Acne and seboregulation: New insights from primary sebocytes

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Introduction

Acne vulgaris, one of the most common skin disorders, is a long-term disease that affects the oil glands present in the skin. With a prevalence of 65-75% among teenagers and the youth, a significant proportion of the human population is prone to acne. Acne lesions can provoke permanent scars and severe scarring can lead to physical and psychological distress, particularly in adolescents. In addition, acne can notably increase the incidence of psychosocial outcomes such as anxiety and reduced self-esteem, possibly leading to suicidal ideation in patients.

It is well established that the primary pathogenic factor for acne is the excessive production of sebum, an oily waxy substance which accumulates within the sebaceous glands, resulting in inflammation. Therefore, the application of cosmetic ingredients that can reduce and/or control sebum production appears to be a popular approach to address this disorder. In this context, the development of reliable methods to assess the effect of such ingredients on seboregulation becomes imperative. The purpose of this work was to compile innovative approaches to evaluate the seboregulatory performance of cosmetic active ingredients using primary sebocytes.

The culturing of human primary sebocytes are generally accompanied by two major challenges: they display slow proliferation (as compared to keratinocytes from the same donor) and they exhibit a strong tendency to spontaneously differentiate *in vitro*. To overcome these limitations, we optimized a specific culture protocol to grow undifferentiated primary sebocytes. We used CK7, a well-known differentiation marker, to validate the differential status of the cultured cells. Next, we evaluated the dynamics of seboregulation in these cells. For this, we investigated the ability of these cells to produce sebum when stimulated by linoleic acid (LA). Following this, we evaluated the ability of dorsomorphin, a popular AMP-activated protein kinase inhibitor to reduce the sebum production. We optimized the procedures to label the lipid contents in the primary sebocytes using BODIPY™, a probe that specifically binds to the fat droplets. With this technique, we showed that LA increased the quantity of lipid droplets by 100 times while dorsomorphin reduced it by 60%. We further investigated the mechanism of action of LA and dorsomorphin by performing a genomic and epigenomic qPCR study of key genes and miRNAs involved in the regulation of sebum production. We highlighted that LA was able to induce the lipid droplet production by increasing PLIN2, MUC1 and mir 29a, a well-known inducer of lipid production and by decreasing ABDH5 and FADS2 by inducing let-7b-5p. In addition, we pointed out that dorsomorphin was able to reverse the LA effect by inducing FADS2 and ABDH5 through a repression of mir-106-5p. Further, dorsomorphin was able to repress MUC1 induction by LA.

Additionally, LA seemed to induce the inflammation pathway in sebocytes by strongly inducing IL6. Dorsomorphin was found to inhibit this induction. Our results revealed that dorsomorphin reduced the proliferation of the primary sebocytes, which could be due to the repression of CCDN1.

Through this study, we have established reliable primary sebocyte culture procedures and optimized genomic, epigenomic and dedicated functionals tests to understand the changes in the cells occurring during sebum repression pathways. Apart from confirming the effect of dorsomorphin on lipid production, we also brought new insights related to its regulation of sebum production. Thus, this innovative compilation of *in vitro* tests can serve as valuable tools to evaluate the seboregulatory performance of various cosmetic active ingredients on human primary sebocytes.

Materials and methods

Primary sebocytes cell culture

Human Primary Sebocytes were provided by our trusted suppliers. The primary cells underwent extensive quality controls which confirmed that the cells were negative for HIV-1, Hepatitis-B and Hepatitis-C. The cells were cultured in sebocyte basal medium supplemented with BPE (Bovine pituitary extract), hrEGF (human recombinant epidermal growth factor) and penicillin/streptomycin/amphotericinB. The cultures were maintained in an incubator equilibrated with 5% CO_2 at 37°C. The cell culture and subculturing procedures were performed according to the supplier's instructions.

Treatment with linoleic acid and dorsomorphin

The sebocytes were plated at 7,000 cells per well in a 96-well plate in complete medium. After overnight incubation, linoleic acid (Merck) was added along with either dorsomorphin (Merck) diluted in medium. The cells were incubated for either 6, 24 or 48 hours to study the lipid droplets production through time. The storage of the products was at room temperature, protected from light. Their solubilities in medium were tested and each compound was filtered through a 0.20µm pore-size membrane before being diluted.

Immunostaining of sebocytes and BODIPY labelling

Human primary sebocytes were fixed with 4% paraformaldehyde (Sigma-Aldrich), and gentle membrane permeabilization procedure was performed using 0.1% saponin (Sigma-Aldrich). The primary antibody incubation was done for 1h with Keratin-7 antibody (Cell Signalling Technology). After rinsing with PBS, the cells were incubated with a Cy3 Anti-rabbit secondary antibody for 1h. Finally, the cells were stained with Hoechst 33342 (Life Technology) and visualised using the CellInsight CX5 high content screening platform (ThermoFisher Scientific) using Cy3 channel and DAPI filters.

For lipid droplet staining, the cells were first fixed and then stained for 30 minutes with BODIPY 493/503 (Life Technologies). The cells were then washed twice with PBS and stained for 10 minutes with Hoechst 33342 (Life Technologies). After rinsing with PBS, the the CellInsight CX5 high content screening platform (ThermoFisher Scientific) was used for the visualization of the BODIPY fluorescence using the FITC channel filter while the DAPI filter was used to view the Hoechst staining.

FACS analysis

The technique of FACS (Fluorescence-activated cell sorting) allows cell-by-cell analysis by measuring the fluorescence of each cell. The Cytokeratin-7 (K7) antibody (Cell Signaling Technology) was used to mark the cells coupled with a CY3 fluorophore. The fluorescence of this fluorophore was detected using a Becton Dickinson FACS (BD Bioscience, Heidelberg, Germany, FACSDiva 6.1.3). For each treatment, a minimum of 1000 cells were analyzed.

HCS cell screening

After staining with K7 or BODIPY, the fluorescence intensity of cells were measured individually using the HCS Studio™ Cell Analysis Software from CellInsight CX5 high content screening platform (Thermo Fisher Scientific). The cells were visualized using the CellInsight 20X objective lens. The cells were segmented using the morphology explorer software as indicated in figure 1 and the labelling area and total intensity were evaluated individually for each cell among ~600 sebocytes for each condition.

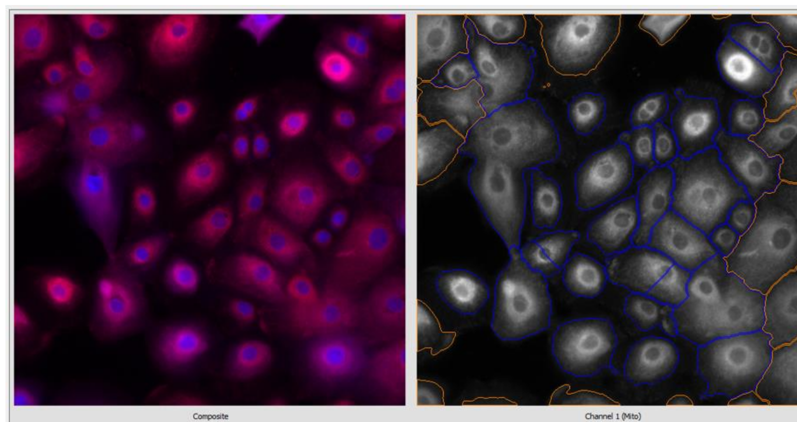


Figure 1: Cell-by-cell analysis using a HCS Studio™ Cell Analysis Software from Cellinsight CX5 high content screening platform (Thermo Fisher Scientific).

WST-1 Cytotoxicity and proliferation assay

The cell proliferation reagent WST-1 was purchased from Roche (Cat. No. 05015944001). The sebocytes were seeded 24h prior to the treatment and were then incubated with either medium plus DMSO or treated with linoleic acid with/without dorsomorphin to study the proliferation.

The WST-1 assays were performed according to the manufacturer's instructions. WST-1 is a tetrazolium salt that is cleaved to a soluble formazan by cellular mechanism involving mitochondrial dehydrogenase. This bioreduction is dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in culture.

RNA extraction

The total RNA from stimulated sebocytes was extracted and purified using RNeasy mini Kit (74104 Qiagen), following the manufacturer's instructions. The quality controls and total RNA quantification were performed with Agilent RNA Nano kit using the Agilent 2100 bioanalyzer and Nanodrop spectrophotometer.

RT-qPCR on mRNA

The total RNA was reverse-transcribed with the Superscript VILO cDNA Synthesis Kit (11754-050 Invitrogen) according to the manufacturer's instructions. The quantitative PCR (qPCR) was performed with a Platinum Quantitative PCR SuperMix-UDG Kit (11733-038 Invitrogen) according to the manufacturer's instructions using the CFX-connect (Biorad). The results were normalized against GAPDH as an endogenous control. The expression levels of RNA were calculated using the 2DD Ct method.

RT-qPCR on miRNA

For qPCR of microRNA, total RNA was reverse-transcribed with a Universal cDNA Synthesis Kit II. The reverse transcription reactions were diluted and a fraction of diluted cDNA was used for each quantitative PCR. Quantitative PCR was performed with an Exilent SYBR Green master mix kit using the the CFX-connect (Biorad). The reference and expression levels of miRNA were calculated using $2\Delta\Delta Ct$ method. For mRNA target quantification, the experiments were performed in duplicates for each biological replicate. The results were normalized against RNU1A as an endogenous control. The target primers and RNU1A primers were computationally designed, bought from Invitrogen and validated (data not shown) before performing the experiments.

Results

1. Validation of primary sebocyte cell culture differentiation

Sebocytes are the main type of cells present in sebaceous glands. They are differentiated cells that accumulate lipids and release them via holocrine secretion. The primary sebocytes used in this study perfectly mimic and are more physiologically relevant as compared to immortalized sebocyte cell lines such as SZ95, SEB-1.

Because the primary sebocytes show slow proliferation, we optimized our cell culture procedures by combining commercial medium and coating plates. The differentiation of primary sebocytes was firstly evaluated through the analysis of cytokeratin-7 expression in cells by FACS and compared to unlabeled control cells. The FACS analysis showed that 97% of cells were cytokeratin-7 positive. Thus, primary sebocytes used in this study, were correctly differentiated.

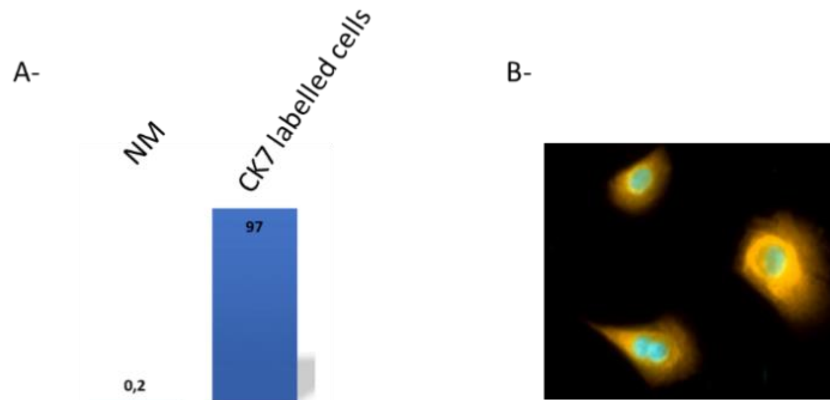


Figure 2: Sebocytes immunostaining using cytokeratin-7 antibody. The sebocytes were fixed in paraformaldehyde and immunostained with cytokeratin-7 antibody. FITC- fluorescence of unlabeled cells (NM) or cells labelled using cytokeratin-7 were determined by FACS analysis (A). Images of immunostained cells (B) were obtained with a Zeiss observer microscope controlled by Zeiss software using 10X objective. The images were processed using Image J software.

2. Dynamic induction of sebum production by linoleic acid and repression of this induction by dorsomorphin

Linoleic acid (LA) is a major fatty acid of lipogenesis process in human sebaceous gland. It has been widely described in the literature that LA treatment is capable of inducing the production of lipid droplets in sebocytes cell line such as SZ95. In this study, the primary sebocytes were used as a model and Linoleic Acid-dependent induction of lipogenesis was optimized on those cells. In this case, Linoleic Acid treatment was able to increase the quantity of lipid droplet production by the primary sebocytes by 100 times after 48 hours of treatment (figure 3).

In the second part, the reversal of this induction was studied. The effect of dorsomorphin, a potent lipogenesis inhibitor was analyzed. It is known to be an AMPK inhibitor. AMPK inhibits the *de novo* lipogenesis by inhibiting ACC1 and controlling the expression of genes involved in fatty acid metabolism [1,2,3]. Here, LA-dependent induction of lipids droplets was decreased by 60% after dorsomorphin treatment (figure 3).

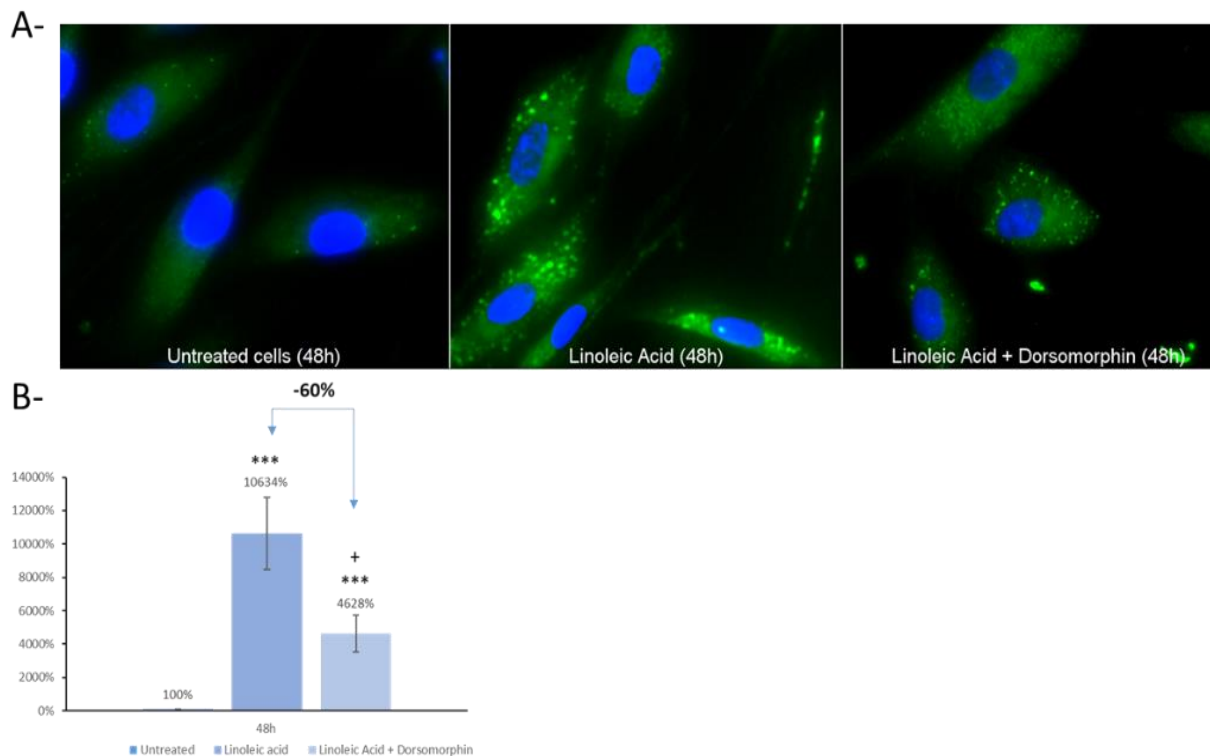


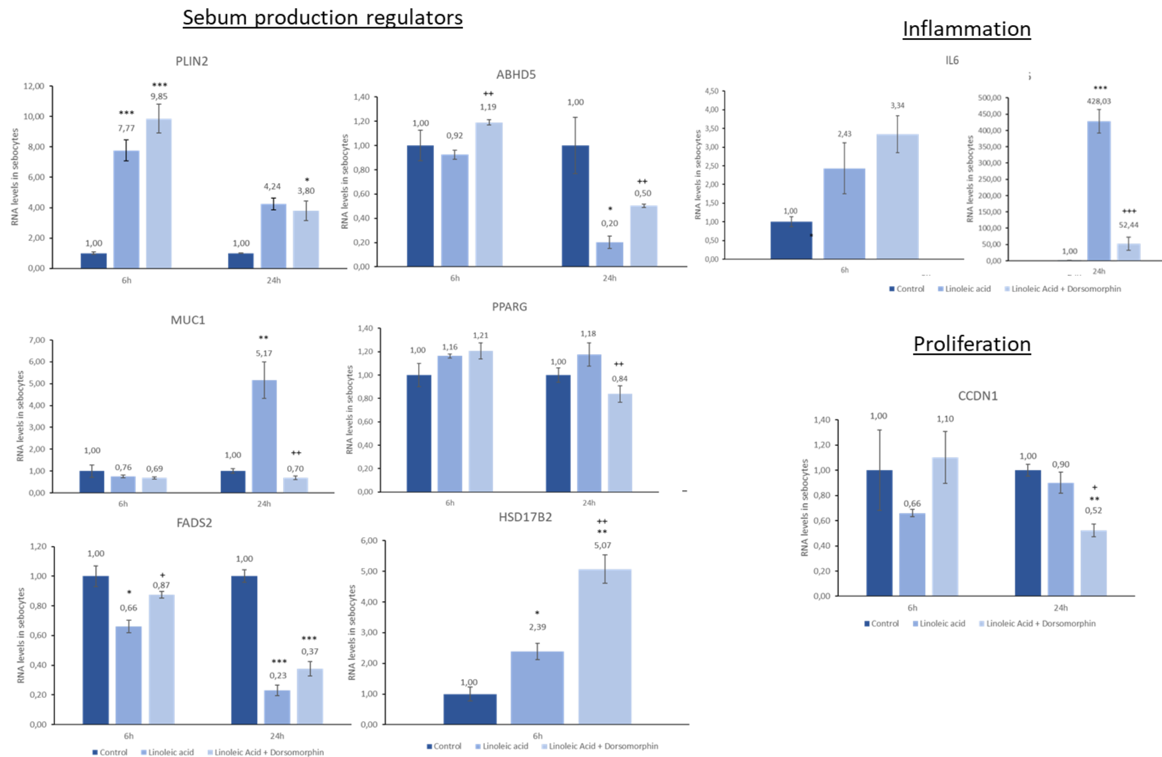
Figure 3: Effect of linoleic acid treatment and Dorsomorphin on primary sebocytes. Primary sebocytes were either incubated with medium or treated with linoleic acid or a mix of linoleic acid and dorsomorphin respectively. The lipid droplets were stained using BODIPY™ according to the manufacturer's instruction after 48h of treatment. The cells were visualized using AxioImager microscope (Zeiss) at a 20X objective. (A) The level of lipid droplets from about 600 sebocytes for each condition was measured using HCS Studio™ Cell Analysis Software from CellInsight CX5 high content screening platform The quantity of induction of lipid droplet production as compared to the untreated cells are indicated in the figure in arbitrary values (B). The graph presents the mean of the induction of at least 3 replicates. The SEM bars are represented in black lines on each histogram. The statistical analysis was performed using two-tailed unpaired T-test (* = vs NT, + = vs LA, *p*-value <0.05) and Wilcoxon cell by cell (cells imaging is described in materials and methods).

3. Effect of LA and LA+dorsomorphin on gene and miR modulation

The effect of Linoleic Acid and Linoleic Acid + dorsomorphin was tested on the gene modulation in primary sebocytes.

First, we analysed the genes that control the sebum production. We measured the expression of PLIN2 and MUC1, two markers of human sebocytes differenciation and enzymes FADS2, ABHD5 and HSD17B2 that control the sebum production and its composition. PPARG plays a major role in human sebum production. We then investigated IL6 cytokine, one that widely regulates the inflammation and because the regulation of sebocyte proliferation is important to reduce the quantity of sebum in the skin, we investigated CCDN1 expression that controls cell proliferation. Indeed, a high expression level of CCDN1 induced a high rate of proliferation [8].

In figure 4, the histograms merging the results from 3 replicates showed that the cells treated with Linoleic Acid as compared to untreated cells strongly induced the expression of PLIN2, MUC1, HSD17B, IL6 and repressed ABHD5 and FADS2 expression. Dorsomorphin treatment could reverse the Linoleic acid-dependant induction of IL6 and MUC1 and Linoleic Acid-repression of FADS2 and ABHD5. Moreover, the dorsomorphin treatment seemed to reduce CCDN1 and PPARG.



<u>Figure 4:</u> Effect of Linoleic Acid and Linoleic Acid + dorsomorphin on primary sebocytes gene expression. The sebocytes were treated for 6h or 24h hours with Linoleic Acid or Linoleic Acid + Dorsomorphin. The untreated cells were used as the control. Each experiment was performed in triplicates. The genomic target expression levels afters treatments were measured by RT-qPCR and compared to untreated cells. The results were normalized to endogenous control GAPDH expression. The SEM bars are represented in black line on each histogram. The statistical analysis was performed using two-tailed unpaired T-test [(* = vs NT, + = vs LA,) (* *p*-value <0.05)].

MicroRNAs are a class of small functional non-coding RNAs that play major roles by targeting the regulation of numerous genes. Thus, microRNAs are master epigenetic regulators of all biological processes. To better understand the regulation of gene expression by LA and Dorsomorphin, we investigated the expression levels of 3 microRNA. Let-7b- 5p is known to control FADS2. Mir29a –5p is induced after oleic acid treatment [5] and other fatty acids. Similarly, mir-106b-5p is known to regulate ABHD5 [9].

In figure 5, the histograms merging the results from the 3 replicates showed that cells treated with Linoleic Acid as compared to the untreated cells strongly induced Mir29a –5p and Let-7b- 5p. LA + Dorsomorphin treatment inhibited 106b-5p.

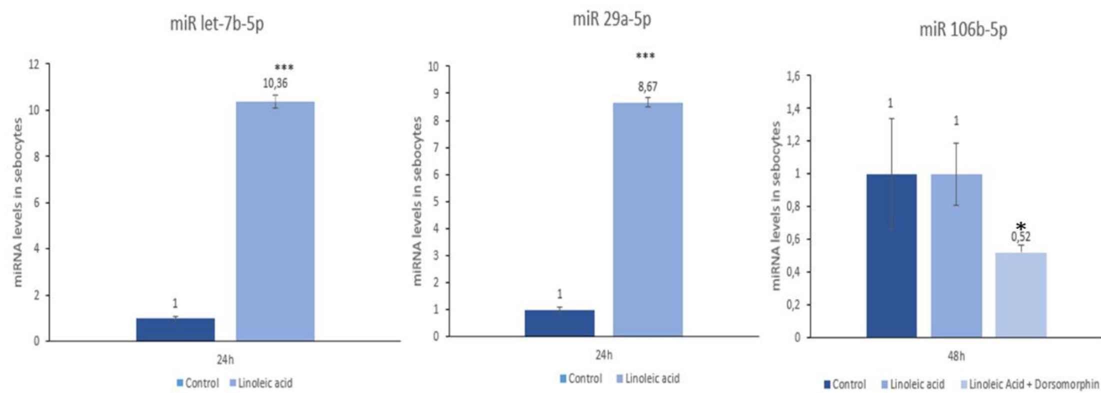


Figure 5: Effect of LA and LA + dorsomorphin on miR expression in primary sebocytes. The sebocytes were treated for 6h or 24h hours with Linoleic Acid or Linoleic Acid + Dorsomorphin. The untreated cells were used as the control. Each experiment was performed in triplicates. The genomic target expression levels afters treatments were measured by RT-qPCR and compared to untreated cells. The results were normalized to endogenous control RNU1A expression. The SEM bars are represented in black line on each histogram. Statistical analysis was performed using two-tailed unpaired T-test [(* = vs NT, + = vs LA,) (* *p*-value <0.06)].

4. Proliferation assay

Since dorsomorphin was able to repress CCDN1 gene expression, we set out to investigate the ability of linoleic acid and dorsomorphin to regulate the process of cell proliferation.

Cell proliferation was measured after 48 hours of treatment using WST-1 reagent. Figure 6 shows that the dorsomorphin treatment significantly decreased proliferation rate of sebocytes by 35%.

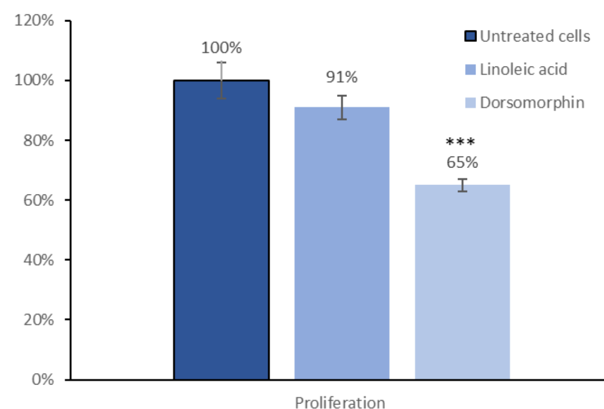


Figure 6: Effect of LA and dorsomorphin treatment on primary sebocytes proliferation. The primary sebocytes were treated for 48h hours with either linoleic acid, dorsomorphin or left untreated. The cell proliferation rate was measured using WST-1 proliferative reagent at OD_{450}nm using a Tecan microplate reader. The SEM bars are represented in black lines on each histogram. The statistical analysis was performed using two-tailed unpaired T-test (* *p*-value <0.05).

Discussion and Conclusion

Acne is a combination of several factors of which inflammation and excessive sebum production play important roles. The development of a model that perfectly mimics these two main processes and the complementary analytical methods to analyse them are of interest.

The method described in this study can be interesting for several reasons. Our model of induction of primary sebocytes by LA and repression by dorsomorphin seemed to mimic the physiopathology of acne. The treatment of primary sebocytes with LA stimulated inflammation through the induction of IL6 and the regulation of key genes that control two main factors involved in sebocyte differentiation and control of lipid production including MUC1 and PLIN2 [4]. Moreover, LA controlled the expression of several enzymes involved in lipid production such as FADS2, ABHD5 and HS. We also showed that mir29a is induced during LA treatment. A previous study that showed that oleic acid treatment induced mir-29a expression validated our results [5].

Dorsomorphin is a potent sebum production inhibitor, because it is a AMPK inhibitor known to inhibit lipid production. However, the mechanism involved in its action are poorly understood. Interestingly, we found that this inhibitor repressed the inflammation in sebocytes, sebum production but also repressed sebocyte proliferation. We bring new insights into the mechanism of action of dorsomorphin. Moreover, we highlighted that dorsomorphin may act on PPARG expression. PPARG is well known to induce sebum production [6].

Finally, in this study, we optimized a procedure to label the lipid contents in the primary sebocytes using BODIPY™ probes. AdipoRedTM reagent which specifically partitions into fat droplets and a specific antibody against Perilipin, a protein that coats the lipid droplets were also used (data not shown). However, dorsomorphin does not act through the regulation of Perilipin 2 and therefore we decided to focus on BODIPY™ labelling that presented a more dynamic and sensible test.

Taken together, the protocols that we have optimized through this study offer a combined and broader approach to further analyze pro- or anti-acne effects of potential active ingredients on primary sebocytes.

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