 were downregulated or upregulated, respectively. In the presence of BIM-A, all four ECM expressions of DIF+ were significantly suppressed. Regarding OMD, similar to BIM-A, the mRNA expression of DIF+ was also suppressed in COL 4, COL 6, and FN, whereas those of COL 1 were increased. Physical properties by a micro-indentation analysis showed that DIF+ caused significant increase in the stiffness of the 3D HOFs organoids. In the presence of BIM-A, the DIF+ induced effect was markedly enhanced, whereas, in contrast, OMD induced a significant decrease of the 3D HOFs organoids stiffness.

Discussion

Prostaglandins (PGs) secreted in a paracrine or autocrine manner, couple to their specific GPCRs on target cells, where they then activate intracellular signaling. The major metabolites of cyclooxygenase (COX) enzymes, PGE2 and PGF2 α are the most abundantly biosynthesized PGs.^{9,19} It is known that both EP2 and FP receptors are often co-expressed in the same cells but are involved in different intracellular signaling pathways; EP2 receptors couple to G α s, resulting in an increase in cAMP levels, and FP receptors couple to G α q resulting in the release of inositol-1,4,5-triphosphate (IP) and diacylglycerol (DAG).^{9,20–22} Because the co-expressed EP2 and

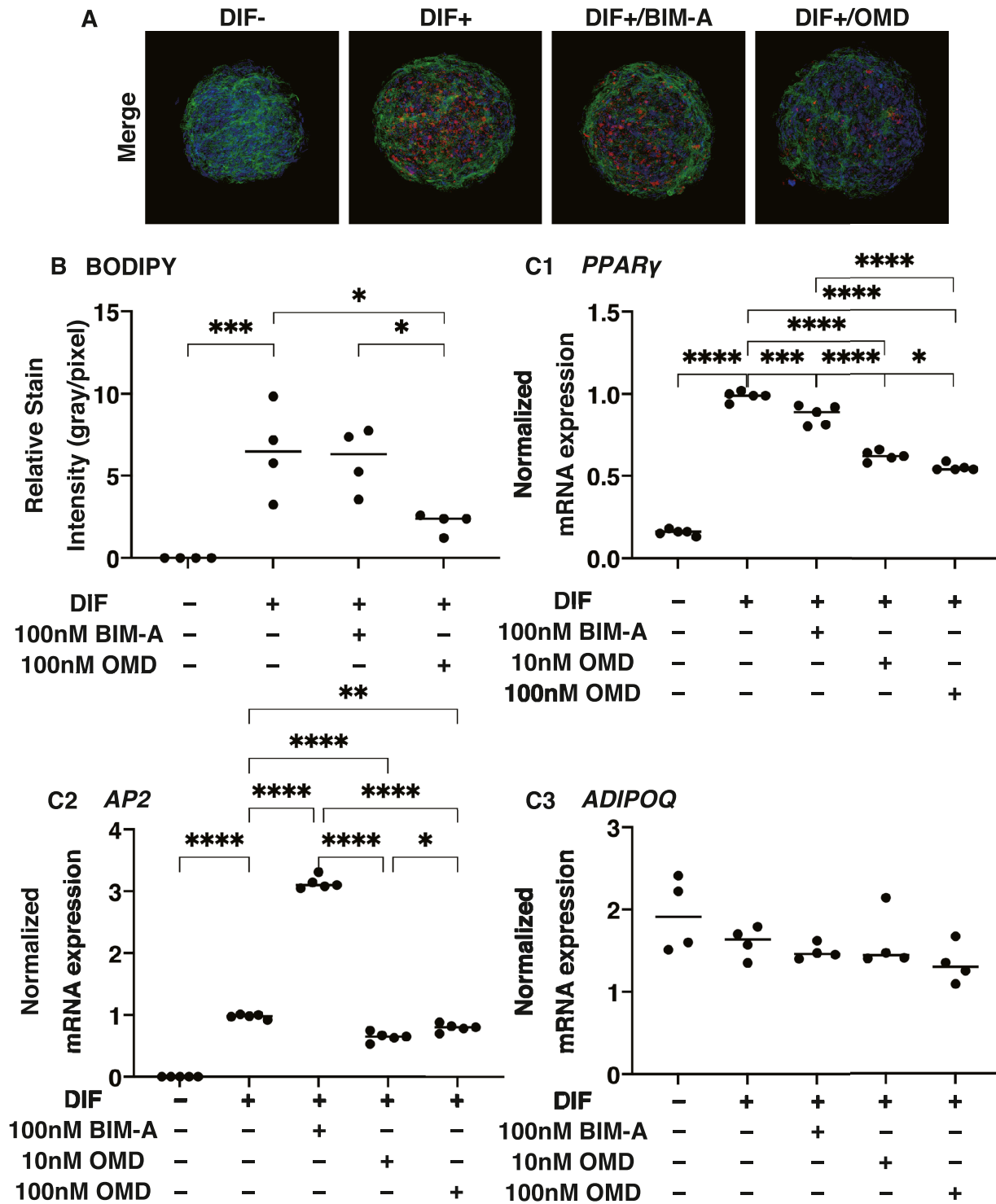


Figure 2. Representative confocal images of lipid staining (BODIPY) and mRNA expression of adipogenesis related genes, *PPAR γ* , *AP2*, and *ADIPOQ* of 3D HOFs organoids under various conditions. The 3D HOFs organoids at day 10 under various conditions; preadipocytes (DIF-) and their adipogenic differentiation (DIF+) without or with 100 nM of BIM-A or 100 nM OMD were prepared for immunostaining by BODIPY (red), DAPI (blue), and phalloidin (green) (A), and their staining intensities by BODIPY were plotted (B). For qPCR analysis to estimate the mRNA expression of adipogenesis related genes, *PPAR γ* , *AP2*, and *ADIPOQ*, 10 nM OMD was also included in addition to the above conditions (C 1-3). All experiments were performed in duplicate using fresh preparations consisting of 10 organoids each for immunostaining or 16 organoids each for qPCR analysis. Data are presented as the arithmetic mean \pm the standard error of the mean (SEM). * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$ (ANOVA followed by a Tukey's multiple comparison test).

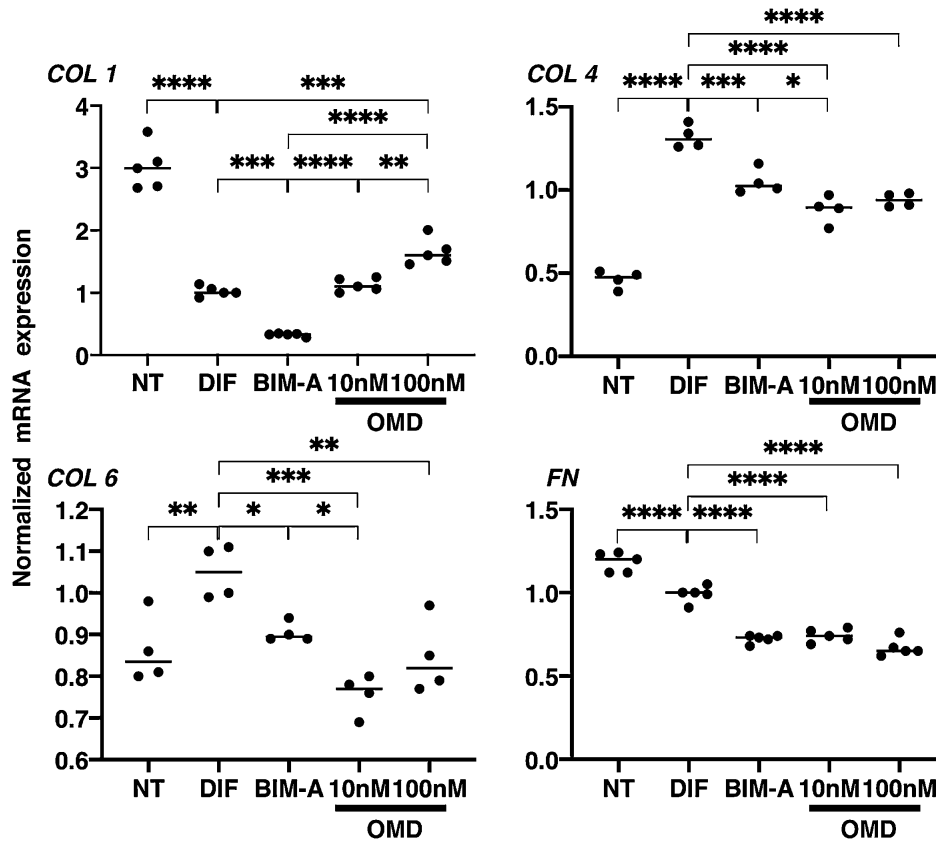


Figure 3. The mRNA expression of ECM in 3D HOFs organoids. At day 10, 3D HOFs organoids of preadipocytes (DIF-) and their adipogenic differentiation (DIF+) without or with 100 nM BIM-A, or 10, or 100 nM OMD were subjected to qPCR analysis to estimate the mRNA expression of ECM (COL 1: collagen 1, COL 4: collagen 4, COL 6: collagen 6, and FN: fibronectin). All experiments were performed in duplicate using fresh preparations consisting of 16 organoids each. Data are presented as arithmetic means \pm the standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ (ANOVA followed by a Tukey's multiple comparison test).

PGF2 α are also recognized by 3T3-L1 preadipocytes, we recently compared effects of OMD on 2D and 3D cultures of 3T3-L1 cells with those of PGF2 α .¹⁸ Although both drugs inhibited adipogenesis in 2D and 3D cultures of 3T3-L1 cells, they caused quite different effects in the physical properties of the 3D 3T3-L1 organoid, that is, OMD caused an increase in size and a decreased stiffness, whereas PGF2 α induced the formation of smaller and stiffer 3D 3T3-L1 organoids. Based upon these results, we speculated that OMD may not induce PGF2 α related orbital fat atrophy, which is referred to as DUES.¹⁸ However, because the physiological properties of human orbital fatty tissues are different from those of 3T3-L1 cells,²³ similar experiments using HOFs instead for 3T3-L1 cells would be needed to elucidate the effects of OMD on human orbital fatty tissues. In the current study using 3D HOFs organoid cultures, we found the following results: (1) both EP2 and PGF2 α receptors were co-expressed in HOFs, and those expressions were downregulated by OMD and BIM-A, respectively (Supplementary Fig. S1); (2) the size of the 3D

HOFs organoids increased remarkably during DIF+, and the sizes were further enhanced in the presence of OMD, whereas BIM-A induced a significant suppression; (3) staining intensities by BODIPY and the mRNA expression of the *PPAR* γ were significantly increased upon DIF+, and these were markedly or slightly inhibited in the presence of OMD or BIM-A, respectively; (4) DIF+ induced the downregulation of COL 1 and FN, or the upregulation of COL 4 and COL 6 expressions, and these effects were all suppressed in the presence of BIM-A, although OMD caused similar effects on COL 4, COL 6, or FN expression, but significantly increased COL 1 expression; and (4) physical stiffness analyses showed that BIM-A significantly increased and OMD decreased the stiffness of the DIF+ induced 3D HOFs organoids. As shown in Supplementary Figure S1, because the expression of EP2 receptors of the 3D HOFs organoid was significantly enhanced by DIF+, and were marked inhibited in the presence of OMD, but not by BIM-A, we conclude that the observed OMD induced effects are exclusively related to the EP2 receptors expressed in

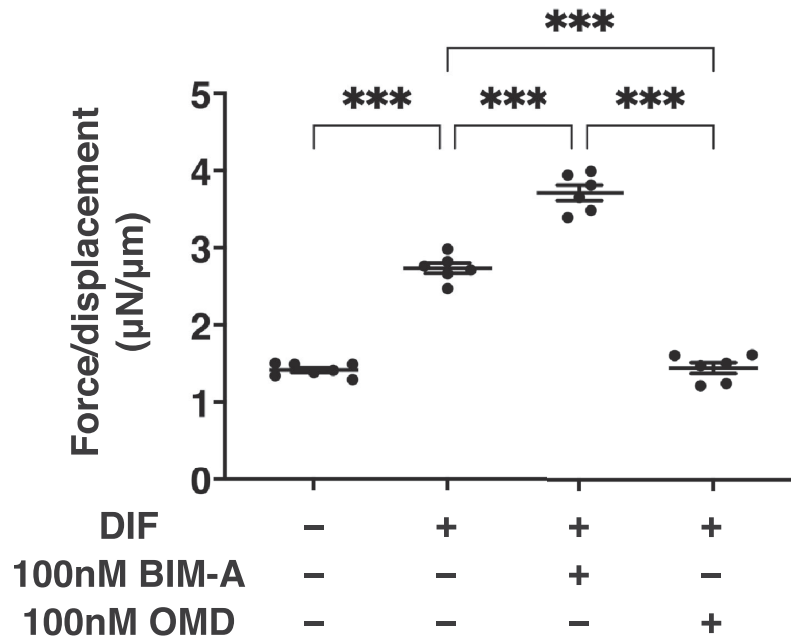


Figure 4. Physical solidity of 3D HOFs organoids. Among the experimental groups under several conditions; preadipocytes of the HOFs (DIF-) and their adipogenic differentiation (DIF+) in the absence and presence of 100 nM BIM-A, or 100 nM OMD, physical solidity of the 3D organoids at day 10 were measured by means of a micro-squeezer ($\mu\text{N}/\mu\text{m}$ force/displacement). All experiments were performed in duplicate using fresh preparations consisted of 12 to 19 organoids. Data are presented as arithmetic means \pm the standard error of the mean (SEM). ns (not significant), *** $P < 0.005$ (ANOVA followed by a Tukey's multiple comparison test).

the HOFs 3D organoids. Taken together, we conclude that the EP2 agonist, OMD, had different effects on 3D HOFs organoids compared to BIM-A, similar to the 3D 3T3-L1 organoid.¹⁸

Consistent with our current observations, a number of studies have previously demonstrated that PGE2 and PGF2 α , both inhibit the development of adipocytes.^{24,25} However, such inhibitory efficacies of the 3D HOFs organoids by OMD and BIM-A were quite different as compared to the 3D 3T3-L1 organoids.¹⁸ That is, the inhibitory efficacies of the 3D HOFs organoids by OMD toward BODIPY staining were higher and *PPAR* γ expression was lower than those of 3D 3T3-L1 organoids, respectively. These difference may be ascribed to the difference in the characteristics of HOFs and 3T3-L1 cells, as previously suggested.²³ In fact, in the present study, the effects of OMD on the physical properties of the 3D organoids, including sizes (see Fig. 1) and stiffness (see Fig. 4), were similar but more evident in HOFs than in 3T3-L1 cells.¹⁸ In addition, in the stiffness analysis, the DIF+ induced effects were also completely different, that is, a significant increase in 3D HOFs organoid (see Fig. 4) vs a significant decrease in 3D 3T3-L1 organoid.¹⁸ These collective findings confirm that that the regulatory mechanisms for adipogenesis and ECM expression were different between HOFs and 3T3-L1

cells, as suggested by previous observation.²³ Thus, this indicates the rationale for the current study for using an appropriate source of materials, HOFs instead 3T3-L1 cell for evaluating the effects of drugs human orbital fatty tissues.

Because ECM provides essential spatial structural support among cells and organs, 3D culture methods, rather than 2D cultures, would be more desirable for such studies in terms of their ECM properties.²⁶ In fact, for this purpose, several studies using 3D organoid culture methods have been developed as suitable ex vivo diseases models so far.¹⁷ In the current study, by using a unique 3D drop organoid culture, we examined the effects of OMD on the expression of these COL 1, COL 4, and COL 6, and FN molecules, which all play pivotal roles during adipogenesis, and found that the downregulation of COL 1 that was induced by adipogenesis was significantly inhibited in the presence of OMD in spite of the further enhancement effects caused by BIM-A. This difference in COL 1 expression between OMD and BIM-A appeared to induce substantial differences in the physical properties of organoids, as evidenced by micro-squeezer measurements. This method for measuring the physical stiffness of a single living 3D organoid is the only currently available method that is available for use in our 3D drop culture system.¹³⁻¹⁵ The issue of why OMD

induces the upregulation of COL 1 expression and the downregulation of adipogenesis, and how large and stiff are the 3D HOFs organoids that are produced currently remain unknown. Because COL 1 is known to provide the framework necessary to sustain the 3D organoid structure,²⁷ the OMD induced upregulation of COL 1 may be attributed to the formation of a larger 3D organoid. If this is correct, we reasonably speculate that, taken together with the OMD induced downregulation of adipogenesis, lower amounts of lipids relative to a larger 3D organoid framework may induce the formation of such large and less stiff 3D HOFs organoids. Alternatively, the possibility that the upregulation of COL 1 and the downregulation of adipogenesis increases the risk of fibrosis in orbital fat cannot be excluded. However, because ECM contributes not only cell support but also the a number of other cellular functions that are critically regulated by several factors, including matrix metalloproteinase (MMP), tissue inhibitors of metalloprotease (TIMP), PGs, and others,^{28,29} to investigate such a possibility, it will be necessary to study additional factors, including MMP, TIMP, and others, using some animal models in addition to the current 3D organoid model. Furthermore, as another limitation to the current study, the effects of antiglaucoma drugs, including OMD, PGF2 α , and others, toward HOFs themselves, that is, without adipogenic differentiation, also represent an important issue that needs to be elucidated. Therefore, further investigations will be required for our next project.

Acknowledgments

Author contributions: F.H. designed and performed the experiments, analyzed the data, and wrote the paper. Y.I. performed the experiments and analyzed the data. Y.O. performed the experiments. M.W. performed the experiments. H.O. analyzed the data and provided conceptual advice.

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