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Research Article

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L-(+)-Tartaric Acid Minimally Affects the Viability or Molecular Signature but Increase the Expression of Selected Hair Growth Associated Genes in Human Dermal Papilla Cells

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Abstract

Objective: Topical application of minoxidil has been adopted as an effective treatment for male and female pattern hair loss. While higher concentration formulas exhibit better efficacy, dissolving and stabilizing minoxidil is technically challenging. L-(+)-Tartaric Acid (LTA) is a colorless crystalline dicarboxylic acid found in plants and has been used as an additive in various hair care products to stabilize the biological functions of active ingredients. LTA has been added to minoxidil to maintain the quality of over-the-counter formula. To date, the influence of LTA on human Hair Follicle (HF) cells has not been sufficiently investigated. As the Dermal Papilla (DP) plays pivotal roles in the regulation of HF regeneration and the hair cycle, we aimed to elucidate the effects of LTA on human Dermal Papilla cells (hDPCs).

Methods: hDPCs were first subject to cytotoxicity and alkaline phosphatase assay after treated LTA. Microarray comparison analysis was performed to elucidate LTA effects on global gene expression on LTA-treated and non-treated hDPCs. Subsequent real-time polymerase chain reaction analysis using multiple donor-derived hDPCs was done to further confirm LTA-induced hair growth genes up-regulation.

Results: LTA exhibited no influence on hDPCs in a cytotoxicity assay. The activity of a representative hDPC marker enzyme, alkaline phosphatase, was not impaired by LTA in cultured hDPCs. Microarray comparison analysis of LTA-treated and non-treated hDPCs elucidated that LTA minimally affected the molecular signature of hDPCs but, intriguingly, up-regulated some hair growth-related genes. Subsequent real-time polymerase chain reaction analysis using multiple donor-derived hDPCs confirmed that LTA indeed increased the gene expression of activin A receptor type 2A (ACVR2A) and Insulin-like Growth Factor Binding Protein 5 (IGFBP5), while down-regulation of hair growth-related genes by LTA was inconsistent among hDPCs derived from respective donors.

Conclusion: These results suggest that LTA minimally affects but potentially supports the biological characteristics of hDPCs. LTA may represent a useful additive to generate efficacious, high concentration formula of active reagents, represented by minoxidil.

Keywords: Androgenetic alopecia • Hair follicle • Human dermal papilla • L-(+)-tartaric acid • Additives • Activin A receptor, type IIA (ACVR2A) • Insulin-like Growth Factor Binding Protein (IGFBP)

Introduction

Hair loss diseases can unfavorably influence the appearance of affected individuals, cause mental stress, and thereby greatly impair their quality of life. Male and female pattern hair loss represent common forms of hair loss diseases, and the demand for efficacious therapeutics is enormous [1]. To date, minoxidil is the only Federal Drug Administration (FDA)-approved topical medication to treat male and female pattern hair loss. At least for male pattern hair loss (androgenetic alopecia), a 5% minoxidil solution has been reported to be more effective than a 2% solution [1,2]. Minoxidil exhibits low solubility in both water and ethanol, and as most topical minoxidil solutions are sold as over-the-counter medications, determination of a formula enabling long-term storage without loss of activity and excessive volume reduction is mandated [3]. Additives, such as solubilizers, pH adjusters, thickeners, and surfactants, are usually adopted to stabilize active ingredients.

L-(+)-Tartaric Acid (LTA) is a colorless crystalline dicarboxylic acid with hydrophilic characteristics which is commonly used in chemical peeling products [4,5]. LTA has shown to be an excellent pH adjuster which stably dissolves minoxidil without causing crystallized precipitation and coloring formation over time in our preliminary study and therefore has been adopted to produce a commercially available minoxidil solution to treat male pattern hair loss. However, how LTA affects biological characteristics of human hair follicle (HF) has not been sufficiently investigated.

The dermal papilla (DP), a specialized group of mesenchymal cells located at the proximal ends of HFs, plays pivotal roles in HF regeneration, hair cycle regulation, and, most importantly, the pathophysiology of male pattern hair loss. In this study, we aimed to assess the influence of LTA on human DP cells (hDPCs) mainly *via* cytotoxicity assays and global gene expression analysis.

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Materials and Methods

Cell culture

Three donor-derived human Dermal Papilla Cell (hDPC) lines were used in this study (Cell Application, Inc., San Diego, USA); details are shown in Table 1. hDPCs were cultured in T75 flasks coated with collagen (Toyobo Co., Ltd., Osaka, Japan) in complete Papilla Cell Growth Medium (Toyobo Co., Ltd., Osaka, Japan). After subculturing to passage 3, hDPCs were seeded in collagen-coated 24-well plates ($5x10_4$ cells/well) for LTA treatment (n=3). Human dermal fibroblasts (hFBs) (Kurabo Industries Ltd., Japan) were cultured in T75 flasks in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Corning, Inc., NY, USA). After culturing to passage 3, hFBs were seeded in 24-well plates ($5x10^4$ cells/well) for subsequent experiments (n=3). All cell cultures were performed at 37°C in 5% CO_2.

Cell Applications, Inc., San Diego, CA, USA			
Lot Number	**Age**	**Sex**	**Race**
2717	66	Male	Caucasian
2598	56	Male	Caucasian
2905	46	Female	Caucasian

Table 1. Cell information of hDPCs.

Alkaline Phosphatase (ALP) staining

After reaching 50% confluence in 24-well plates, hDPCs (lot 2717) were treated with 1 mM or 3 mM LTA (CAS# 87-69-4) in DMEM supplemented 0.1% FBS for 24 hours. The cells were then fixed with a 4%-paraformaldehyde phosphate buffer solution (Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) at 4°C, incubated with an ethanol/acetone mixed solution (50:50 v/v) at -20°C, and stained with an ALP staining kit (Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer's instructions. The cells were examined under a BZ-X810 microscope (Keyence Corp., Osaka, Japan; magnification ×20), and the number of ALP-positive cells was finally analyzed with Image.

Cell proliferation assay

After reaching 70% confluence in 24-well plates, hDPCs (lot 2717) were incubated with 1 mM or 3 mM LTA in DMEM containing 0.1% FBS for 24 hours, and Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was utilized to assess cell proliferation according to the manufacturer's instructions. In brief, CCK-8 solution was diluted 10 times with medium and added to the cells. After 1-2 hours of incubation at 37°C, 100 µl of medium was collected in a 96-well plate, and the absorbance was measured at 450 nm by a microplate reader (Molecular Devices, Tokyo, Japan). Data were analyzed as percentages relative to the control.

RNA isolation and real-time PCR analysis

hDPCs (lots 2717, 2598, and 2905) were incubated in 24-well plates until reaching 70% confluence. The cells were then treated with 1 mM or 3 mM LTA in DMEM containing 0.1% FBS. After 24 hours of incubation, total RNA was isolated from hDPCs using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). For real-time PCR analysis, cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix (with gDNA remover) kit (Toyobo Co., Ltd., Osaka, Japan). PCR was then carried out using TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Santa Clara, CA, USA) and the QuantStudio Real-Time PCR System (Applied Biosystems). The qRT-PCR were performed under the following conditions: 2 min at 50°C, 20 sec at 95°C, and 45 cycles of 1 sec at 95°C and 20 sec at 60°C. The primer sets were purchased from Applied Biosystems (Table 2). Target mRNA expression levels were normalized to that of ACTB using the ΔCt method and calculated as $2^{-\Delta Ct}$.

Gene Symbol	Gene Name	Thermo Fisher TaqMan® probe ID
ACTB	actin beta	Hs01060665_g1
A2M	alpha-2-macroglobulin	Hs00929971_m1
ACVR2A	activin A receptor, type IIA	Hs00155658_m1
BAMBI	BMP and activin membrane-bound inhibitor	Hs03044164_m1
FGFR2	fibroblast growth factor receptor 2	Hs01552918_m1
GREM1	gremlin 1, DAN family BMP antagonist	Hs01879841_s1
IGFBP3	insulin-like growth factor binding protein 3	Hs00181211_m1
IGFBP5	insulin-like growth factor binding protein 5	Hs00181213_m1
IGFBP7	insulin-like growth factor binding protein 7	Hs00266026_m1
LTBP1	latent transforming growth factor beta binding protein 1	Hs01558763_m1
SMAD1	SMAD family member 1	Hs00195432_m1
SOCS2	suppressor of cytokine signaling 2	Hs00919620_m1
THBS1	thrombospondin 1	Hs00962908_m1
ACVR1	activin A receptor, type I	Hs00153836_m1
BMP6	bone morphogenetic protein 6	Hs01099594_m1
FGF1	fibroblast growth factor 1	Hs01092738_m1

Table 2. The primer information for real-time PCR.

Microarray

hDPCs (lot. 2717) were used for microarray analysis. Total RNA was isolated as described above, and microarray analysis was performed using the SurePrint G3 Human GE microarray 8x60K Ver3.0 (Agilent Technologies, Palo Alto, CA, USA) by DNA Chip Research Inc. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer. Labeled cRNA was prepared using standard Agilent protocols. The microarray slide was scanned using an Agilent SureScan Microarray Scanner (G4900DA), and the scan images in .tiff format were then analyzed using Agilent Feature Extraction Software version 11.5.1.1. The raw intensity values were log_2-transformed to the median of all samples and normalized to the 75th percentile. Data were analyzed and visualized with R (http://www.r-project.org).

Results

LTA minimally affected the morphology, viability, and biochemical properties of hDPCs

hDPCs were distinguished from hFBs by their polygonal morphology (Figure 1A), and the addition of 1 mM and 3 mM LTA to the hDPC culture did not alter their morphological characteristics (Figure 1A). Cytotoxicity analysis revealed that the viability of hDPCs was not affected by LTA, regardless of the concentration in culture (Figure 1B). ALP is an established biomarker of hDPC activity [6], and its enzymatic reactivity was not detectable in hFBs (Figure 1C). The ALP activity in hDPCs treated with 1 mM and 3 mM LTA was almost equivalent to that in non-treated hDPCs, as supported by quantitative analysis (Figures 1C and 1D). These data suggested that LTA minimally affected the morphology, viability, and biochemical properties of hDPCs *in vitro*.

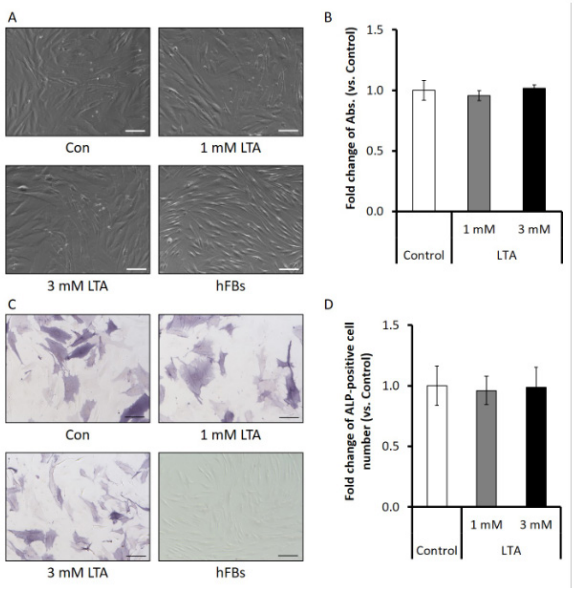

Figure 1. LTA minimally affected morphology, viability, and biochemical properties of hDPCs. (A) Phase contrast images of hDPCs treated with 1 mM or 3 mM LTA. Scale bar:100 µm. (B) Relative cell viabilities of hDPCs treated with or without LTA (1 mM or 3 mM). (n=3, mean ± SD. Dunnett's test). (C) Alkaline phosphatase (ALP) staining of hDPCs treated with 1 mM or 3 mM LTA. Scale bar:100 µm. (D) Relative ALP-positive cell number. (n=12, mean ± SD. Dunnett's test).

LTA treatment exerted little influence on global gene expression in hDPCs

TTo investigate the effect of LTA on global gene expression patterns in hDPCs, microarray analysis was performed with vehicle- and LTA (1 mM or 3 mM)-treated hDPCs. hFBs were also subjected to this analysis for comparison. The A_{260}/A_{280} values of harvested total RNA were all in the range of 1.9-2.0, indicating the sufficient quality of RNA purity for microarray analysis. Detectable probe sets ranged 28959 (49.6%) - 32345 (55.4%), showing reasonable standard detection availability of microarrays.

In the hierarchical clustering analysis, the gene expression profiles of hFBs and hDPCs were clearly distinct; however, there were no obvious differences in their expression patterns in LTA-treated and control hDPCs (Figure 2A). Similarly, scatter plot analysis showed tightly correlated global gene expression between control and LTA-treated hDPCs (Con vs 1LTA, R^2 = 0.998; Con vs 3LTA, R^2 = 0.992), while those between hDPCs and hFBs were relatively weak (R^2 = 0.945) (Figure 2B). These results suggested that LTA exerted little influence on fundamental gene expression in hDPCs.

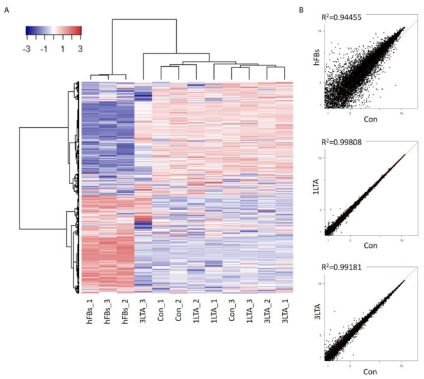

Figure 2. Heat map and scatter plot of microarray analysis. For microarray analysis, total RNA was isolated from LTA-non-treated control hDPCs (Con), 1 mM or 3 mM LTA treated hDPCs (1LTA or 3LTA) and human dermal fibroblast (hFB). (A) Hierarchical clustering of samples based on probes detected in all samples (n=3). (B) Scatter plot and correlation coefficients from microarray analysis of Con vs. 1LTA, Con vs. 3LTA and Con vs. hFB (n=3).

LTA up-regulated HF-associated growth factor-related genes

Although LTA treatment had only minor effects on global gene expression in hDPCs, we next examined the effects of LTA on specific genes associated with hair growth. To identify genes that were up-regulated after LTA treatment, those with increased expression levels in hDPCs versus hFBs were selected (18,343 probes), and those with a raw signal intensity over 100 were further selected (7,658 probes). Next, the genes that included the words "Growth factor" in the GO molecular function annotation of the Gene Ontology Consortium were filtered and chosen (108 probes) (Figure 3A). Finally, the genes were classified into three groups: 1LTA up/3LTA up (32 probes), 1LTA down/3LTA down (35 probes), and 1LTA up/3LTA down or 1LTA down/3LTA up (41 probes) (Figure 3B). After removing the repeated genes, 27 growth factor genes were up-regulated in both the 1 mM LTA and 3 mM LTA treatment groups, with 23 genes being up-regulated in a LTA dose-dependent manner. These included ACVR2A, BAMBI, and SMAD1, which are associated with HF development and differentiation [7-9]. These results suggested that LTA might be an effective adjuvant to support hair growth.

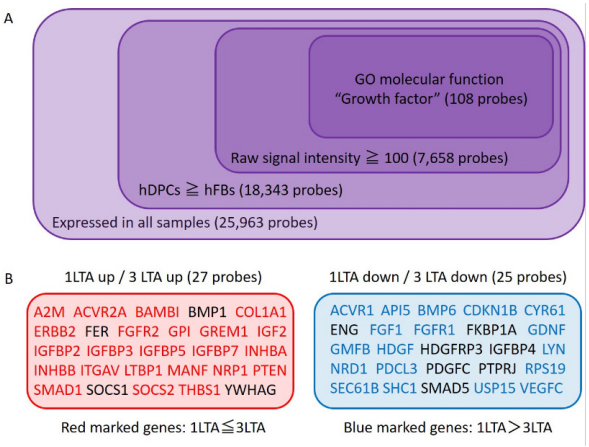

Figure 3. Diagram of selection process of up- and down-regulated genes from gene profiling results. (A) From 25,963 probes which detected in all samples, hDPCs ≥ hFB were picked up (18,343 probes). Furthermore, the genes that raw signal intensity was over 100 were selected (7,658 probes), and finally, the genes which included the word of "Growth factor" in GO molecular function Annotation from Gene Ontology Consortium (108 probes). (B) Selected 108 probes divided into three groups, 1LTA up/3LTA up (32 probes), 1LTA down/3LTA down (35 probes) and 1LTA up/3LTA down or 1LTA down/3LTA up (41 probes). The genes shown in red and blue letters were changed by LTA concentration dependently. Fold change >1.00 was defined as up-regulated and <-1.00 was defined as down-regulated.

LTA consistently increased the expression of hair growth-related genes in multiple donor-derived hDPCs

To verify the microarray analysis results, real-time PCR was performed to evaluate the expression of the up-regulated genes. hDPCs of the same lot used for microarray analysis (lot 2717) were first examined, revealing that the gene expression levels of ACVR2A, BAMBI, FGFR2, IGFBP3, IGFBP5, IGFBP7, SMAD1, and SOCS2 were elevated in a LTA dose-dependent manner (Figure 4, left row). To further confirm the universal effect of LTA, two additional hDPC cell lines (lots 2598 and 2905) were examined (Figure 4, middle and right row). Among the genes analyzed, the expression levels of ACTR2A and IGFBP5 were tendentiously increased in both hDPC cell lines with dose-dependency. Based on the average gene expression data of the three hDPC cell lines, significant increases in the gene expression levels of ACTR2A, BAMBI, IGFBP5, IGFBP7, and SOCS2 were observed in 3mM LTA-treated hDPCs in comparison to those in 1mM LTA-treated and control hDPCs (Figure 5).

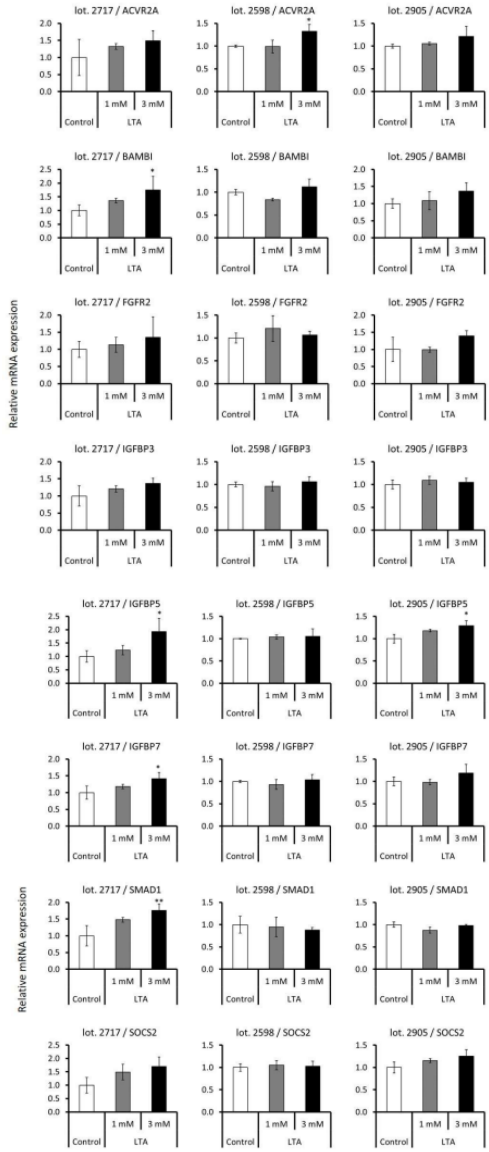

Figure 4. Real-time PCR analysis of each hDPCs cell lines (n=3, mean ± SD.*P < 0.05, **P < 0.01, Dunnett's test).

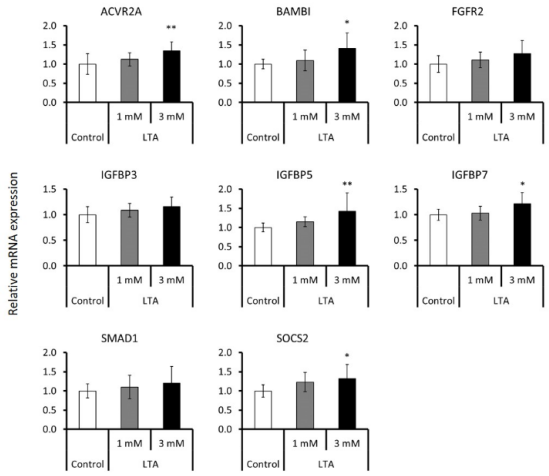

Figure 5. Average data of selected genes confirmed by real-time PCR in multiple donor-derived hDPCs. The genes 1LTA ≤ 3LTA were picked up. ACVR2A, BAMBI, GREM1, IGFBP3, IGFBP5, IGFBP7, SMAD1, and SOCS2 were slightly up-regulated in hDPCs (lot 2717). The same evaluation was performed with these 8 kinds of genes using other two different hDPCs cell lines (lots 2598 and 2905). The average data was shown (n=9, mean ± SD. *P < 0.05, **P < 0.01, Dunnett's test).

In addition to the aforementioned up-regulated genes, 25 down-regulated genes, 18 of which expression was decreased in a dose-dependent manner (Figure 3B), were also examined by real-time PCR. Among these genes, the expression levels of ACVR1, BMP6 and FGF1 were confirmed to be down-regulated in lot 2717 hDPCs by real-time PCR but not in the other two hDPC cell lines (Figure 6). The other 15 genes were also not consistently down-regulated (Figure 7). These findings implied that LTA might preferentially support hair growth via pathways related to growth stimulation.

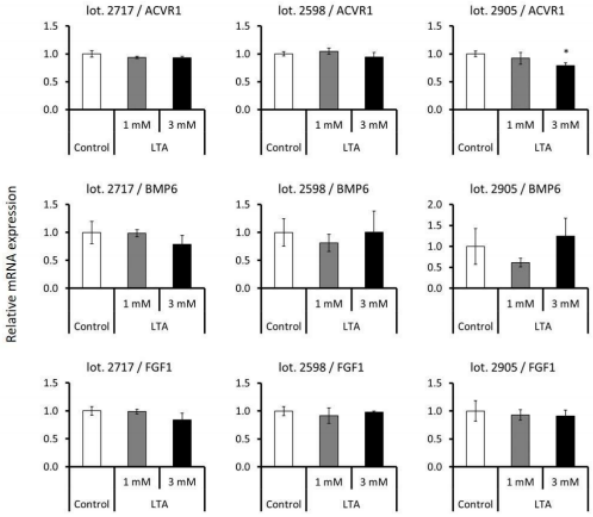

Figure 6. Real-time PCR analysis which shown dose-dependently suppression by LTA in hDPC (lot 2717) (n=3, mean ± SD. *P < 0.05, **P < 0.01, Dunnett's test).

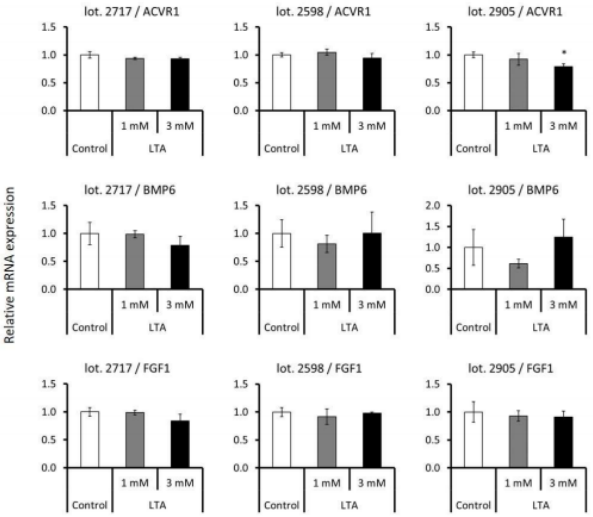

Figure 7. Real-time PCR analysis which showed gene down-regulated expression by microarray but did not show dose-dependent change after LTA treatment (n=9, mean ± SD. *P < 0.05, **P < 0.01, Dunnett's test).

Discussion

AAdditives are essential to improve the stability or solubility of chemical and pharmacological reagents, however, their bioreactivities tend to be disregarded. LTA is well-known as an α-hydroxy acid [5] and also as a pH stabilizer used in a minoxidil solution on the market. In our previous study, we found that LTA increase FGF-7 production, a factor that is well-related to hair growth, in dermal sheath cells (Rohto Pharmaceutical Co., Ltd. 2019 news release (https://www.rohto.co.jp/news/release/2019/0124_01). To further assess the possible supportive role of LTA in hair growth promotion, its biological effects on hDPCs, key element governing

epithelial–mesenchymal interactions to maintain hair follicle homeostasis and regeneration [10,11] was attempted in this study.

Despite that the biological properties of cultured hDPCs are distinct from those *in vivo* intact hDPCs and therefore the outcomes obtained by *in vitro* experimentations may not directly relevant to *in vivo* hDPCs, however, based on the findings obtained in cell proliferation and ALP enzymatic activity assay, it may be reasonable to conclude that LTA does not markedly impair biological characteristics of cultured hDPCs. This is further supported by high correlation coefficients between LTA-treated and untreated hDPC global gene expression profile as detected by microarrays. As supplementation of WNT, BMP, and FGF ligand to hDPC culture or cell aggregation of cultured hDPCs have been shown to ameliorate biological properties [12,13], adopting such techniques should enable more accurate assessment of the biological influence of LTA on *in vivo* hDPCs, which represent important next step in future studies.

Among those detected as up-regulated by LTA in a dose-dependent manner in microarray analysis, an extracellular matrix transporter A2M, IGBP3, and THBS1 have been reported to be hDPC specific when compared to dermal fibroblasts by secretome analysis [14]. The expression levels of LTBP1 and BAMBI have been suggested to correlate with hair inductive capacity [12,15]. GREM1 has been shown to play a role in skin appendage formation. Albeit some genes were not consistently up-regulated in all three hDPC-lines, the average up-regulation levels of the genes, such as ACTR2A, BAMBI, IGFBP5, IGFBP7, and SOCS2 were found to be statistically significant when compared to 3mM LTA-treated hDPC group than those in 1mM LTA-treated and control hDPC group. Considering that biological properties of cultured hDPCs are variable, depending on the passage number or medium condition [12], and that inconsistency in the tendency of down-regulated genes among respective hDPC lots, these findings suggested that LTA mildly ameliorated hDPC properties *in vitro*.

ACVR2A and IGFBP5 were the most significantly up-regulated by LTA in hDPCs. ACVR2A is a receptor belonging to the transforming growth factor-beta (TGF-β) superfamily and has been reported to be expressed in HF [16]. Bmpr2/Acvr2a-deficient mice exhibit rapid hair generation cycles and eventual loss, implying its fundamental role in HF homeostasis [7]. Igfbp5, in conjunction with Krox20, has been shown to exert influence on hair shaft differentiation in mice [17]. As these genes rather seem to be involved in HF morphogenesis than elongation or enlargement, their roles in augmentation of hair loss in humans are still unclear. Still, given their essentially in HF biology, the effect of LTA on hDPC to up-regulate these genes can be considered to be beneficial.

We are aware of the limitations of this study. The effect of LTA was evaluated using hDPC alone. Integration of keratinocyte-hDPC co-culture system [12] should enable more in-depth dissection of the influence of LTA on HF. The relevance of adopted two concentrations of LTA to absorbed LTA concentration *in vivo*. In addition, biological significance of up-relguation in identified hDPCs genes were not evaluated by functional asssays.

Conclusion

Considering that LTA is an additive used for the adjustment of pH for the maintenance of the efficacy of main agent, the data obtained in this study can favorably support the use of LTA for hair care products, including minoxidil solution.

Conflicts of interest

Ohyama M is a scientific advisor for Eli Lilly Japan, Janssen Pharmaceutical (Japan) Co., Pfizer Japan Inc., and Rohto Pharmaceutical Co., and Taisho Pharmaceutical Co. and receives research grants from Shiseido Co. and Maruho Co. not related to this study. Other authors are employees of Rohto pharmaceutical Co., Ltd.

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