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Three-dimensional culture of sebaceous gland cells revealing the role of prostaglandin E_2-induced activation of canonical Wnt signaling

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ABSTRACT

Background: Prostaglandin E_2 (PGE_2) is a proinflammatory mediator and activates the canonical Wnt–β-catenin signaling pathway in hematopoietic stem cells. The SZ95 cell line was established from human sebaceous gland cells and is studied as a model system for these cells. Given that 2D culture of SZ95 cells does not recapitulate the organization of sebaceous glands in situ, we developed a 3D culture system for these cells and examined the effects of PGE_2 on cell morphology and function.
Results: SZ95 cells maintained in 3D culture formed organoids that mimicked the organization of sebaceous glands in situ, including the establishment of a basement membrane. Organoids exposed to PGE_2 were larger and adopted a more complex organization compared with control organoids. PGE_2 activated the canonical Wnt signaling pathway as well as increased cell viability and proliferation, mitochondrial metabolism, and lipid synthesis in the organoids.
Conclusions: Culture of SZ95 cells in 3D culture system recapitulates the structure and susceptibility to PGE_2 of sebaceous glands in situ and should prove useful for studies of the response of these glands to inflammation and other environmental stressors. Our results also implicate PGE_2-induced activation of canonical Wnt signaling pathway in regulation of the morphology,proliferation, and function of "semi-vivo" sebaceous glands.

1. Introduction

Sebaceous glands are present in hair-covered areas of human skin, where they are connected to hair follicles. Hair follicles contain a region known as the bulge area, which is the tissue stem cell niche. Transit-amplifying cells are produced from the stem cells located in the niche and maintain homeostasis of the sebaceous gland by giving rise to its differentiated cells [1]. All human sebaceous glands possess a similar structure and secrete sebum in a holocrine manner. Sebum contains triglycerides, wax esters, and squalene, all of which are products of lipid metabolism.

SZ95 cells are normal human sebaceous gland cells that have been immortalized by expression of the large T antigen of SV40 [2]. These cells form a gland-like structure with a lumen in 2D culture [2–4]. Sebaceous glands in situ, however, are composed of a heterogeneous cell population that includes clear cytoplasm-bearing differentiated sebocytes and surrounding stem-like basal cells. This heterogeneous structure is thought to be important for homeostasis of sebaceous glands in the skin.

The proinflammatory mediator prostaglandin E_2 (PGE_2) activates the canonical Wnt signaling pathway through interaction with its EP receptors and consequent activation of protein kinase A (PKA) and inhibition of the GSK3β-Axin-CK1-APC axis, the latter of which is responsible for phosphorylation of cytoplasmic β-catenin and its consequent degradation by the ubiquitin–proteasome system [5–7]. The stabilization and nuclear translocation of β-catenin induced by activation of Wnt–β-catenin signaling are followed by its association with members of the TCF/LEF family of transcription factors and regulation of target gene expression [5–7]. Although regulation of this signaling pathway by PGE_2 has been demonstrated in hematopoietic stem cells [8], it remains unclear whether PGE_2 exerts a similar action in epithelial cells of the skin during inflammation.

CD44 is an adhesion molecule whose expression is regulated in large part by the Wnt–β-catenin signaling pathway [9]. Various ligands including osteopontin and hyaluronic acid have been found to bind to CD44 [10]. CD44 exists in various isoforms as a result of alternative splicing of its mRNA, with these isoforms being broadly

Abbreviations: PGE_2, prostaglandin E_2; PKA, protein kinase A; EGF, epidermal growth factor; TCA, tricarboxylic acid cycle; ROS, reactive oxygen species; PPARγ, peroxisome proliferator-activated receptor γ; PGC1α, PPARγ coactivator 1α; SREBP1, sterol response element-binding protein 1; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix.

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classified as standard (CD44s) or variant (CD44v) on the basis of the exclusion or inclusion of variant exons. CD44v is not only an epithelial marker but also a stem cell marker and thus also identifies cells in an undifferentiated state. The epithelial-mesenchymal transition is associated with down-regulation of CD44v expression and up-regulation of CD44s [11]. CD44v was recently shown to protect cells from oxidative stress by promoting the uptake of cysteine and thereby increasing the synthesis of glutathione [12].Thus, in terms of protection from oxidative stress, CD44v8-10 is recognized as a stem cell marker in epithelial tumors, but the function in normal cells is still elusive.

Inflammatory skin disorders often manifest hyper-proliferation of sebaceous glands. Little is known of the biological response of sebaceous glands to the inflammatory microenvironment, however. We hypothesized that PGE_2-induced activation of canonical Wnt signal might contribute to the hyper-proliferative response of sebaceous glands in the skin. To mimic the inflammatory microenvironment of sebaceous glands in situ, we established a 3D culture system for SZ95 cells that promotes the formation of sebaceous gland organoids.

2. Materials and methods

2.1. Cell culture

The immortalized human sebaceous gland cell line SZ95 (kindly provided by C. C. Zouboulis, University of Münster, Germany) was cultured in 2D under at atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum. For 3D culture of the cells, 50–100 μl of Matrigel (BD Technology) was added to glass-bottom dishes with a diameter of 1 cm and was allowed to set during incubation for 20 min on ice. SZ95 cells in 400 μl of DMEM supplemented with recombinant human EGF (5 ng/ml) (SUPPLIER) and 2% Matrigel were then added to the Matrigel layer. The overlay medium was replaced twice a week during culture of the cells for 2 weeks. Where indicated, the cells were also exposed to PGE_2 (Tokyo Chemical Industry) during 3D culture for the latter 7 days.

2.2. Immunoblot analysis

Cells in 2D culture were washed with phosphate-buffered saline (PBS) and lysed directly in 2× SDS sample buffer supplemented with phosphatase inhibitors (PhosSTOP, Roche) and proteinase inhibitors (Complete Mini EDTA-Free, Roche). ECM in 3D culture was lysed in Cell Recovery Solution (BD Technology) and cells were recovered by centrifuge (400× G for 5 min) and washed with PBS. The lysates were subjected to ultrasonic treatment, heated at 95 °C for 5 min, and assayed for protein concentration with the use of a Pierce BCA protein assay kit (Thermo Scientific) before electrophoresis. All samples were fractionated by SDS–polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated for 15 min at room temperature with PBS containing 0.5% Tween 20% and 3% dried skim milk before exposure to primary antibodies. Antibodies included those to CD44 (1:200 dilution, BD550392, BD Technology), to β-catenin (1:500, sc7199, Santa Cruz Biotechnology), to β-actin (1:1000, sc-47778, Santa Cruz Biotechnology), to Axin-2 (1:1000, ab32197, Abcam), to SREBP1 (1:200, sc17755, Santa Cruz Biotechnology), to lipin-1 (1:500, #5195S, Cell Signaling Technology), to PPARγ (1:500, #2435S, Cell Signaling Technology), to PGC1α (1:500, sc-13067, Santa Cruz Biotechnology). Immune complexes were detected with alkaline phosphatase– or horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) at a dilution of 1:5000 and with appropriate chemiluminescence substrates (nacalai tesque). Bands were detected with an LAS3000 imaging system (Fujifilm).

2.3. Fluorescent immunohistochemical staining

The slides (SKD2421, Biomax) were depleted of paraffin by exposure to xylene for 15 min, rehydrated with a graded series of ethanol solutions (100–70%), and then heated in a microwave oven at 750 W for 10 min in citric acid buffer (0.01 M, pH 6.0) for antigen retrieval. They were then washed with PBS for 10 min before exposure for 5 min to PBS containing 0.1% Triton in order to increase the permeability efficiency of the antibody. After an additional wash with PBS for 5 min, the slides were immersed in blocking buffer (see above) for 1 h at room temperature before incubation overnight at 4 °C with primary antibodies; mixture of, with specific antibody to mouse-specific CD44v8-10 (1:100) [12], and, to β-catenin (1:100, sc7199, Santa Cruz Biotechnology). Immune complexes were detected with the use of a fluorescent probe-conjugated secondary antibodies; Alexa-Fluor 594 donkey anti-rat IgG (1:200, A-21209, Invitrogen) and Alexa-Fluor 488 goat anti-rabbit IgG (1:200, A-11034, Invitrogen). Nuclei were stained with mounting medium containing DAPI (H-1500, VECTOR). The slides were observed with a fluorescence microscope (BZ-9000, Keyence).

2.4. Flow cytometry

Cells ($0.5–1 \times 10^6$) in 2D culture were harvested with the use of Enzyme-Free-Cell-Dissociation Buffer (Life Technologies) and centrifugation, resuspended in 100 μl of PBS, and incubated in a final volume of 100 μl for 15 min on ice with allophycocyanin-conjugated antibodies to human-specific CD44v8-10 [12], human CD326 (EpCAM)-FITC (130-080-301 MACS), rat IgG2-APC conjugate isotype control (BD Technology), mouse IgG1-FITC conjugate isotype control (Thermo Scientific) at a final dilution of 1:200. As a negative control, cells were incubated with an isotype control antibody (BioLegend). The cells were then washed three times with PBS and stained with propidium iodide before analysis with a FACS Calibur flow cytometer (BD Technology). Data were analyzed with the use of FlowJo software (Digital Biology).

3. Results and discussion

3.1. Neither 2D culture nor xenograft model has limitations to study the biology of sebaceous glands

Flow cytometry analysis after double-staining with antibodies to CD326 (EpCAM), universal marker for epithelial cells, as well as to CD44v8-10 showed that expression pattern between EpCAM and CD44v8-10 was parallel with each other. Double-positive population (62%) and double-negative population (31%) consist mostly of SZ95 cells (Fig. 1A). This result strongly suggests that CD44v8-10 is useful to detect epithelial characteristics in sebaceous gland cells.

We examined localization of CD44v and β-catenin in a normal sebaceous gland. Sebaceous glands are composed of clear cytoplasm-bearing sebocytes that are rich in lipid droplets as well as surrounding basal cells that manifest a high level of CD44v and β-catenin expression (Fig. 1B). Thus, differentiated sebocytes and the surrounding basal cells are distinguished on the basis of differences both in the level of CD44v and β-catenin expression and in cellular morphology. The stem-like basal cells surrounding sebaceous glands are expected to provide enough number of transit

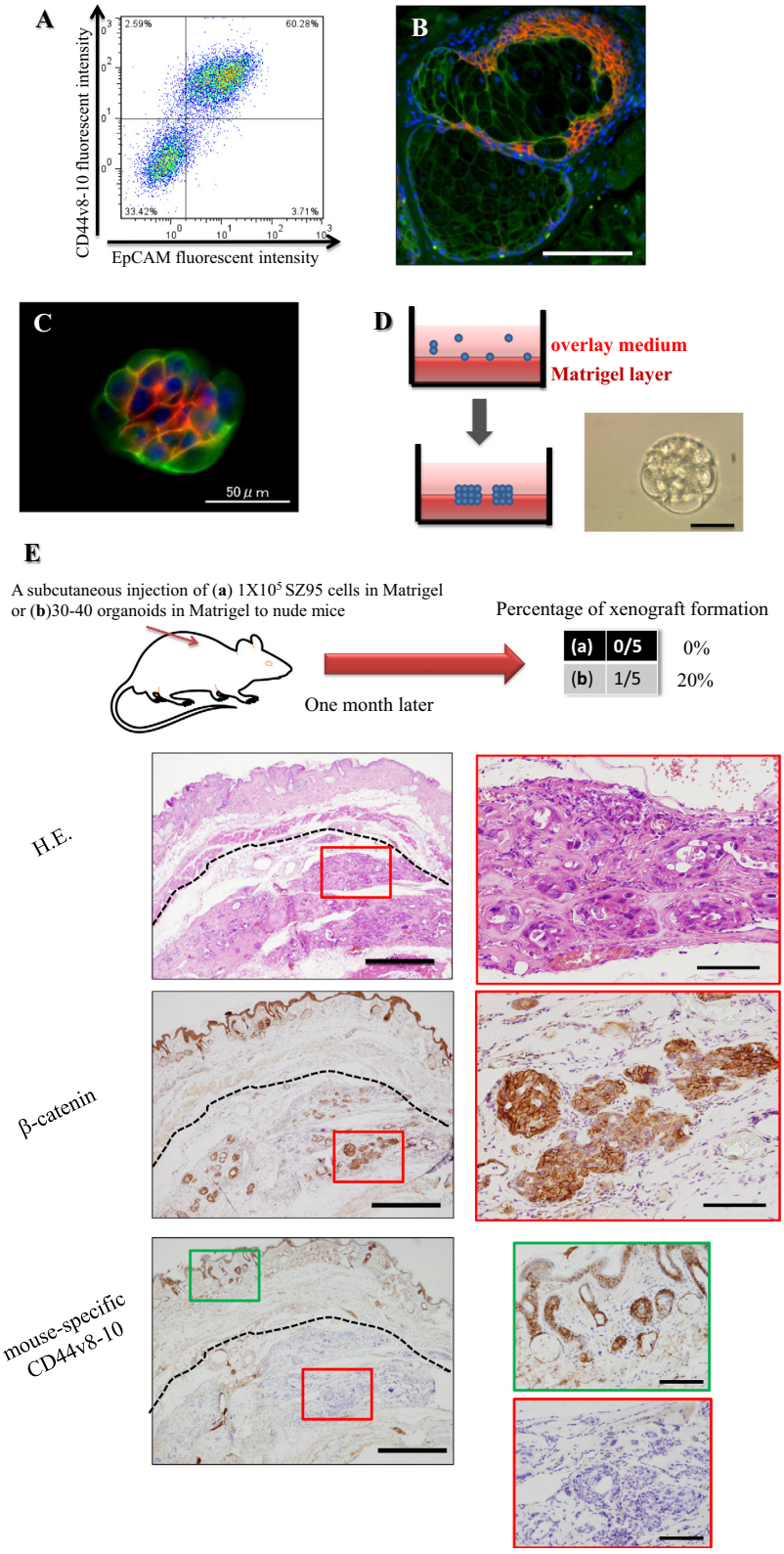

Fig. 1. Limitations of xenograft model approach to study the sebaceous gland. (A) Flow cytometry analysis after double-staining with antibodies to CD326 (EpCAM) and to CD44v8-10. Single-positive populations were nearly absent. (B) Specimen of normal sebaceous gland in the skin tissue array was subjected to fluorescent immunohistochemical analysis with antibodies to CD44v8-10 (red) and to β-catenin (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar, 100 μm. (C) Immunofluorescence analysis of CD44v8-10 (red) and β-catenin (green) in a colony derived from a single SZ95 cell in 2D culture. Scale bar, 50 μm. (D) Experimental protocol for 3D culture of SZ95 cells. The cells are seeded on a layer of Matrigel and maintained in 400 μl of DMEM supplemented with EGF (5 ng/ml) and 2% Matrigel, with this overlay medium being changed every 3–4 days over 2 weeks. Organoids were subjected to confocal microscopy and z-stack images were assembled. Phase-contrast microscopic picture of the organoid was shown. Scale bar, 100 μm. (E) A subcutaneous injection of (a) 1×10^5 SZ95 cells in Matrigel or (b) 30–40 organoids in Matrigel to nude mice were performed. One month later, there was palpable xenograft only 1 out 5 nude mice in the protocol (b). Black dotted line showed the borderline between xenograft and dermal muscle layer of nude mouse. H.E. staining was shown (upper panels). Immunohistochemical analysis with antibodies to β-catenin (middle panels), to mouse-specific CD44v8-10 (lower panels). Scale bar, 500 μm (left), 100 μm (right).

amplifying cells to hyper-proliferate sebaceous glands in response to inflammation.

To examine the localization of β-catenin and CD44v in this cell line, we seeded a single cell in a glass-bottom dish and subjected the resulting single cell-derived colony to immunofluorescence analysis. Whereas both CD44v and β-catenin were detected at cell–cell attachment, the localization of the two proteins differed. CD44v8-10 was thus detected mostly at the internal surfaces of cells in the colony, whereas β-catenin was expressed predominantly at the external surfaces (Fig. 1C). This finding suggested that 2D culture does not recapitulate the localization patterns of these two proteins observed in sebaceous glands in situ (Fig. 1A).

This fact strongly suggests that the biology of sebaceous glands should be evaluated in other way than 2D culture system. Accordingly, we attempted 3D culture of SZ95 cells to establish organoids that mimic the organization and heterogeneous cell population of sebaceous glands. Culture of the cells on a layer of Matrigel in medium containing epidermal growth factor (EGF) at 5 ng/ml and Matrigel at 2% for 2 weeks indeed resulted in the formation of organoids (Fig. 1D). Matrigel is derived from extracellular matrix of mouse sarcoma cells [13] and consists largely (~90%) of laminin, the major component of basement membrane [14,15]. In the case of PGE_2 treatment, organoids were exposed to the cytokine for latter half of 2 weeks (PGE_2 100 ng/ml for 7 days).

Subcutaneous injection of 2D-cultured SZ95 cells or 3D-cultured SZ95 organoids was performed However, All of the nude mice subcutaneously transplanted 1×10^5 SZ95 cells failed to form xenograft after one month, while a small xenograft was formed from 30 to 40 organoids inoculated in only one mouse. (Fig. 1E). Immunohistochemistry of the xenograft with an accumulation of β-catenin in the nuclei revealed the activation of canonical Wnt signal (Fig. 1E). There was not clear formation of basal membrane to maintain cellular polarity (Fig. 1E). None of the SZ95 cells in xenograft were positive for mouse-specific CD44v8-10, which confirmed that the origin of xenoraft was not the endogenous inflammation of nude mouse, but SZ95 cells-derived organoids (Fig. 1E).

In this experimental protocol, however, we could not stably analyze sebaceous glands nor mimic persistent inflammation in situ. That was why we determined to change layer medium containing inflammatory cytokine PGE_2 in 3D culture system. Besides, replacement of animal experiments with 3D culture system is expected to meet the trend for 3R agenda (refinement, reduction, and replacement) [16].

3.2. Sebaceous glands in "semi-vivo" and in vivo resemble to each other

Phase-contrast microscopy revealed that the cells within each organoid had differentiated into mature sebocytes containing lipid droplets, and that each organoid was surrounded by a basement membrane–like structure (Fig. 2A). The formation of basement membrane majorly composed of laminin was expected to be specific to 3D-cultured, but not in 2D-cultured SZ95 cells. Laminins are trimeric proteins that contain an α-chain, a β-chain, and a γ-chain, and interact with integrin on the cellular membrane. Which an α-chain consists of laminin depends on each tissue or organ; laminin-332, also referred to as laminin-5 is composed of $\alpha3\beta3\gamma^2$, secreted and distributed in the skin [17]. Quantitative RT-PCR revealed that mRNA expression of laminin-γ^2 was much higher in 3D cultured SZ95 cells than 2D-cultured SZ95 cells (Fig. 2B).Condition of 3D culture promotes the expression of laminin-γ^2, leading to the formation of basement membrane.

We compared protein expression pattern between 2D-cultured and 3D-cultured SZ95 cells by western blotting analysis. Up-regulation of β–catenin was significantly observed, indicating increased activity of canonical Wnt signal (Fig. 2C). Both CD44s and CD44v were expressed higher in 3D-cultured SZ95 cells than in 2D-cultured SZ95 cells. PGC1α and PPARγ the molecules involved in mitochondrial biosynthesis [18,19] and oxidative phosphorylation metabolism [20,21], respectively, were up-regulated (Fig. 2C). Given that CD44 is a downstream target molecules of β-catenin [22], 3D-cultued SZ95 cells showed the higher Wnt signal activity than 2D-cultured cells. In terms of lipid synthesis, both SREBP1 and lipin1 were up-regulated in 3D-cultured SZ95 cells compared with 2D-cultured SZ95 cells (Fig. 2C).

Of note, CD44v was significantly up-regulated probably due to the influence of 3D culture microenvironment on the alternative splicing machinery of CD44. The expression change in epithelial splicing regulatory protein1/2 (ESRP1/2) was not detected (data not shown). Therefore, some other epigenetic regulation might contribute to CD44v up-regulation in 3D-culture system. However, we could not find out what kind of epigenetic regulation was responsible for the increased expression of CD44v in 3D cultured-organoids.

3.3. PGE_2 promotes the growth of "semi-vivo" sebaceous glands

Confocal immunofluorescence analysis revealed that the basement membrane–like structure surrounding each SZ95 cell organoid contained laminin, and that cells in the outer layer of the organoid expressed CD44v8-10 at a much higher level than those located in the interior of the organoid (Fig. 3A).

We examined the effects of PGE_2 on organoids derived from SZ95 cells in 3D culture. The cells were cultured for 7 days without PGE_2, after which the overlay medium was replaced with one containing PGE_2 (100 ng/ml). Culture of the cells in the presence of PGE_2 (100 ng/ml for 7 days) resulted in the formation of organoids with a more complex architecture, which we referred to as a "gland-in-gland structure" and might be indicative of a PGE_2-induced hyper-proliferative response (Fig. 3B). The number of protrusions at the organoid surface was increased by exposure to PGE_2, and the cells became more heterogeneous with regard to the level of CD44v8-10 expression. Balloon-shaped cells with a high level of CD44v8-10 expression were found to protrude the organoid surface in bleb-like structures. Invasion of cancer cells into surrounding tissue is associated with high levels of CD44 and laminin expression [23]. Although SZ95 cells are derived from normal (not malignant) tissue, the pattern of SZ95 cell growth and protrusion was reminiscent of that observed at the invasive front of tumor tissue in the presence of PGE_2.

The expression of CD44v in cells at the surface of SZ95 organoids might thus reflect a proliferative and undifferentiated state of these cells, similar to that of basal cells in sebaceous glands, and expression of CD44v might protect both the organoids and sebaceous glands from environmental stressors. Again as in sebaceous glands, the cells generated at the perimeter of the SZ95 organoids may subsequently move into the interior of these structures and acquire differentiated characteristics such as the potential to produce lipid.

3.4. PGE_2 activates canonical Wnt signal as well as mitochondrial metabolism, and lipid synthesis in 3D-cultured organoids

After culture of the cells for 7 days in the presence of PGE_2, we examined the size of the organoids. PGE_2 induced a marked (~10fold) and significant increase in the superficial area of organoids. Organoids smaller than 1000 μm^2 were excluded because it was difficult to distinguish between SZ95 cell-aggregates and organoids smaller than 1000 μm^2 (Fig. 4A). Previous studies have examined the concentration of PGE_2 in vivo [24–26]. For instance, human non–small cell lung cancer cells are thought to be exposed

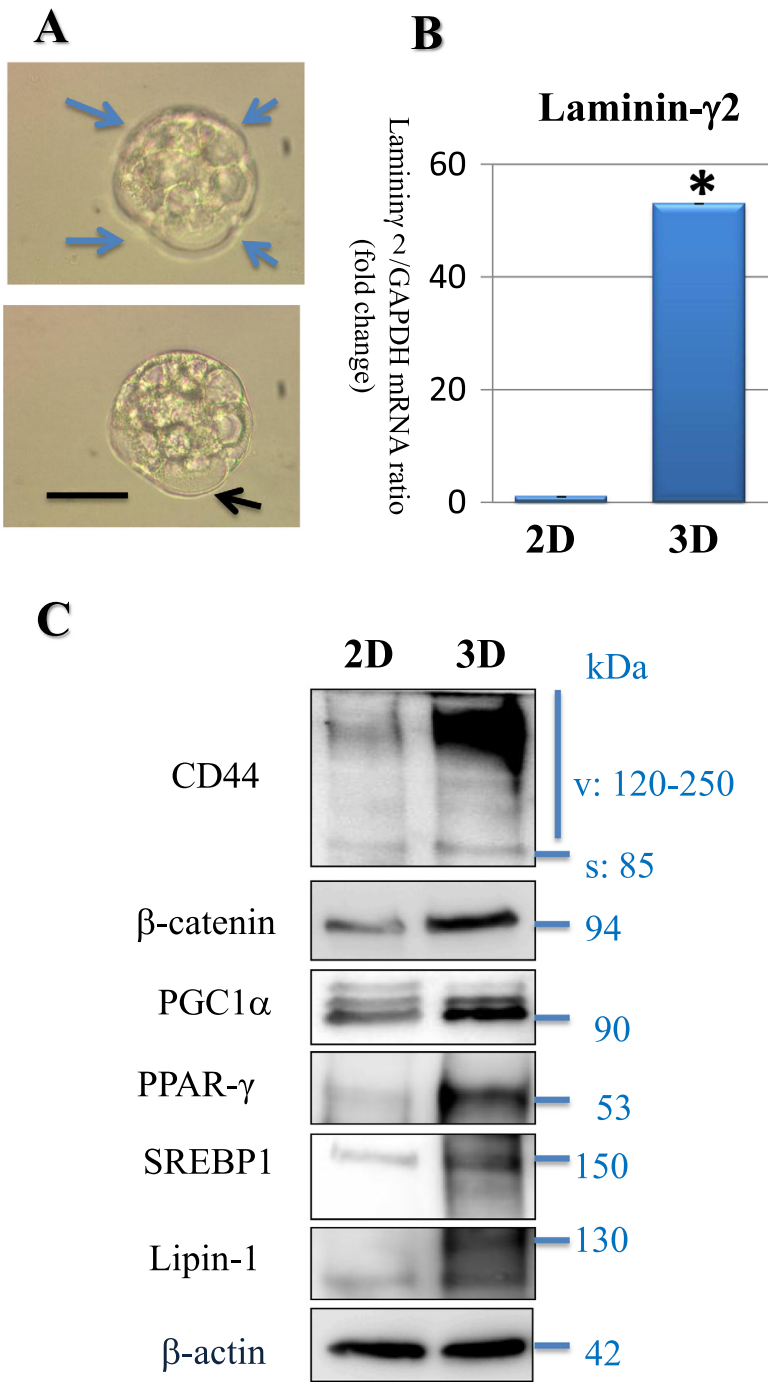

Fig. 2. The similarity between organoids in 3D culture of SZ95 cells and sebaceous glands in vivo. (A) Phase-contrast microscopy of organoids formed by SZ95 cells after 14 days in 3D culture. The black arrow indicates a lipid droplet, and blue arrows indicate a basement membrane–like structure. Scale bar, 100 μm. (B) Quantitative RT-PCR analysis of laminin-γ^2 expression of 2D- and 3D-cultured SZ95 cells (fold change). *$P < 0.001$.The P value was determined by Student's t test. (C) The comparison of gene expression patterns between 2D- and 3D-cultured SZ95 cells. Molecules involved of β-catenin/Wnt signal, mitochondrial metabolism, and lipid synthesis were analyzed.

to PGE_2 at ~2 ng/ml, a concentration about twice that to which normal lung cells are exposed [24]. In addition, synovial fibroblasts in rheumatoid arthritis produce PGE_2 that achieves a concentration of 2–10 ng/ml in the extracellular fluid depending on their exposure to adiponectin [26]. As far as we are aware, however, the PGE_2 concentration associated with the increase in the number of sebaceous glands in inflammatory skin conditions has not been examined. Results obtained with our 3D culture system now suggest that the concentration of PGE_2 in the inflammatory microenvironment is on the order of 100 ng/ml, if we assume that PGE_2 is alone responsible for the proliferation of sebaceous glands associated with inflammation in situ. Canonical Wnt signaling pathways promote cell survival and proliferation [27,28], and their activation by PGE_2 likely contributes to the proliferation of sebocytes associated with inflammation.

Susceptibility of 3D-cultured SZ95 cells to PGE_2 became resembled to in vivo. Indeed, treatment of SZ95 cells in 3D-cultured system with low concentration of PGE_2 (100 ng/ml) promoted the growth and proliferation of organoids, which mimicked in vivo response to PGE_2 (Fig. 4A).

We performed quantitative RT-PCR to detect the transcriptional change in CD44, Axin-2, PGC1α, and PPAR-γ all of which were sig-

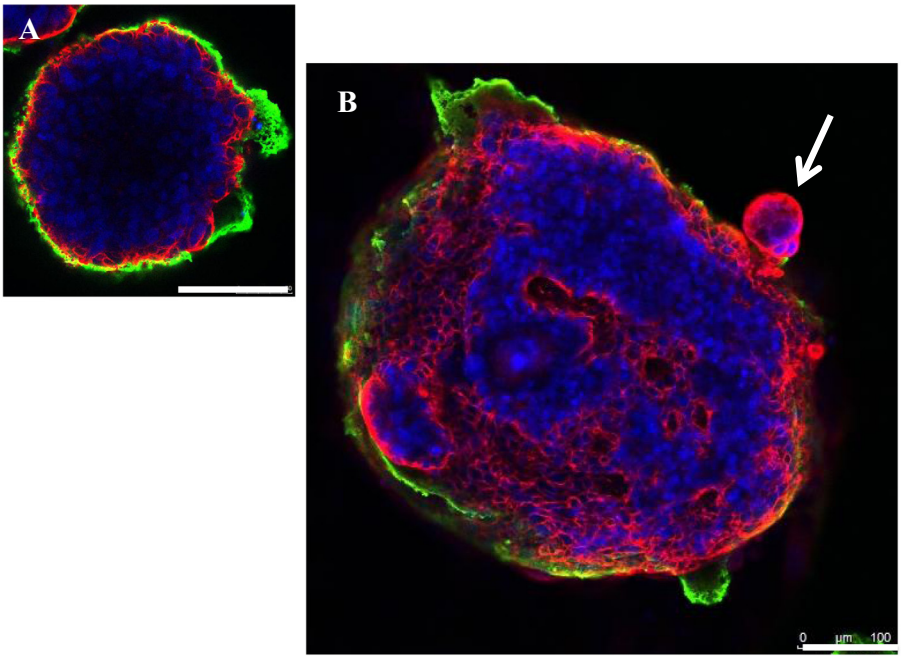

Fig. 3. Influence of PGE_2 on the growth of organoids in 3D culture of SZ95. Immunofluorescence staining of CD44v8-10 (red) and laminin (green) in organoids formed by SZ95 cells in the absence (A) or presence (B) of PGE_2 (100 ng/ml for 7 days). Nuclei were stained with Hoechst 33342 (blue). The arrow in (B) indicates a growing bleb. Scale bars, 100 μm.

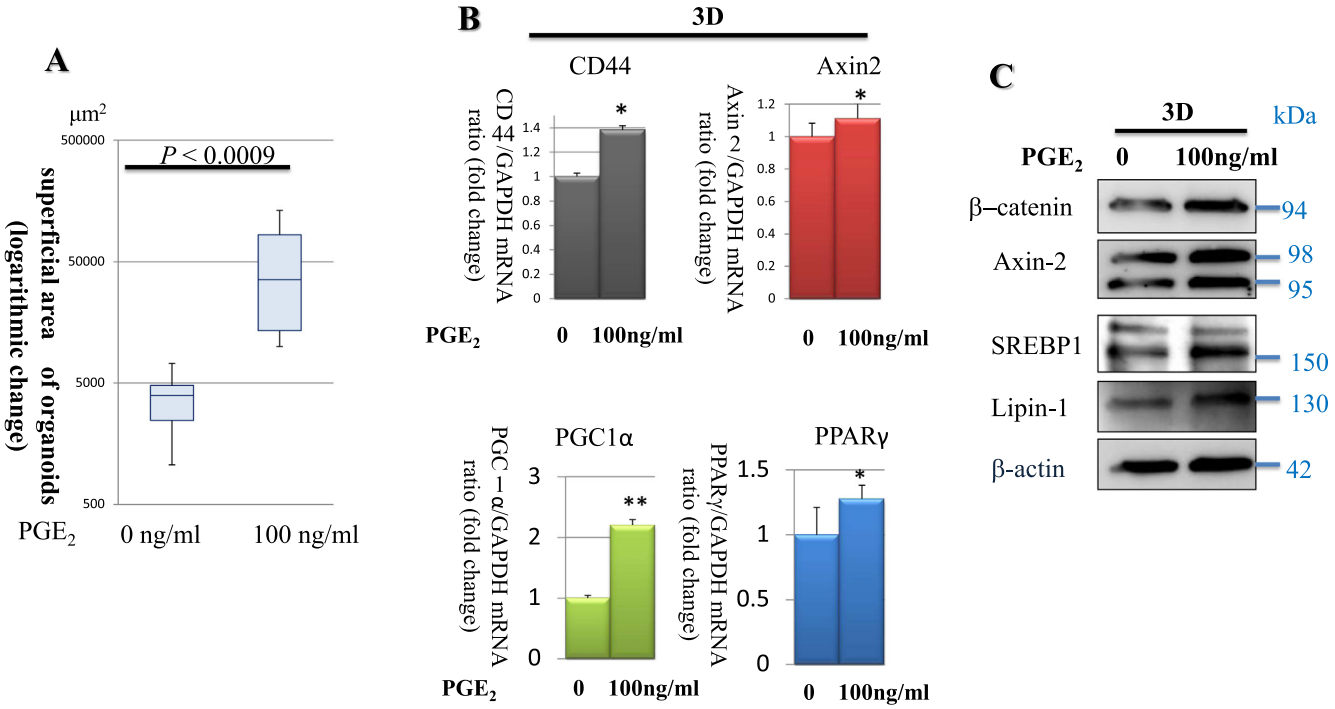

Fig. 4. Activation of canonical Wnt signal as well as up-regulation of molecules involved in mitochondrial metabolism, and lipid synthesis by PGE_2 in SZ95 cells-derived organoids. (A) Box-plot analysis of 30–40 organoids superficial area examined in 3 independent experiments. Organoids smaller than 1000 μm^2 were excluded because it was difficult to distinguish between SZ95 cell-aggregates and organoids smaller than 1000 μm^2. The *P* value was determined by Student's *t* test. (B) Quantitative RT-PCR analysis of CD44, Axin-2, PGC1α and PPARγ to compare 3D-cultured SZ95 cells in the absence or presence of PGE_2 (100 ng/ml for 7 days). $^{*}P < 0.05$, $^{**}P < 0.005$. (C) The comparison of gene expression patterns of control organoids and PGE_2-treated organoids. SZ95 cells were treated with PGE_2 at the concentration of 100 ng/ml for 7 days.

nificantly up-regulated in mRNA level (Fig. 4B). When exposed to PGE_2 in 3D culture, canonical Wnt signal was activated in SZ95 cells, because up-regulation of Axin-2, a well-known target molecule of β-catenin, was recognized (Fig. 4B and C). The transcription of PGC1α was up-regulated, resulting in the robust mitochondrial biogenesis [18,19]. The transcription level of PPARγ which promotes tricarboxylic acid (TCA) cycle [20,21], was also increased. Both lipin1 and SREBP1, transcriptional factors regulating the lipid synthesis, were up-regulated after PGE_2 treatment (Fig. 4C).

Taken together, PGE_2 activates both canonical Wnt signaling pathway, mitochondrial metabolism, and lipid synthesis in the organoids composed of SZ95 cells.

4. Conclusions

We established 3D culture of SZ95 cells and investigated the effect of inflammatory cytokine PGE_2 in organoids, also referred to as "semi-vivo" glands. In parallel with Wnt signal activation, activated gene expression involved in mitochondrial metabolism and lipid synthesis was recognized. It will be necessary to better understand the exact mechanism of the way Wnt signal contributes to the proliferation and lipid metabolism of differentiated sebocytes and undifferentiated basal cells, which heterogeneously configure sebaceous glands.

Author contributions

GJY conceived, designed, and performed the experiments, analyzed the data, and wrote the manuscript. HS conceived the experiments and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.129.

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