AN IN VITRO METHOD FOR IDENTIFYING AGENTS WITH LIPOFILLING-LIKE ACTIVITY: PARALLEL ASSESSMENT OF LIPID ACCUMULATION, TOXICITY AND GENE EXPRESSION

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ABSTRACT

An *in vitro* assay was developed to facilitate the identification and characterization of agents demonstrating lipofilling-like activity. The method involves exposing replicate cultures of primary human pre-adipocytes to various concentrations of test agents during cell differentiation. The cultures are then divided into groups for analysis of lipid accumulation using AdipoRed, cell viability using Cell Titer Blue, and collection of RNA for gene expression analysis. Agents for which enhanced lipid accumulation is observed relative to vehicle controls without adverse effects on cell viability are flagged for gene expression analysis to characterize the metabolic pathways affected.

In the initial test of the assay, the effect of three materials purported to enhance lipid accumulation in adipocytes was evaluated. Two of the test agents were found to promote lipid accumulation in differentiating pre-adipocytes as evidenced by AdipoRed signals that were approximately 3.5 times and 8 times the corresponding vehicle controls, respectively, with no obvious toxicity (gene expression in progress). In contrast, the third agent failed to enhance lipid accumulation relative to the corresponding vehicle control.

Data obtained from a subsequent test of the method involving the same three agents, along with 12 additional test agents, revealed significant inter-assay variability that is likely related to the use of different batches of primary human pre-adipocytes in each assay run. Despite the need for further assay optimization, the method described here represents an important first step towards developing a reliable *in vitro* method for identifying and characterizing compounds with lipofilling-like activity for further study.

INTRODUCTION

Human skin aging occurs at different rates, with some individuals showing fewer signs of aging than others, despite environmental insults. Corrective measures mainly focus on smoothing the surface microtexture, decreasing perceived pigmentation irregularities, and reducing the appearance of lines and wrinkles. For deeper lines and wrinkles and macro folds of the skin, the use of fillers has come into common use.[1,2] The upper and lower thirds of the face, along with the neck, have received the most attention with procedures now being developed for the midfacial areas.[3,4,5,6,7] Injectable fillers and stimulators are used mainly to replace volume in the aging face with autologous fat transfer and grafting becoming more common.[8,9,10] Enrichment of grafts for adipocyte-derived stem cells is now seen as a step toward better long-term success, but given their multipotent nature, a certain degree of caution may be needed in attempting to stimulate differentiation for enhancement of adipocyte number and lipid accumulation.[11,12] In addition to filling voids in the face, a comprehensive anti-aging approach includes resurfacing with chemicals, lasers, and microdermabrasion as well as stimulating the restructuring of the extracellular matrix with topical active ingredients and energy sources such as thermal, light, ultrasound, and radiofrequencies.[13] Methods developed to document the aging face primarily focus on the shape, color, and texture and the facial skin and its thickness.[14,15] However, one aspect of aging facial skin remaining to be adequately targeted is pan-facial subcutis lipoatrophy, or the loss of bulking supportive fat lying beneath the dermis in general.[16,17] In this study, we investigated the applicability of standard *in vitro* cell culture methods for screening topical cosmetic ingredients for use in supporting the maintenance or enhancement of adipocyte number and lipids in the subcutis.

OBJECTIVE

Optimize an *in vitro* cell culture system for the evaluation of cosmetic ingredients capable of supporting or enhancing facial subcutis adipose tissue.

METHODS

CELL CULTURE AND COMPOUND TREATMENTS: Primary human facial preadipocytes were obtained from individual subjects or as pools from multiple subjects from Zen-Bio, Inc. The cells were cultured to confluence in 96-well plates (for lipid accumulation and toxicity assessments) and in 12-well plates (for RNA isolation) using Preadipocyte Medium (Zen-Bio, Cat. #PM-1) before being differentiated into adipocytes over 14 days by replacing the Preadiocyte Medium with Adipocyte Differentiation Medium (Zen-Bio Cat. #DM-2). Test compounds or control vehicles (described in the figures below) were included in the Adipocyte Differentiation Medium during the first seven days of differentiation. After seven days, the differentiation medium and compounds were removed and replaced with Adipocyte Maintenance Medium (Zen-Bio, Cat. #AM-1) and the cells were allowed to continue differentiation for an additional seven days.

ASSAY ENDPOINTS: Cultures treated with each test compound and related vehicles were processed to evaluate lipid accumulation (AdipoRed) and compound toxicity (Cell Titer Blue) using triplicate cultures for each endpoint.

For lipid assessment by AdipoRed, the medium was removed from the cultures and the cell monolayers were rinsed with phosphate-buffered saline (PBS). PBS (200 µL) and AdipoRed (5 µL, Lonza, Cat. #7009) were added to each well, mixed, and incubated for 15 minutes at room temperature. Fluorescence was then read for each well using excitation/emission wavelengths of 485/535 nm.

For toxicity (viability) assessment by Cell Titer Blue, 5 µL of the Cell Titer Blue reagent (Promega, Cat. #TB317) was added to each well containing 100 µL medium. The cultures were then incubated for two hours at 37°C before measuring fluorescence using excitation/emission wavelengths of 560/590 nm.

Cultures were collected for RNA isolation after differentiation by first removing the culture medium and washing the monolayers PBS. After removing the PBS wash, the cells were lysed using 350 µL Buffer RLT (Qiagen) containing 10 µL/mL β-mercaptoethanol with repeated pipetting. The resulting cell lysates were transferred to storage tubes, immediately frozen using a dry ice/isopropanol bath, and stored at -80°C for subsequent analysis.

RESULTS

PROOF-OF-CONCEPT STUDY—Three cosmetic ingredients, purported by the manufacturer to have lipid enhancing activity, exhibited differential effects on lipid accumulation in pooled primary human facial adipocytes from donor pool #1 (Figure 1). The ability of three cosmetic ingredients (A, B, C) to affect lipid accumulation in differentiating human facial adipocytes (donor pool #1) was evaluated using the AdipoRed assay. Two of the three ingredients (A, C) induced significant enhancement of lipid accumulation relative to their respective vehicle controls.

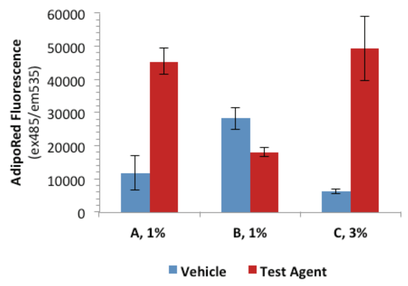


FIGURE 1. Lipid accumulation in pooled primary human facial adipocytes (donor pool #1) exposed to three cosmetic ingredients. Data shown represents mean values from three replicates with error bars equaling one standard deviation.

EXPANDED STUDY—The positive results from the proof-of-concept study (Figure 1) warranted the evaluation of an expanded panel of cosmetic ingredients with respect to enhancement of lipid accumulation by differentiating human adipocytes (Figure 2). In this analysis, performed using a second cell donor pool (donor pool #2), none of the ingredients caused notable enhancement of lipid accumulation in the adipocytes relative to vehicle controls. Interestingly, ingredients A and C, each of which promoted marked enhancement of lipid accumulation in the proof-of-concept study, failed to show activity in this follow-up study.

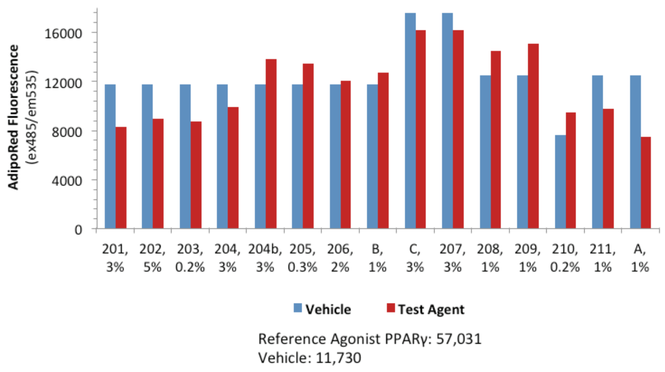


FIGURE 2. Fifteen cosmetic ingredients with purported lipid enhancing activity exhibited minimal effect on lipid accumulation by primary human facial adipocytes from donor pool #2.

INGREDIENT TOXICITY EVALUATION—To assess the possible impact of ingredient toxicity on the cultured adipocytes, the viability of human facial adipocytes differentiated from preadipocytes (originating from donor pool #2), after exposure to same 15 cosmetic ingredients (Figure 2) during differentiation, was assessed using the Cell Titer Blue cytotoxicity assay (Figure 3). This analysis revealed that one ingredient and its base formulation (204 and 204b) caused significant loss of cell viability, while two others (A, C) each demonstrated moderate toxicity. It is interesting to note that in the proof-of-concept (Figure 1), ingredients A and C stimulated lipid accumulation.

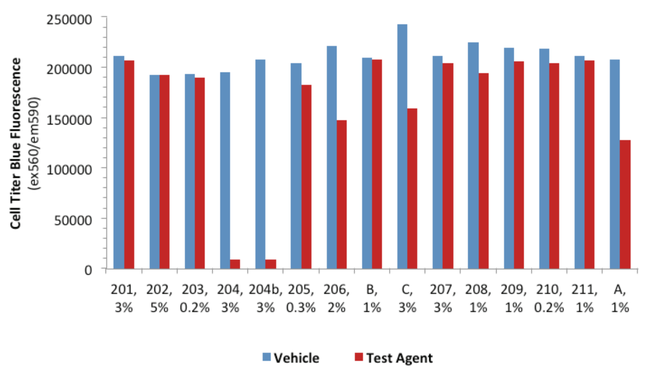


FIGURE 3. *In vitro* cosmetic ingredient toxicity as measured in a Cell Titer Blue cytotoxicity assay.

DONOR/ANATOMICAL SITE VARIABILITY—The variable lipid accumulation results observed for ingredients A and C across studies involving preadipocytes from different donor pools (Figures 1 and 2) prompted a study of variability related to individual preadipocyte donors. For this study, preadipocytes from six individual donors were used to assess adipocyte-stimulating activity of three cosmetic ingredients (B, C, 202), three vehicle controls, and a positive control (PPARγ). The results reveal significant variation in the lipid accumulation potential of differentiating preadipocytes from different donors (Figure 4). None of the cosmetic ingredient test agents produced significantly more lipid accumulation than their vehicle controls, and none exhibited significant toxicity with this set of donors.

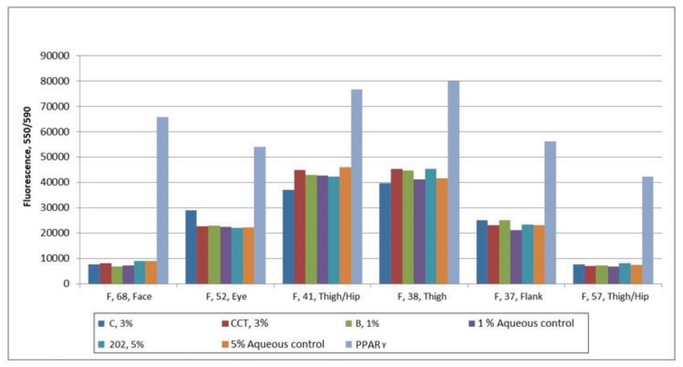


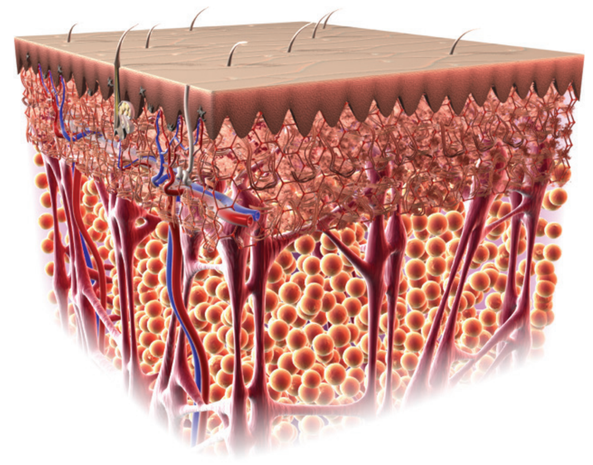
FIGURE 4. Three ingredients (B, C, 202), three controls (5% water, 1% water, and caprylic/capric triglyderide (CCT)) and a positive control (PPARγ) were applied to preadipocyte cultures isolated from differing anatomical sites of six human subjects (F, female) of varied age.

DISCUSSION

The initial proof-of-concept study described here provided encouraging results that suggested that the methods described have significant potential for identifying test agents with lipofilling activity. Variation imparted by differences in preadipocytes obtained from different donors and anatomical sites suggests that to avoid inter-study variability, tissue used for the isolation of preadipocytes should be from subjects of the same sex and age and from a similar anatomical site. Gene expression studies used to evaluate cosmetic ingredient lipofilling activity should be carefully designed to select the preadipocyte tissue most comparable to the *in vivo* site being targeted for treatment.

CONCLUSION

Despite the need for further assay optimization, the method described here represents an important first step towards developing a reliable *in vitro* method for identifying and characterizing compounds with lipofilling-like activity targeted to specific anatomical sites.



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