

Article

Addressing Human Skin Ethnicity: Contribution of Tissue Engineering to the Development of Cosmetic Ingredients

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Abstract: Recent publications describe various skin disorders in relation to phototypes and aging. The highest phototypes (III to VI) are more sensitive to acne, with the appearance of dark spots due to the inflammation induced by *Cutibacterium acnes* (previously *Propionibacterium acnes*). Dryness with aging is due to a lower activity of specific enzymes involved in the maturation of lipids in the stratum corneum. To observe and understand these cutaneous issues, tissue engineering is a perfect tool. Since several years, pigmented epidermis with melanocytes derived from specific phototypes allow to develop in vitro models for biological investigations. In the present study, several models were developed to study various skin disorders associated with phototypes and aging. These models were also used to evaluate selected ingredients' ability to decrease the negative effects of acne, inflammation, and cutaneous dryness. Hyperpigmentation was observed on our reconstructed pigmented epidermis after the application of *C. acnes*, and pollutant (PM10) application induced increased inflammatory cytokine release. Tissue engineering and molecular biology offer the capability to modify genetically cells to decrease the expression of targeted proteins. In our case, GCase was silenced to decrease the maturation of lipids and in turn modify the epidermal barrier function. These in vitro models assisted in the development of ethnic skin-focused cosmetic ingredients.

Keywords: ethnic skin; reconstructed human pigmented epidermis; acne; pollution; dry skin; PIH



Citation: Capallere, C.; Arcioni, M.; Restellini, L.; Imbert, I. Addressing Human Skin Ethnicity: Contribution of Tissue Engineering to the Development of Cosmetic Ingredients. *Cosmetics* **2021**, *8*, 98. <https://doi.org/10.3390/cosmetics8040098>

Academic Editor: Enzo Berardesca

Received: 8 September 2021

Accepted: 8 October 2021

Published: 15 October 2021

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1. Introduction

Since years, cosmetic industries have developed suitable solutions to deliver skin benefits to a large range of skin tones in mass market products. More recently, products have been developed to focus on specific skin tones according to the skin phototype. To target more precisely the needs of consumers, two marketing concepts have emerged based on ethnic habits or geographic location, respectively named Ethno-cosmetic and Geo-cosmetic. To answer to consumer needs regarding skin benefits, the study and the classification of skin according to their phototype has been an initial step. Several scientists classified skins according to their phototype such as Fitzpatrick in 1975 (classification from I to VI, from clear skins to dark skins), Lancer added a parameter related to racial background and Goldman added to this scale a geographic parameter [1–3]. Then, technology has made possible to measure a skin score with the determination of L^*a^*b (L^* indicates lightness, a^* is the red/green coordinate, and b^* is the yellow/blue coordinate) [4]. All these scales are still considered as references to classify skin types. However, nowadays, it's challenging to classify skin according to ethnic background only, indeed, geographical location and lifestyle are also parameters which must be considered [5]. Across all skin types, the aging process involves photodamage, fat redistribution, and the loss of connective tissue [6,7]; however, it has been reported that individuals with darker skin have firmer and smoother skin than individuals with lighter skin thanks to a higher concentration of collagens and lower activity of collagenases in the dermis [8–10]. It has been reported that individuals with darker skin have firmer and smoother skin than individuals with lighter skin due to a higher concentration of collagen and less collagenase activity in the dermis [8–10]. On the

opposite, some skin diseases (e.g., acne and dry skin) are more pronounced—based on both clinical and prevalence observation—in individuals with certain phototypes. Acne is more prevalent in African Americans than in Hispanic, Asian, Caucasian, or Indian people (37%, 32%, 30%, 24%, and 23%, respectively) [11] and, with age, differences between ethnicities become more and more pronounced. The development of acne (a chronic inflammatory disease involving *Cutibacterium acnes*) is mainly driven by an increased production of sebum, increased proliferation and reduced desquamation of keratinocytes in the pilosebaceous unit [12]. Hyperpigmentation is one of the consequences of inflammation-induced diseases such as acne and environmental factors such as pollution [13]. Several scientific publications investigating differences between skin types found that dark skins (phototypes IV to VI) have high sensitivity to hyperpigmentation [14,15]. Indeed, dark skins are characterized by a high inflammatory basal level which affects skin hyperpigmentation, blemishes, and scars [16]. The severity of post inflammatory hyperpigmentation (PIH) is determined by the inherent skin color and the degree and depth of inflammation [17,18]. Moreover, pollution represents another external factor which can exacerbate skin disorders. It was evidenced that pollutants (particulate matter (PM) or benzo(a)pyrenes) increase the consequences of acne [19] by the activation of cell metabolism and inflammatory processes via AhR pathways. For example, in normal human epidermal keratinocytes (NHEKs) exposed to O₃ (a well-known atmospheric pollutant), activation of the AhR pathway in NHEKs has been linked to the induction of proinflammatory molecules such as interleukin-8 [20,21]. As observed clinically, dark skin is also more prone to dryness with age [22] than lighter skin is. The enzyme glucocerebrosidase (GCCase) plays a central role in the maturation of ceramides, which are the major component of the “brick and mortar” structure of the stratum corneum and allow the epidermal permeability barrier to function correctly [23]. Inhibition of GCCase activity reduces formation of the permeability barrier, while a complete lack of GCCase results in a disease phenotype with fatal skin abnormalities [24].

An appropriate understanding of skin ethnicity with the technical support of tissue engineering is required to develop new in vitro models in order to evaluate cosmetic ingredients tailored to specific skin ethnicities and related disorders. More than 40 years ago, cutaneous in vitro models were developed to evaluate the sensibility of skin ex vivo, including reconstructed epidermis (developed on insert or feeder layers), dermis (collagen matrix in combination with fiber proteins), full thickness (dermis and epidermis), etc. [25–32]. These in vitro skin models can also be used to better understand mechanisms linked to pigmentation, inflammation, and aging in order to develop cosmetic ingredients capable of decreasing or slowing these processes. Indeed, reconstruction of epidermis using specific keratinocytes (isolated according to their phototype) is possible and, in combination with “ethnic” melanocytes, the reconstruction of pigmented epidermis offers various possible ways to study “ethnic” disorders. Focusing on dark skin-specific disorders, several in vitro models linked to these ethnic issues have been developed on melanocytes and reconstructed pigmented epidermis (phototypes V–VI) in order to assess the efficacy of cosmetic ingredients. Based on ethnic clinical observations, we developed *specific* in vitro models using classic or pigmented epidermis in order to mimic the effects of acne, pollution, or cutaneous dryness, thereby allowing one to assess the ability of ingredients to counteract the negative impact of these conditions.

2. Materials and Methods

2.1. Ethical Compliance

Ex vivo skin samples were obtained from anonymous healthy donors after abdominal plastic surgery. Surgical residues were harvested according to French regulations (agreement DC 2011-1323) and after procurement of written informed consent from patients.

2.2. Isolation of Ethnic Cells (Keratinocytes and Melanocytes)

Normal human primary epidermal keratinocytes (NHKs) and melanocytes (NHEMs) were isolated following surgery. A first enzymatic digestion (Dispase II, Sigma, St Quentin

Fallavier, France, overnight at 4 °C) was used to dissociate the epidermis from the dermis, followed by a second enzymatic digestion (Trypsin/EDTA, Sigma, St Quentin Fallavier, France, 10 min at 37 °C) to obtain isolated epidermal keratinocytes and melanocytes. The cells were centrifugated and the pellet was resuspended in MCDB153 modified medium complemented with Bovine pituitary extract [33]. After several days of culture, NHEMs were separated from NHKs by differential trypsinization. NHEMs were cultivated in M2 growth medium complemented with Supplement Mix (Promocell, Germany, Hedelberg) and NHKs were cultivated in a proprietary low-calcium medium. The NHKs and NHEMs were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ [34].

2.3. Reconstruction of Pigmented Epidermis

After isolation of NHKs and NHEMs, the cells were seeded onto a 0.5 cm² inert polycarbonate membrane (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) in a proprietary chemically defined media (MCDB153 complemented with growth factors) at the air–liquid interface for 10 days at 37 °C in a humidified atmosphere containing 5% CO₂.

2.4. Silencing

GBA siRNA (HS 282997, Thermo Fisher Scientific, Waltham, MA, USA) was prepared according to the manufacturer's recommendation and was transfected into human keratinocytes using lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA). Some of the cells were harvested and analyzed by quantitative polymerase chain reaction to validate the silencing procedure, while the rest was placed into cell culture inserts to reconstruct epidermis. Stealth RNAi Negative Control Duplex (Thermo Fisher Scientific, Waltham, MA, USA) with medium GC content was used as a negative control as recommended by the manufacturer.

2.5. Histology and Immunohistochemistry

For histological analysis, reconstructed human epidermises were fixed in 10% formalin buffer (Sigma, Sigma, St Quentin Fallavier, France) for at least one hour at room temperature. After successive dehydration steps, the tissues were embedded in paraffin. Paraffin sections (4 µm) were deposited on glass slides, deparaffinized, and rehydrated in xylene baths, alcohols of different percentages, and water. Hematoxylin and eosin staining (H&E) was performed by placing the sections in a hematoxylin bath, rinsing them with water for 5 min at room temperature, and placing them in an eosin bath. The tissues were then dehydrated in successive baths of absolute alcohol and xylene. After mounting a coverslip with Eukitt Mounting Medium (O. Kindler), pictures were acquired with a Qimaging Retiga 2000R Fast 1394 camera and processed using the Q-Capture Pro 7 (QImaging) acquisition software.

For immunohistochemistry (IHC), embedded sections of RHE were obtained as described above. Exposure of the antigens was realized by treatment of the sections with 0.01 M citrate buffer pH6 (Sigma) and 0.25% pepsin or 0.05% trypsin (Zymed, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at 37 °C. Saturation of nonspecific sites was performed with 5% BSA buffer (Sigma) for 30 min before primary antibodies (mouse monoclonal pmel17Abcam, mouse monoclonal Tyrosinase; Santa Cruz, Dallas, TX, USA) were applied at room temperature for 1 h. Sections were rinsed in PBS and the secondary antibody was applied at room temperature under dark conditions for 1 h. Finally, the slides were incubated with 0.3 µM 4',6'-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA) for 5 min at room temperature in the dark and mounted with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA). The photos were taken with a Qimaging EXI blue camera paired with the Volocity acquisition software (Improvision, Coventry, England).

2.6. Fontana-Masson Staining

For histological analysis, the reconstructed human pigmented tissues were fixed in 10% formalin buffer (Sigma, St Quentin Fallavier, France) and embedded in paraffin [35]. Paraffin section (4 μm) were cut and used for Fontana-Masson staining. The Fontana-Masson stain is based on melanin's ability to reduce ammoniacal silver nitrate to metallic silver (brown) without the use of an external reducing agent. Sections were deparaffinized and rehydrated (as described above) before a 10% solution of ammoniacal silver nitrate was added to each section and slides were incubated for 45 min at 60 °C. After washing with distilled water, the tissues were incubated with 5% sodium thiosulfate (Sigma). Slides were washed in a distilled water bath and finally dehydrated in several alcohol and xylene baths. The slides were mounted with a coverslip using Eukitt Mounting Medium (O. Kindler), pictures were acquired with a Qimaging Retiga 2000R Fast 1394 camera and processed using the Q-Capture Pro 7 (QImaging, Teledyne Photometrics, Tucson, AZ, USA) acquisition software.

2.7. Statistics

Statistical analysis was performed using Student's *t*-tests for independent samples with one-tailed direction of rejection. * $p \leq 0.05$ was considered significant, ** $p \leq 0.01$ as very significant, and *** $p \leq 0.005$ as highly significant using JMP software (version 14, SAS France).

3. Results

Tissue engineering affords unique opportunities to develop specific in vitro models focusing on skin ethnicity and related disorders. Indeed, isolation of specific ethnic cells such as keratinocytes or melanocytes offers the possibility to reconstruct "ethnic" tissues. The techniques of keratinocyte isolation and epidermis reconstruction allowed us to obtain three-dimensional human tissues with characteristics close to those of in vivo epidermis in terms of morphology, protein expression, and physiological responses to chemical stress such as irritation [21]. The next step was to include other cell types, such as melanocytes, to obtain pigmented epidermis. From keratinocytes and melanocytes isolated from human skin following plastic surgery, a pool of cells can be deposited onto the culture insert. After 10 days of culture, a pigmented epidermis (RHPE) was obtained with all characteristic layers (basal, spinous, granular, and corneal layers) and functional melanocytes were identified in the basal layer by immunodetection of pmel17 and tyrosinase (two specific proteins expressed by melanocytes) (Figure 1).

From morphological observation, RHPE is comparable to native epidermis with the presence of characteristic epidermal layers. In our in vitro model (epidermis reconstructed with melanocytes), melanocytes (phototypes V to VI, according to Fitzpatrick classification) are visible with H&E staining. The presence of melanocytes was confirmed by Fontana-Masson staining (a dye specific to melanin) in the basal layer. The functionality of melanocytes was detected by the immunodetection of two specific proteins involved in the synthesis of melanin (pmel17 and tyrosinase).

After the development of specific human epidermal tissues, these tissues could be used to observe, in vitro, specific skin disorders such as hyperpigmentation induced by acne. Indeed, the inflammation induced by *C. acnes*, which is more pronounced in darker skin of higher phototypes, induces hyperpigmentation caused by an increase in melanin. An in vitro model was developed to observe the effect of *C. acnes* on the pigmentation of reconstructed human pigmented epidermis (Figure 2) and to evaluate the effect of a cosmetic ingredient designed to prevent this inflammation-induced hyperpigmentation.

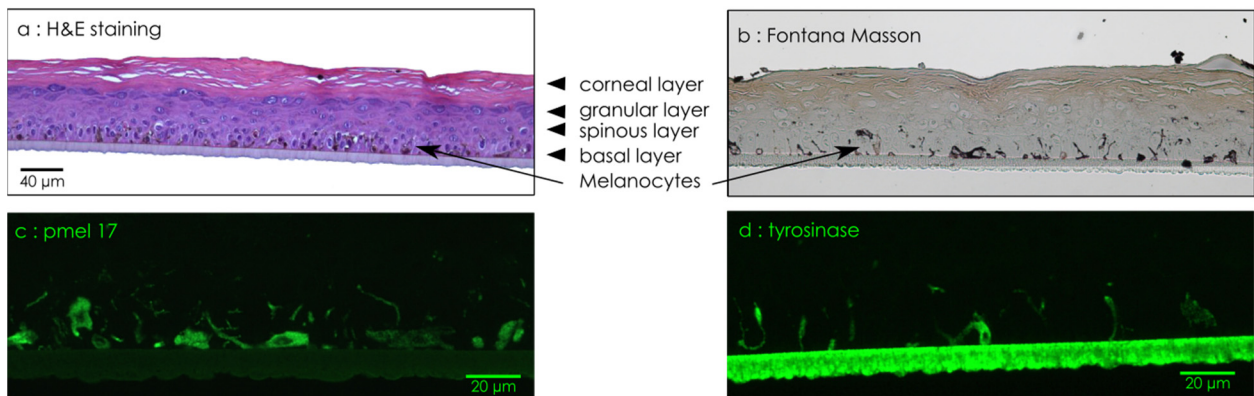


Figure 1. Microscopic observation and immunodetection of proteins in RHPE. Microscopic observation after hematoxylin/eosin staining (a) and melanocyte observation (dark arrows) after Fontana-Masson staining (b). Immunodetection of pmel17 (c) and tyrosinase (d) in melanocytes present in the basal layer of pigmented epidermis (at day 10).

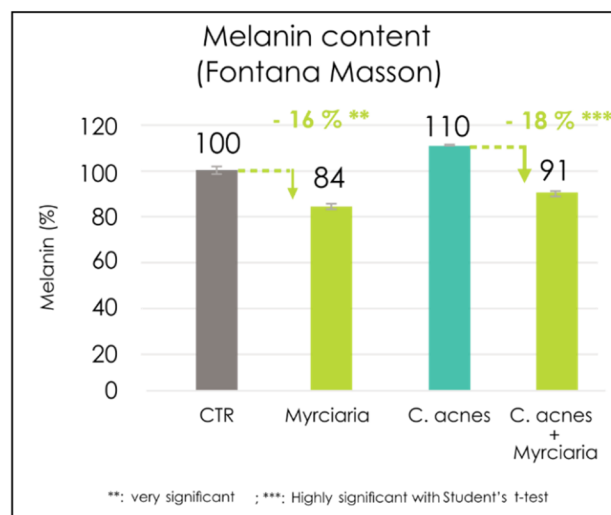
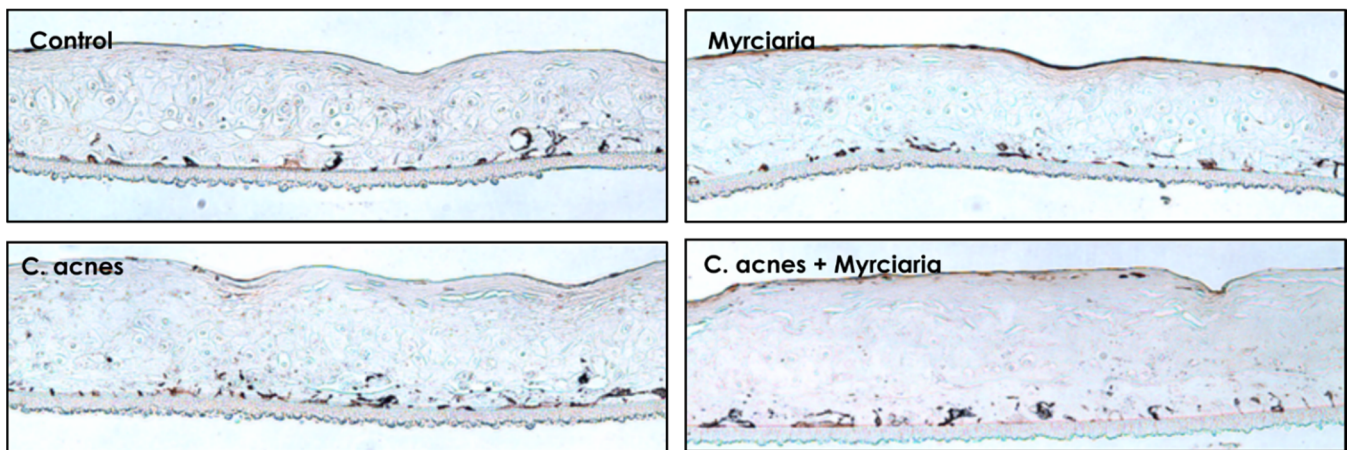


Figure 2. Effect of *Myrciaria Dubia* fruit extract on hyperpigmentation induced by *C. acnes*. Melanin was stained by Fontana-Masson staining. RHPE were pretreated with *Myrciaria dubia* fruit extract once a day for 2 days from Day 10. *C. acnes* was applied at day 12 for 24 h. Staining was performed on RHPE at day 14 (three pictures per condition were analyzed).

At day 10, *Myrciaria Dubia* fruit extract was topically applied to RHPE for 48 h before *C. acnes* was applied to the top of the RHPE for 24 h. Melanin quantification was determined 96 h after the beginning of the treatment (D14). *Myrciaria Dubia* fruit extract treatment induced a 16% ** decrease in skin pigmentation compared with the control. Application

of *C. acnes* induced an increase in pigmentation and Myrciaria Dubia fruit extract was associated with the protection of epidermis against this hyperpigmentation (-18% ***, compared with the *C. acnes* condition).

Recently, researchers demonstrated the relationship between pollution and increased acne stigma [15]. To further investigate the impact of pollution, a second in vitro model was developed to analyze the effect of pollutants on RHPE—more precisely, the effect of pollutants whose particulate size is less than $10\ \mu\text{m}$ (PM 10)—and to quantify the expression of the inflammatory cytokine IL8 (Figure 3).

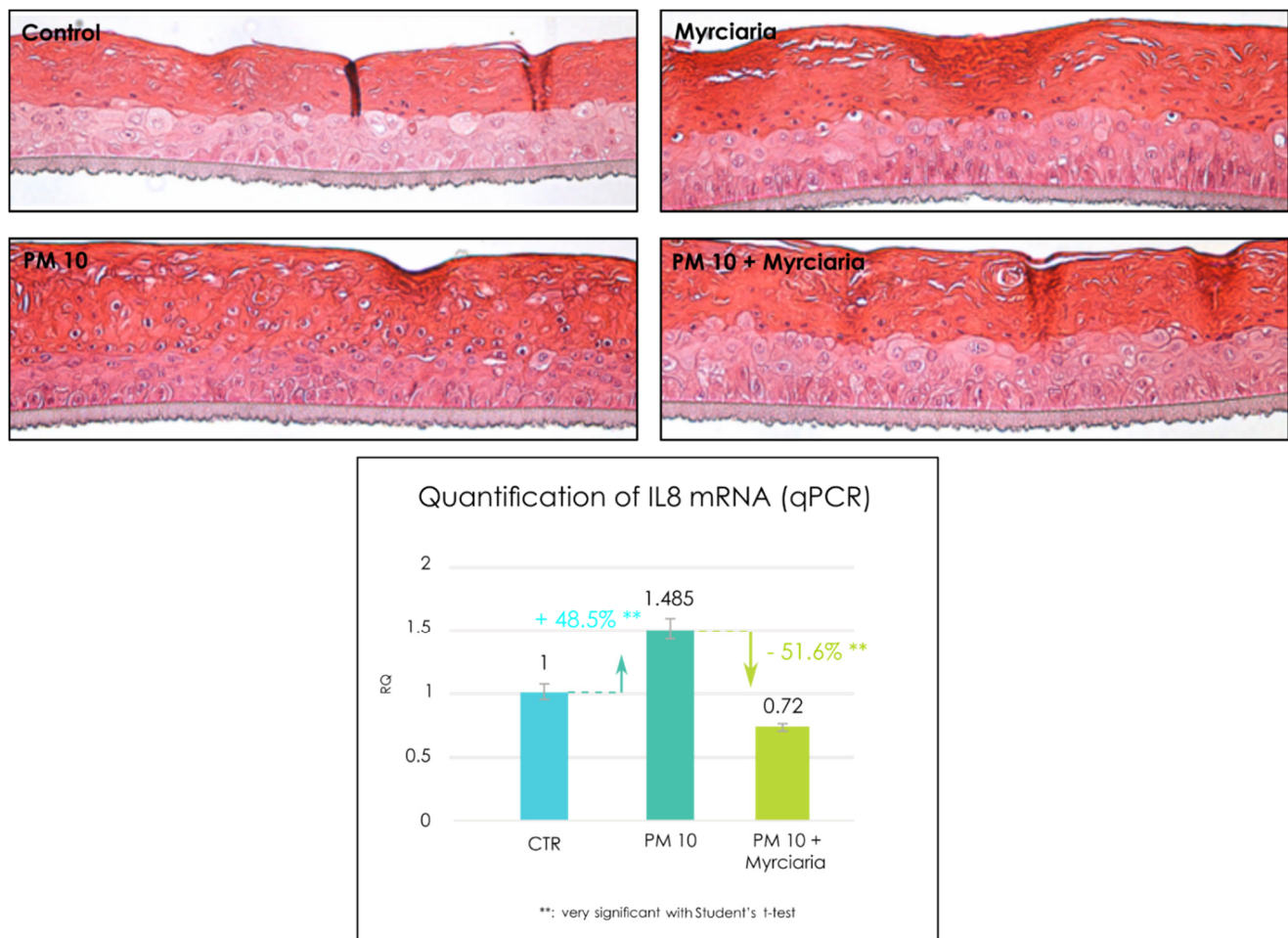


Figure 3. Effect of Myrciaria Dubia fruit extract on inflammation induced by pollutant (PM10) on RHPE. Histological observation was performed on RHPE after hematoxylin/eosin staining. RHPE were pretreated with Myrciaria Dubia fruit extract once a day for 2 days from Day 10. PM10 (particulate matter $< 10\ \mu\text{m}$) were applied at day 12 for 24 h. Staining was performed on RHPE at day 14.

In this experiment, inflammation ($+48.5\%$ **) was induced by pollutants quantified by the expression of IL8 mRNA on RHPE. Prevention with Myrciaria Dubia fruit extract treatment for 48 h before the application of pollutants was associated with a 51.6% ** decrease in inflammation. Hyperpigmentation was not observed on RHPE after the application of pollutant in this protocol (data not shown).

As previously discussed, an increased potential for darker skins to develop dry skin with aging due to a weak level of lipid content in the stratum corneum has been reported. Glucocerebrosidase enzyme (GCCase) is an enzyme involved in the synthesis of ceramides from glucosylceramides. A GCCase-deficient in vitro model was developed to mimic aged epidermis displaying low activity in this enzyme. Using molecular biology tools such as the silencing of specific RNA, it was possible to study the consequences of this enzyme

deficiency. Before the reconstruction, keratinocytes were silenced with siGBA, harvested, and placed at the air–liquid interface inside the insert for several days.

Silencing of siGBA in keratinocytes before epidermal reconstruction induced a 27% decrease in lipid expression, as detected by Nile red staining (Figure 4). Myrciaria Dubia fruit extract application for 2 days was associated with an increase in lipid expression in both normal RHE (+83.5%) and in GCCase-deficient RHE (+552%).

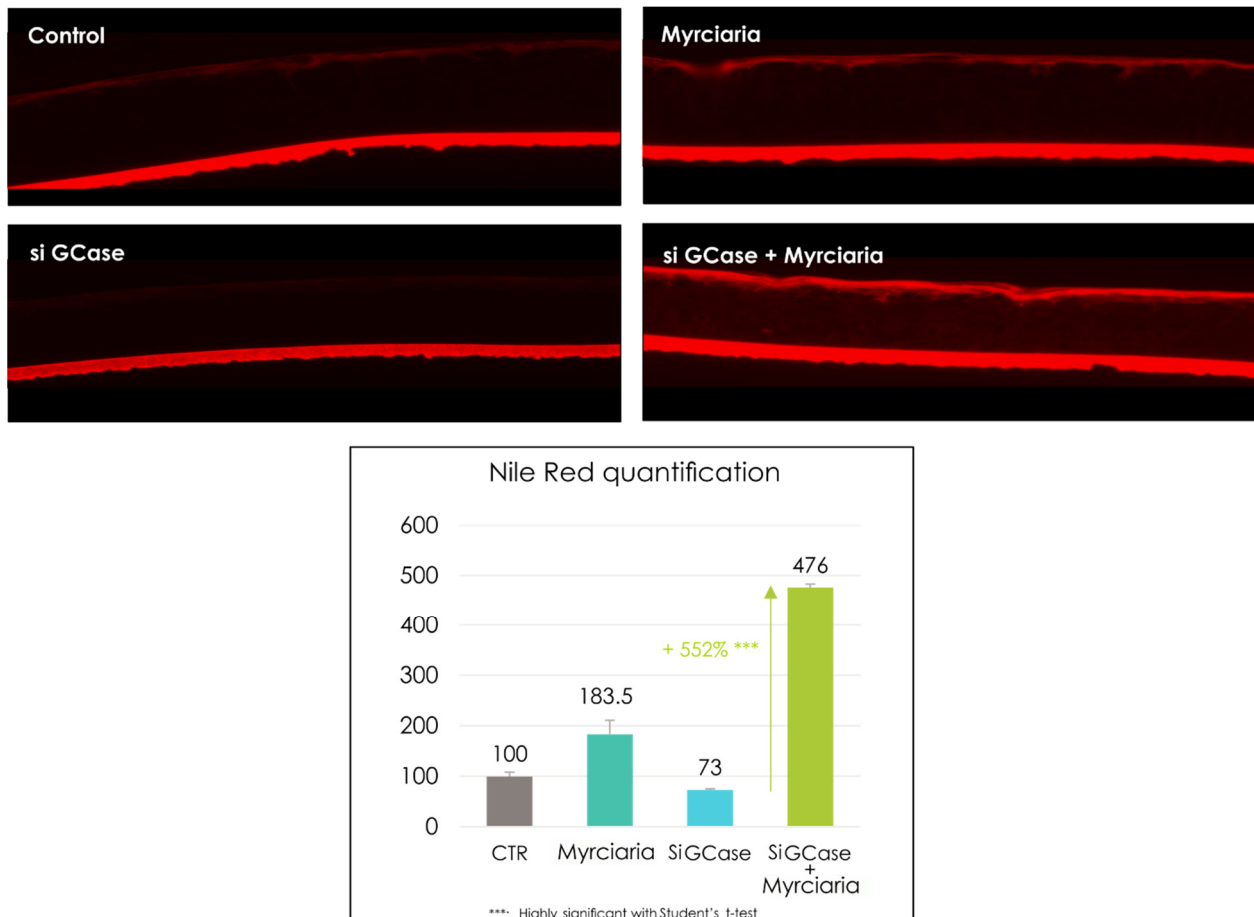


Figure 4. Effect of Myrciaria dubia fruit extract on lipid synthesis in GCCase-deficient RHE. Keratinocytes were silenced with siGBA before the reconstruction. At day 6, RHE were treated with Myrciaria Dubia fruit extract for 2 days. Nile red staining was performed on RHE at day 8 (three pictures per condition were analyzed).

4. Conclusions

With each skin being unique in terms of its biological parameters (tone, thickness, sun reaction, and sensitivity), each skin also demonstrates unique responses to environmental exposure. Similarly, individuals within the same ethnic group can have different skin types and thus different predispositions toward skin conditions.

Interestingly, skin tone classification allows one to determine potential skin disorders that may arise with age. Skin disorders commonly reported in dark skins—such as inflammation, post-inflammatory hyperpigmentation, and dryness—were studied with the support of tissue engineering and, more precisely, the reconstruction of epidermis including melanocytes to mimic different skin tones.

The goal of this publication was to demonstrate that tissue engineering, specifically reconstructed human epidermis, can assist in the study of disease related to ethnic skin. Reconstructed human epidermis has been commercially available for several years [36] and can be used to study skin functions, to mimic some cutaneous diseases, or to evaluate toxicity [37–42]. In the present study, several in vitro models were developed to observe

hyperpigmentation induced by *C. acnes* stress on RHPE, inflammation by specific pollutants (PM10), or dryness by silencing of a specific enzyme (glucocerebrosidase). The first model developed focused on acne and its primary consequence: post-inflammation induced hyperpigmentation. Based on reconstructed pigmentary epidermis (RHPE), reconstruction of epidermis containing keratinocytes and melanocytes (selected according to their phototype) allowed us to study the effect of the application of the main bacteria involved in acne: *Cutibacterium acnes*. After 48 h of *C. acnes* topical application, pigmentation was increased in RHPE. This hyperpigmentation resulted in the appearance of dark spots after skin inflammation [38] due to the activation of the TLR pathway by *C. acnes* [43]. *C. acnes* was recognized by TLR2, which induces the expression of inflammatory cytokines via NF κ B pathways [43].

A second in vitro model was developed to study the impact of pollution on the skin and its ability to exacerbate the consequences of acne. Pollution can penetrate to the deeper layers of the skin inducing ROS production and antioxidant depletion, activation of aryl hydrocarbon receptors, and induction of inflammatory responses [44]. In our model, the application of PM10 pollutants induced morphological modifications resulting in impaired barrier function and an inflammatory response [45]. This inflammatory response exacerbates the hyperpigmentation of acne [46].

Another characteristic of higher skin phototypes is that, with aging, the skin becomes drier due to the accumulation of stratum corneum, resulting in lower enzymatic activities [47]. Thus, a third model was developed to evaluate the physiological consequences of the silencing of a key enzyme (glucocerebrosidase) involved in this process of lipid maturation [48]. Silencing of the glucocerebrosidase gene in keratinocytes used to reconstruct human epidermis induced a downregulation of both loricrin and filaggrin (two main proteins involved in epidermal differentiation) (data not shown) and total lipid synthesis [48,49].

A large range of models developed using tissue engineering techniques allow for the investigation of various topics of interest regarding skin ethnicity and associated disorders. They can also be used to evaluate cosmetic ingredients and to put forward solutions focused on specific skin types. In this study, Myrciaria Dubia fruit extract was studied using these in vitro models and was shown to prevent the appearance of hyperpigmentation induced by acne and to limit the inflammation induced by pollutants. It also demonstrated the interesting benefit of increasing the expression of lipids to counteract aging in terms of skin dryness.

Author Contributions: Writing—original draft preparation, C.C., M.A. and L.R.; Supervision, I.I. All authors have read and agreed to the published version of the manuscript.

Funding: No funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Authors wish to thank C. Plaza and C. Meyrignac (Ashland Specialties France) about their technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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