The studies of skin aging and anti-aging

−New mechanism and efficacious biofunctional ingredients−

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DOCTORAL DISSERTATION

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by

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概要

皮膚は人体最大の臓器であり、多くの重要な機能を果たす。外傷、紫外線および赤外線、熱、環境汚染、病原性微生物など、外部刺激や環境因子から生体内部を防御する上で重要な役割を担う。

　生理学において皮膚老化とは、皮膚細胞における表現型の変化に伴う組織の再構築・再構成が、毛穴や肌理(きめ)の乱れ、シワやたるみ、色素沈着によるシミやくすみなどとして現れるものであると広く認識されている。ヒトの皮膚は、表皮と真皮によって構成される。表皮においては、角化細胞が主要な細胞でありその構成要素である。表皮は皮膚の最表層を形成することから、外部刺激や環境因子の影響を直接受ける。これらの外的要因は、角化細胞において酸化ストレスや炎症を引き起こすことで表皮の老化を促進し、結果として肌理の乱れ、毛穴の目たちなどを生じさせる。さらに、色素細胞も表皮基底層に生存し、その色素を生成する活性がおもに角化細胞によってコントロールされていることが明らかにされており、皮膚の色素沈着も表皮角化細胞が大きく関わっていることが知られている。一方、真皮においては、線維芽細胞が主要な細胞であり、線維芽細胞により合成される細胞外マトリックスが主な構成要素である。老化の過程では、内因性および外因性因子により線維芽細胞の老化が起こることで、細胞外マトリックスの量および質が低下し、それが皮膚のシワやたるみの原因になることが知られている。したがって、表皮における角化細胞、および真皮における線維芽細胞は、皮膚老化を制御する上で最も重要な標的となる。

　近年、人々の平均寿命が延びる一方で、皮膚老化が大きな関心事となっている。それは、皮膚老化が外見に大きく影響を与えるだけでなく、個人の生活の質（QOL; Quality of Life）に影響を及ぼす一連の病患を引き起こすためであり、皮膚老化の進行を制御することがますます重要となる。しかしながら、皮膚老化のメカニズムに関する知見は未だ乏しく、さらなる解明を要する。また，皮膚に対するアンチエイジング効果を持つ生体機能性成分がいくつも知られており、それらは様々な薬や健康食品などに使われ、人体にも安全だと思われている。しかし、その作用機序については不明な点が

多く、それら生体機能性成分についての研究も同様に不可欠である。

本研究ではまず、経年的に被験者の皮膚老化過程を疫学的に調査し、その結果、肌理の状態およびシワの有無、そして色素沈着によるシミの有無の三点が皮膚の見た目の老化にとって最も重要な要素だと認めた。上記のように、これらの皮膚老化要素が表皮角化細胞および真皮線維芽細胞と密接に関わっていることが知られていることから、三つの皮膚老化要素を抑制するために、角化細胞および線維芽細胞を標的とした細胞生物学的、分子生物学的解析、およびバイオインフォマティクスを用いた研究を行うことで、新たな皮膚老化に関するメカニズムを明らかにすることを目的とした。さらに、生体機能性成分の作用機序を解明することにより、有効であり、かつ安心・安全な皮膚アンチエイジング戦略の構築を目指している。

はじめに、ヒト角化細胞のアリール炭化水素受容体（AhR）および NF-E2 p45 関連因子（Nrf2）の関与するシグナル伝達における Opuntia ficus-indica 抽出物（OFIE）の抗酸化作用について in vitro 試験を行った。その結果、OFIE は AhR-Nrf2-NQO1（NAD(P)H 酸化還元酵素、キノン 1）経路を活性化することで抗酸化作用を持つことが示された。実験結果はさらに、OFIE が AhR 依存的にフィラグリン（Filaggrin; FLG）およびロリクリン（Loricrin; LOR）の発現を増強することを示した。これは、OFIE が皮膚バリアの破壊を防ぐことを示唆する。

次に、ヒトの老化した真皮および若い真皮におけるマイクロ RNA の発現を網羅的に比較するとともに、細胞老化抑制への有効性が実証された生体機能性成分による、マイクロ RNA 発現様式への影響を調べた。その結果、miRNA 34 および 29 が、線維芽細胞の老化および細胞外マトリックスの再構築を制御する上で中心的な役割を果たすことが明らかとなった。

最後に、表皮角化細胞におけるメラニンの生物学的作用を明らかにするため、単純化細胞モデルを構築することで角化細胞によるメラニンの取り込みを調べ、さらにこのモデルにおけるナイアシンアミドの有効性を評価した。その結果、ヒト表皮角化細胞によるメラニンの直接的な取り込みを示す証拠が得られたほか、ナイアシンアミドおよびトリプシン阻害剤が表皮角化細胞におけるメラニン取り込みを抑制することが

示された。取り込まれたメラニンは角化細胞における細胞周期を停止させ、その増殖を抑制した。さらにマイクロアレイ解析では、メラニンによる表皮角化細胞における Dickkopf 1（DKK1）遺伝子の発現低下が明らかとなった。

　これらの結果は、肌理の状態、シワの有無、色素沈着によるシミの有無という皮膚老化要素に影響を与える角化細胞および線維芽細胞の働きについて新たな知見を提供するものであり、ヒトにおける皮膚老化の抑制に向けた新たな戦略を立てるうえで大きな示唆を与えている。さらに、生体機能性成分が持つ皮膚老化要素に対する有効性をより強固にサポートしている。これらの成果は、今後の新たな皮膚アンチエイジング技術の開発を通して、肌理の乱れ、シワやシミといった多くの人の皮膚老化への懸念を解消し、高齢化社会における人々の QOL の向上に資するものと期待される。

1. BACKGROUND

1.1   Skin, the largest organ of human body

1.2   Skin aging, clinical and physiological change, the causes of skin aging

1.3   Skin care, the primary approach to maintain the healthy and youthful skin

2. INTRODUCTION

2.1   Longitudinal study of skin aging progress

2.2   Comprehensive mechanism of skin aging

- Mechanism of epidermis aging: overall and AhR-Nrf2 signaling

- Mechanism of dermis aging: overall and miRNA's role

- Mechanism of skin hyperpigmentation: overall introduction and melanin uptake

2.3   Several biofunctional ingredients

2.4   Purpose of the series of the studies

3. MATERIALS AND METHODS

3.1   Longitudal study of skin aging progress

- Los Angles facial wrinkling longitudinal study

- Akita facial skin longitudinal study

3.2   Study of OFIE and activation of AgR-Nrf2 signaling

- Reagents and antibodies

- Cell culture

- Reverse transcription-polymerase chain and quantitative real-time polymerase chainreaction analyses

- Detection of ROS production by microscopy

- Statistical analysis

3.3　Study of age-dependent miRNAs in human dermis

- Tissue collection from donors

- Primary cultures of human dermal fibroblast and the induction of senescence

- RNA extraction

- miRNA microarray and data analysis

- miRNA target gene prediction, annotation and function analysis

- Real-time quantitative reverse transcriptase PCR

- Senescence-associated β-galactosidase staining

- Statistical Analysis

3.4　Study of specific pattern of miRNAs modulated in human skin fibroblasts by a biofunctional complex

- Properties of the tested natural ingredient complex

- miRNAs of interest

- Target genes

- qPCR array experiments

- Interaction network of miRNA and genes

3.5　Study of melanin uptake of keratinocyte by a newly established cell model

- Cell Culture

- Measurement of melanin uptake

- Examination of melanin uptake in cultured keratinocytes and solar lentigo tissues

- MTT Assay

- Immunofluorescent staining

- Immunohistochemistry

- Flow cytometry

- RNA isolation and RT/Real-time PCR

- Western blot analysis

4. RESULTS

4.1   Longitudinal study of skin aging progress

- Los Angles facial wrinkling longitudinal study

- Akita facial skin longitudinal study

4.2   Study of OFIE and activation of AhR-Nrf2 signaling

- Upregulation of Nrf2 and NQO1 by OFIE

- Anti-oxidation activity of OFIE was Nrf2 dependent

- OFIE activated the AhR-Nrf2 pathway

- AhR-dependent upregulation of epidermal barrier proteins by OFIE

- OFIE prevented Th2 cytokine-induced downregulation of epidermal barrier proteins

4.3   Study of age-dependent miRNAs in human dermis

- Differential expression of miRNAs in human aged dermis

- miRNA target gene prediction, annotation and function analysis reveals multiple processes involving dermis aging

- Construction of miRNA-Gene-Network

- Alteration of miRNAs expression associated with cultured replicative senescent fibroblasts

4.4   Study of specific pattern of miRNAs modulated in human skin fibroblasts by a biofunctional complex

- List of studied miRNAs

- Relative expression of each miRNAs and potential target genes

- Predictive results on gene expression

- New genes closely linked with miRNAs modulated by ingredient treatment

4.5   Study of melanin uptake of keratinocyte by a newly established cell model

- Direct uptake of melanin in human epidermal keratinocytes

- Niacinamide and trypsin inhibitor suppressed melanin uptake in epidermal

keratinocytes

- Melanin inhibited cell proliferation and Ki-67 expression in epidermal keratinocytes

- Melanin arrested the cell cycle progression of epidermal keratinocytes

- Microarray study revealed the downregulation of DKK1 gene expression in human epidermal kerationocytes by melanin

5. DISCUSSION

5.1 Longitudinal study of skin aging progress

5.2 Antioxidant OFIE activates AhR-Nrf2 signaling and upregulates filaggrin and loricrin expression in human keratinocytes

5.3 miRNA-34 and miRNA-29 families may play major roles in dermis aging

5.4 Modulation of a Specific Pattern of microRNAs, Including miR-29a, miR-30a and miR-34a, in Cultured Human Skin Fibroblasts, in Response to the Application of a Biofunctional Ingredient that Protects against Cellular Senescence in Vitro

5.5 Melanin uptake reduces cell proliferation of human epidermal keratinocytes

5.6 Overall discussion

6. CONCLUSION

1. BACKGROUND

1.1 Skin

The skin is the largest organ in the human body in terms of surface area and mass, and it fulfills many important functions. First, the skin has a number of protective functions. The primary function of the skin is to act as a barrier that plays an important role in protecting the inner organism from external environmental factors such as heat, UV radiation, pollution, pathogens, or mechanical stress. Secondly, the skin has regulatory functions. It regulates numerous physiological properties, such as body temperature (through sweating and the presence of hair) or peripheral circulation and fluid balance (through sweating). Moreover, the skin is also an important organ for the synthesis of vitamin D. Thirdly, the skin has functions in the perception of sensations. The skin contains an extensive network of nerve cells that detect and relay changes in the environment; there are separate receptors for heat, cold, touch, and pain.

The skin consists of two layers: the epidermis and the dermis (Fig. 1.1.1). Between the epidermis and dermis is the basement membrane, and beneath the dermis lies subcutaneous fatty tissue. Each layer has specific characteristics and functions.
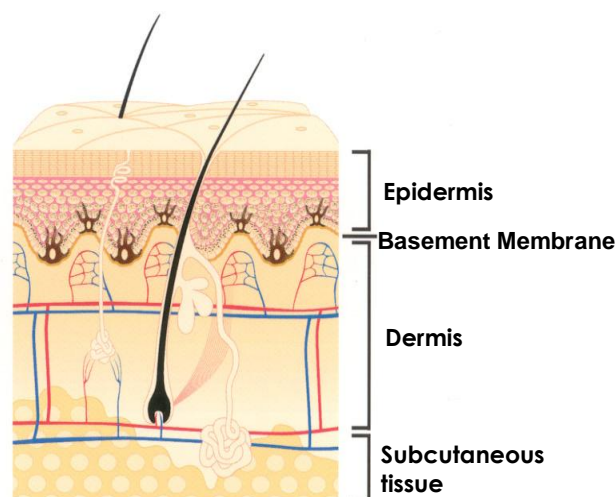

Fig. 1.1.1.   Illustration showing the structure of skin

(Xianghong Yan; Copyright P&G)

The epidermis is the outermost layer of the skin and is about 0.1–0.2 mm thick. The epidermis is important to the appearance of the skin, because it contributes to the texture and moisture of the skin and it defines the skin color. The epidermis consists of four layers; from innermost to outermost, these are the basal layer (*stratum basale*), the spinous layer (*stratum spinosum*), the granular layer (*stratum granulosum*), and the horny layer (*stratum corneum*) (Fig. 1.1.2). Keratinocytes form the majority of cells in the epidermis. The epidermis is a dynamic layer that undergoes constant turnover. In this turnover process, keratinocytes are initially produced in the basal layer and undergo a series of changes in form as they move upward through the epidermis and become mature before they finally lose their nuclei and granules and are shed (desquamated) from the surface. Each layer is important to the epidermis, and each plays a different role in terms of its biological functions.
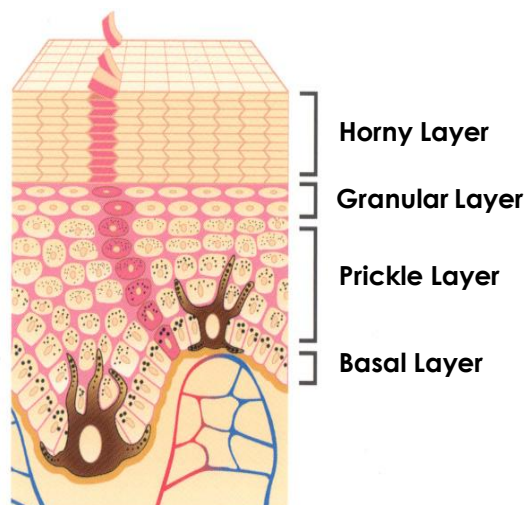

Fig. 1.1.2.   Illustration showing the structure of the epidermis

(Xianghong Yan; Copyright P&G)

The dermis lies between the epidermis and the subcutaneous fatty tissue. It is responsible most of the thickness (1–2 mm) of the skin (Fig. 1.1.3). The dermis is rich in nerves, blood vessels, sweat glands, sebaceous glands, and the like. Unlike the

epidermis, which is mainly composed of cells, the dermis consists mainly of extracellular matrix materials. Fibroblasts, the primary cells in the dermis, synthesize collagen, elastin, glycosaminoglycans, and other matrix proteins. Collagens compose 70–80% of the components of the dermis. The upper layer of the dermis is called the papillary dermis and consists of small collagen bundles, whereas the inner dermis is called the reticular dermis and is composed of larger collagen bundles. Another important structural material of the dermis is elastin; together with collagen, elastin imparts elasticity, firmness, durability, and resilience to the skin.
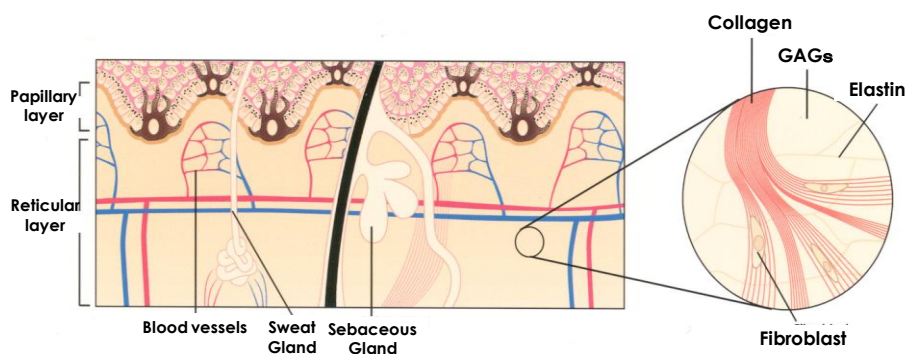

Fig. 1.1.3.   Illustration of the structure of the dermis

(Xianghong Yan, Copyright P&G)

1.2   Aging of skin

It is well documented that aging of skin is a process of structural and compositional remolding that manifests itself in the form of wrinkling, sagging, hyperpigmentation, and the like. Facial appearance is regarded as a typical index of aging. On the basis of unpublished original research by P&G, we know that, on average, the onset of the signs of aging of the facial skin vary with age, as shown in the Fig. 1.2.1 below.

Signs of Skin Aging

| 20's | 20's – 30's | 30's – 40's | 40's – 50's |

●Shine ●Dryness
●Fine Wrinkle

●Pore ●Fine Wrinkle
●Persisting Wrinkle
●Dullness ●Dryness

●Wrinkle ●Dullness
●Dryness ●Partial Sagging

●Wrinkle ●Dullness
●Dryness ●Sagging

Fig. 1.2.1.   Signs of skin aging in various age groups, based on original unpublished research by P&G.

(Xianghong Yan, Copyright P&G)

Fig. 1.2.1 shows that women of various ages have different issues and concerns related to the appearance of their facial skin. However, people of a given age do not necessarily all show the same degree of aging of the facial skin, which can vary hugely from individual to individual.

From the point of view of the physiology of the skin, during skin aging, the epidermis becomes thinner and the duration of its turnover cycle increases. Keratinocyte proliferation and differentiation processes alter between the young skin and the aged skin. In dermis, during aging of the skin, the vitality of dermal fibroblasts decreases and there is a decline in the quantity and quality of extracellular matrix components such as collagen and elastin (Fig. 1.2.2).
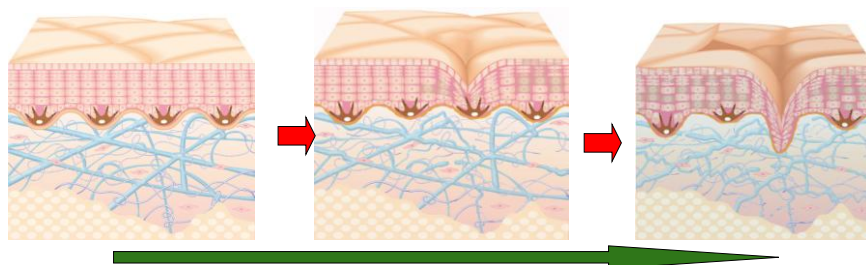

Fig. 1.2.2.   Illustration showing the progress of skin aging

(Xianghong Yan, copyright P&G)

Taking the example of collagen, more than 25 years ago, Imayama and

Braverman (1989) investigated the modulation of collagen fibers by comparing the skin of mice *in vivo* by means of transmission electron microscopy (Fig. 1.2.3). Their result clearly showed that collagen bundles became thinner and more irregular as the skin ages. Although many scientists have since examined age-related changes in skin morphology by more-advanced techniques, the visuals produced by Imayama and Braverman in their study remain the clearest.
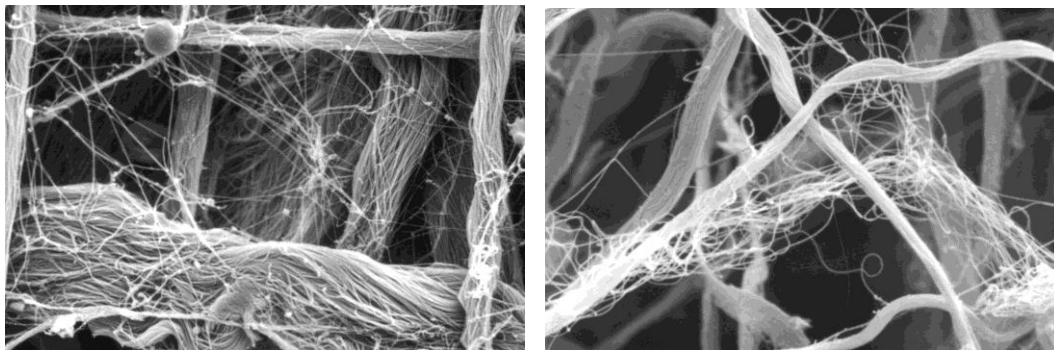

Fig. 1.2.3.   Changes in collagen fibers in aged skin (right) compared with those in young skin (left).

[Imayama S, Braverman IM: *Am J Pathol* 134, 1019–1025, 1989]

The complicated alterations in skin by aging, which include modulation of essential components such as proteins and lipids, as well as complicated enzymatic activities and signaling pathways, need to be further elucidated.

On the other hand, causes of skin aging have also been extensively studied. In general, it is widely recognized that skin aging is affected by intrinsic factors, such as cell senescence or DNA programming, and by extrinsic factors, such as UV exposure, air pollution, or smoking (Fig. 1.2.4). Because UV exposure has the greatest effect among the various extrinsic factors, skin aging can also be classified in terms of intrinsic aging and photoaging.
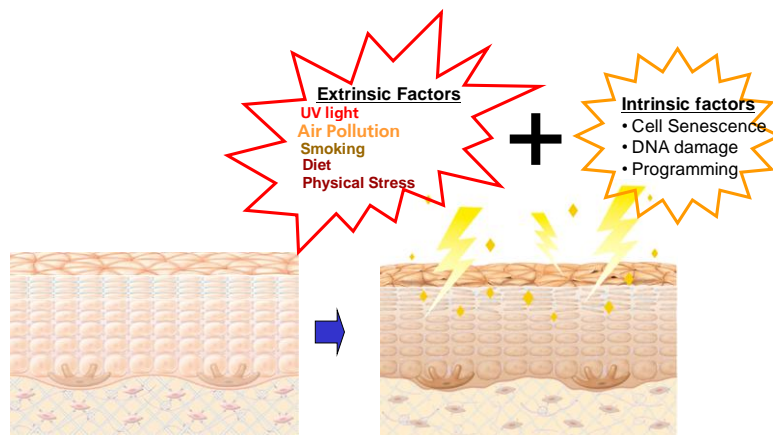
Fig. 1.2.4.   Illustration of the causes of skin aging. (Left-hand side) Extrinsic factors such as UV radiation, air pollution, or smoking; (right-hand side) intrinsic factors, such as cell senescence, DNA damage, or DNA programming.

(Xianghong Yan, copyright P&G)

By adopting an epidemiological approach, P&G investigated the impact of UV exposure on skin by comparing the skins of two groups of women living in Kagoshima and Akita, respectively; these groups represent women receiving large amounts of UV exposure and small amounts of UV exposure, respectively (Figs. 1.2.5 and 1.2.6). Our results beautifully proved that UV exposure significantly impacts aspects of skin aging, including wrinkling and hyperpigmented spots (Hillebrand et al. 2001).
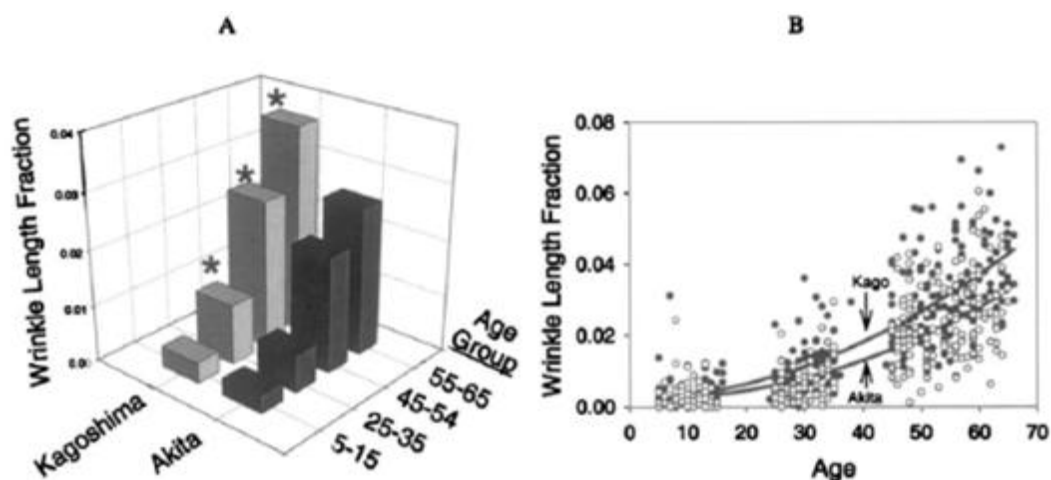
Fig. 1.2.5. Wrinkle length fraction by age group and study location. Kagoshima bars marked by '*' are significantly different (P<0.05) than the corresponding Akita age group. (B) Wrinkle length fraction by age and study location. Each point represents a subject in the study (● Kagoshima, ○ Akita). Lines are second order polynomial regression fits to Kagoshima (r^2=0.74) and Akita (r^2=0.58) data.

[Hillebrand et al. *J Dermatol Sci* 27 (Suppl. 1), S42–S52, 2001]
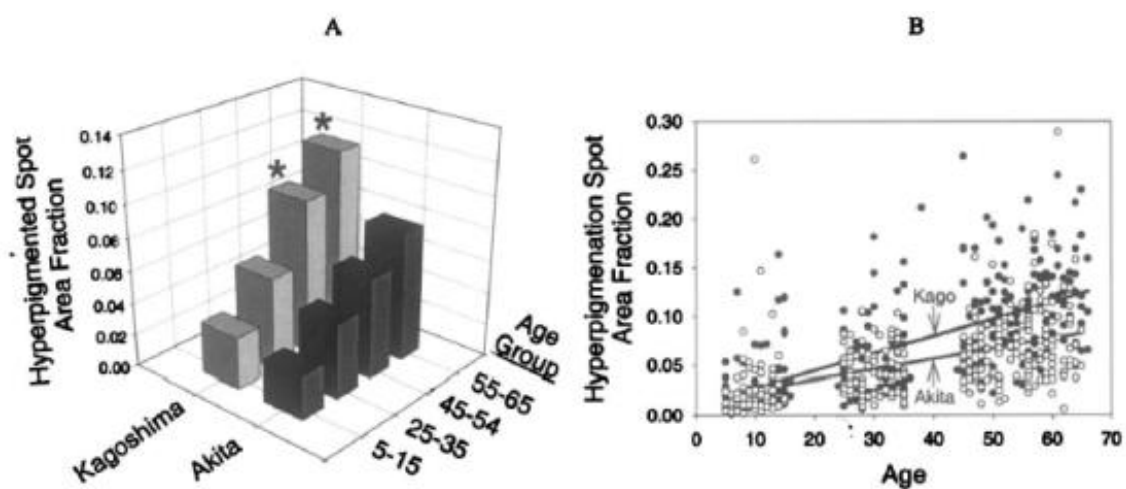

Fig. 1.2.6. (A) Hyperpigmentation spot area fraction by age group and study location. Kagoshima bars marked by '*' are significantly different (P<0.05) than corresponding Akita age group. (B) Hyperpigmentation spot area fraction by age and study location. Each point represents a subject in this study (● Kagoshima, ○ Akita). Lines are linear regression fits to Kagoshima (r2=0.49) and Akita (r2=0.35).

[Hillebrand et al. *J Dermatol Sci* 27 (Suppl. 1), S42–S52, 2001]

Furthermore, by adopting a genomic approach, we compared gene expression in aged skin with that in young skin for UV-exposed and nonexposed areas (Fig. 1.2.7). Our results demonstrated that among the genes that showed significant changes, more than 80% occurred in the UV-exposed area, thereby confirming that extrinsic factors have a greater effect on skin aging than do intrinsic factors.
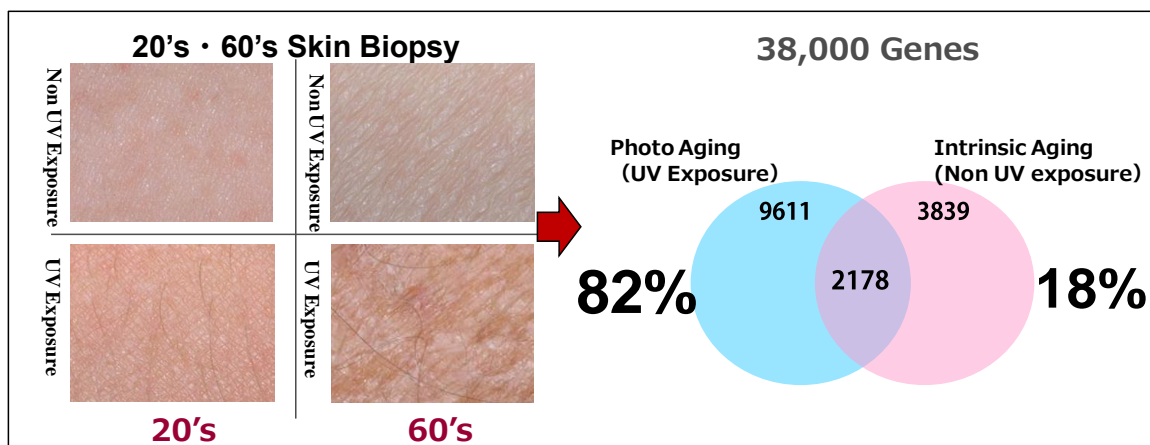
Fig. 1.2.7. Gene expression in aged skin versus young skin for UV-exposed and non-UV-exposed areas is compared. Of 38,000 genes, the expression of 9611 showed changes in UV-exposed areas, whereas the expression of 3839 showed changes in non-UV-exposed areas. Among these two groups of genes, 2178 genes showed changes in both groups. Therefore, the expression of 82% of genes was changed as a result of photoaging, and that of 18% was changed as a result of intrinsic aging.

(P&G unpublished data; Xianghong Yan, copyright P&G)

1.3 Anti-aging by skin care

In recent years, as the lifespan of people in general has extended, skin aging has become a growing concern, not only because it significantly affects a person's appearance, but also because it can lead to a series of diseases that affect the person's quality of life. In particular, the needs and demands of women to maintain a healthy and youthful skin have increased. Not only does beautiful skin reflect a woman's attractiveness, but also many women perceive that beautiful skin is an important indicator of social status. Hence, modulating the progress of skin aging is becoming more and more important. Approaches to retaining the appearance of a youthful skin are diverse and vary from individual to individual. A healthy diet and a balanced lifestyle are fundamental to the maintenance of a healthy skin; furthermore, in addition to physical factors, mental and emotional conditions also play an important role in

respect of skin condition. However, signs of an aged skin, such as wrinkles, sagging, or hyperpigmentation, are difficult to avoid completely, and many women who manifest such signs wish to eliminate them. Consequently, skin beauty clinics that offer drastic treatments such as laser, Botox, chemical peeling, or collagen or hyaluronic acid fillers have become increasingly popular and acceptable, particularly in countries such as South Korea and Taiwan. Even in Japan, the receipt of such drastic treatments has become more acceptable. However, across the various countries, skin care by the use of cosmetics products remains the primary approach to skin maintenance and rejuvenation that is adopted by most women. In particular, among the numerous claims made for skin-care products on the market, 'anti-aging' and 'botanical' are two key terms. (Result of a study on market claims for all skin-care products for the face, neck, lips, and eyes and for eye cleansers performed by the global research company Mintel in 2012; Fig. 1.2.8.)
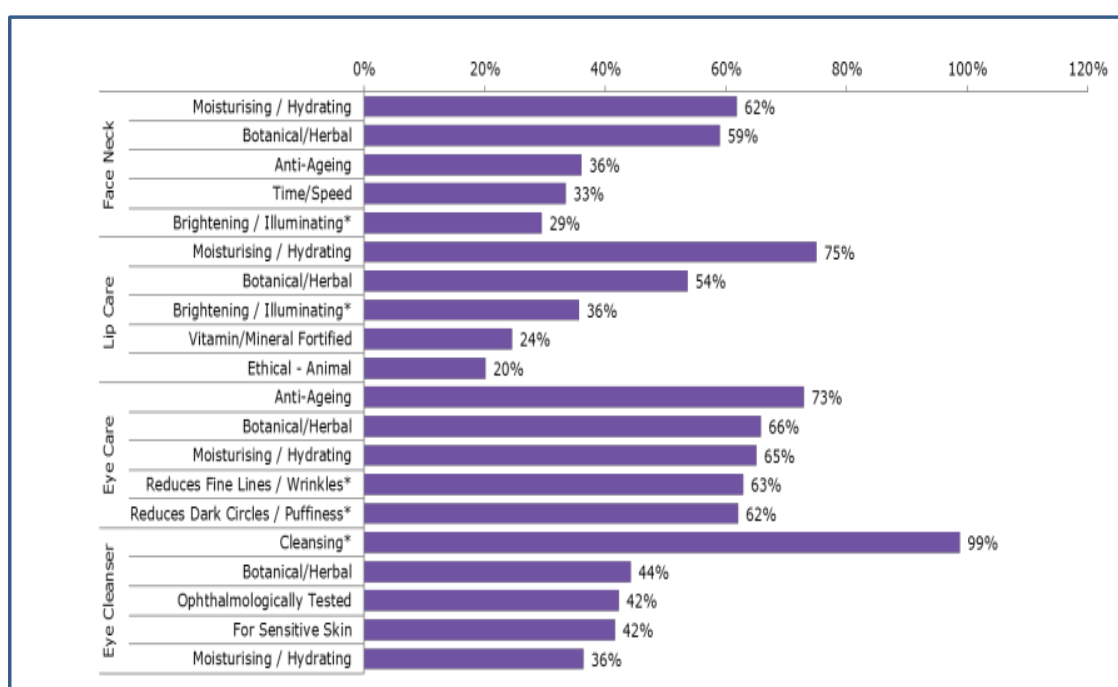

Fig. 1.3.1.   Claims for skin-care products on the market (Mintel, 2012)

If we take all these factors into consideration, the investigation of the parameters, mechanism of skin aging and the development of effective functional ingredients for skin-care products to modulate the skin aging progress is clearly a growing interest for both dermatologists and cosmetics scientists.

2. INTRODUCTION

2.1   Longitudinal study of skin aging progress

Though different individuals may have different issues and concerns to the appearance of their facial skin, as common sense, facial skin problems, such as wrinkles, hyperpigmented spots, texture roughness, pores, wrinkles, and skin tone are considered significantly impact the impression of facial aging. In order to comprehensively understand women's facial parameters of skin aging, since 1999, P&G scientists have conducted epidemiology studies and measured women's skin by utilizing an original imaging system, until now we have captured more than 4000 images of women ranging in age from 10-70, representing various ethnicities and geographies (Chinese, Japanese, Caucasian, Indian, and African American; Fig. 2.1.1).

Fig. 2.1.1.   The database of P&G skin epidemiology studies

(Xianghong Yan, copyright P&G)

As one example, in 2006, we conducted epidemiology study by using this

imaging system in Beijing. In the study, we enrolled 452 subjects, ages 10-70, we measured subjects' parameters of wrinkles image, facial skin hydration, elasticity, firmness. Interesting, we observed a insightful indication from the wrinkling analysis that is statistically the average age of onset for visible expressive lines around the eye precede that of persistent wrinkles by several years (Fig. 2.1.2), which implies that the expressive lines that you see today might grow to the persistent wrinkles you see several years later.

Facial Wrinkle Area Fraction
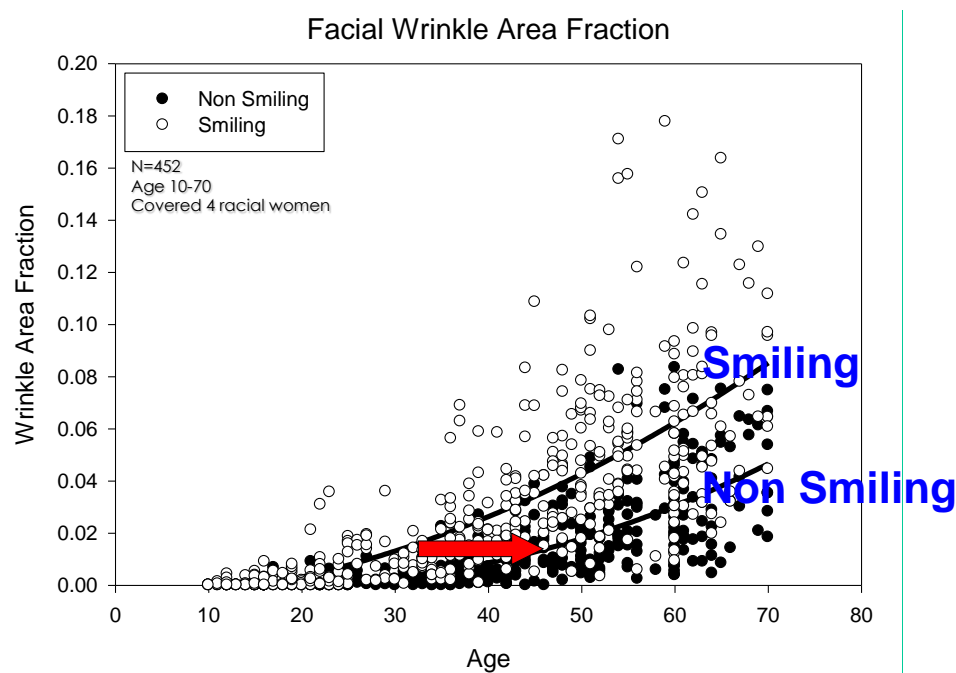
Fig. 2.1.2.   Statistical analysis: current expression line grows to permanent wrinkle in the future

(Xianghong Yan, copyright P&G)

Epidemiology study is an important tool to understand various aspects of human skin condition by age group, however, study results could not avoid the variability caused by individual living environment, lifestyle, and genetic background. It is well known that the aging of facial skin proceeds relatively slowly and therefore requires long term follow up to elucidate its progress. Taking together, in order to investigate the skin aging progression, we think follow up of the same subjects for long term

23

might be needed. Therefore, in 2008, taking the advantage of P&G global database, as the 1st trial, we followed up Los Angeles panels which we measured their skin in 1999, and which cover multiple ethnicities across from Caucasian, Hispanics, African American to Eastern Asian. In 2008, we studied the same subjects of LA panels and quantified their changes in wrinkles by age. This study indicated that longitudinal follow up of the same subjects was a unique approach to investigate skin-aging progression, which possibly eliminated the individual variability. Therefore, in order to further investigate Asian women's skin aging progression more precisely, as the 2nd trail, we conducted skin longitudinal study by choosing Japanese panels. In March 1999, we conducted an epidemiology study of skin photoaging with 602 Japanese females who lived in Akita and Kagoshima, Japan, as the first phase (Hellibrand et al, 2001). Eleven years later, in March 2010, we attempted to conduct a study on skin aging by follow up of the same subjects from Akita, under the same measurement conditions. The purpose of this study was to clinically identify facial skin parameters contributing the subjective impression of the overall aging and characterize the degree of skin aging during 11 years.

2.2 Comprehensive mechanism of skin aging

As shown above, in order to understand the important parameters of skin aging, clinical measurement by epidemiological and longitudinal approach is essential. On the other hand, in order to develop the effective approaches to control those skin-aging parameters, investigation of the molecular mechanism of skin aging is crucial. Human skin mainly composes epidermis and dermis, it is well known that the keratinocyte in epidermis and fibroblast in dermis are two primary types of cell in skin, their activation modulate skin aging parameters such as texture roughness and wrinkles. In addition, as another significant skin aging parameter, hyperpigmentation is associated with the comprehensive interactions between keratinocytes and melanocytes in the epidermis,

in fact, recent studies have shown that keratinocytes play a primary role in control of melanogenesis in melanocyte and melanosome transfer from melanocyte to keratinocyte. Taken together, keratinocyte and fibroblast are two most important targets to investigate molecular mechanism of skin aging. Moreover, by revealing the new mechanisms, we aim to develop the effective strategies to modulate the central parameters of skin aging such as texture roughness, wrinkles and hyperpigmented spots which we found in the skin aging longitudinal study.

● **Mechanism of epidermis aging: overall and AhR-Nrf2 signaling**

Epidermis is the outmost layer of skin, its most important role is to protect the inner body against external and environmental factors by its barrier function. Precisely, the barrier of human epidermis is composed of stratified squamous keratinocytes which integrity depends on the outer cornified envelope, thus cornified envelope is essential for the maintenance of barrier function (Marshall et al. 2001). Cornified envelope maturation is accomplished by sequential and coordinated cross-linking of ceramides and various barrier proteins such as involucrin (IVL), loricrin (LOR), filaggrin (FLG), small proline-rich proteins (SPRRs) and late cornified envelope proteins (LCEs) through transglutaminase-1 and -3 (Kypriotou et al. 2012). Those proteins are important to the barrier function of skin.

On the other hand, epidermis directly encounters external and environment factors which induce oxidation in epidermis and accelerate its aging. Various studies by P&G have proven that skin aging is mainly caused by extrinsic factors; in particular, UV exposure plays the largest role in skin extrinsic aging by inducing oxidative stress in keratinocytes. Specifically epidermis aging is mainly driven by ROS induced by UV irradiation, the sources of ROS include the mitochondrial ETC, peroxisomal, ER localized proteins, and enzymes such as cyclooxygenases, lipoxygenases, xanthine oxidases, and NADPH oxidases. On the other hand, anti-oxidative enzymes such as

superoxide dismutases, catalases, peroxiredoxins, and GSH peroxidases, in addition, anti-oxidative organic compounds such as L-ascorbate, α-tocopherol, beta-carotene, uric acid, and glutathione are also synthesized in the skin. Therefore, anti-oxidation is an important function for ameliorating of skin aging (Rinnerthaler et al. 2015). On the other hand, oxidative stress induces modifications of proteins, lipids and DNA in epidermis, as the consequence, oxidation causes a disruption of the epidermal calcium gradient and change in the composition of the cornified envelope such as ceramides and the important proteins. The modified cornified envelope also leads to an altered anti-oxidative capacity and a reduced barrier function of the epidermis (Rinnerthaler et al. 2015). Taken together, in epidermis, the reduction of anti-oxidation capability and barrier disruption impact each other which can cause a vicious cycle, as the result, this vicious cycle can lead the acceleration of epidermis aging. Therefore, anti-oxidation and barrier function are regarded as two most important functions to modulate epidermis aging. Clinically, oxidation and barrier disruption of epidermis by aging can lead skin surface dryness and texture roughness, moreover, they also can induce the aging of inner skin layers.

The skin is also a highly sophisticated sensory organ, and the sensing of external and environmental stimuli plays key roles in self-defense and homeostasis. Aryl hydrocarbon receptor (AhR, also called dioxin receptor) is a chemical receptor that responds to exogenous and endogenous chemicals by inducing/repressing the expression of several genes with toxic and shows protective effects in a wide range of tissues (Denison et al. 2011). As reviewed previously (Ikuta et al. 2009; Furue et al. 2014), keratinocytes harbor abundant AhR, which exerts multi-functional effects on skin homeostasis and pathology.

On the other hand, a recent study by Fischedick et al. has demonstrated that certain phytochemicals activate nuclear factor erythroid 2-related factor (Nrf2: Butera et al. 2002; Fischedick et al. 2012) which is a key transcription factor that upregulates

a series of antioxidant enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) (Jaiswal. 2004; Ma. 2013). Moreover, there is compelling evidence that one of the major pathways of Nrf2 activation is mediated through the AhR (Tsuji et al. 2012; Ramyaa et al. 2014). AhR is assumed to undergo a conformational change and it subsequently translocates into the nucleus. The ligand/AhR complex then binds to its specific DNA sequence, namely the xenobiotic-responsive element or dioxin-responsive element, upregulating the transcription of a series of responsive genes, including cytochrome P450 1A1 (CYP1A1) (Ikuta et al. 2009). In addition to polycyclic aromatic hydrocarbons, various phytochemicals, Malassezia metabolites, particularly tryptophan photoproducts as the consequence of UV exposure can also bind to AhR with a wide range of affinities (Furue et al. 2014).

Tsuji et al. identified the antifungal agent ketoconazole as an inducer of AhR signaling and the Nrf2 antioxidant response in human keratinocytes. Ketoconazole-stimulated nuclear translocation of Nrf2 and its cytoprotective effects against oxidative stress strongly depend on functional AhR (Stemmann et al. 2012). The finding of the effects of ketoconazole through AhR-Nrf2 signaling drew our interest to investigate other biofunctional ingredients, since this might provide new opportunities for modulating skin aging via the activation of AhR-Nrf2 signaling in keratinocyte.

- **Mechanism of dermis aging: overall and microRNAs' role**

In aged dermis, the quantity and quality of the extracellular matrix (ECM) decrease. Remarkably, owing to the complicated nature of the components of the ECM, it is well known that the dermis plays a dominant role in the skin structural remodeling, which involves abnormal synthesis of matrix proteins, increased degradation of collagen and elastin, and senescence of dermal fibroblasts. These molecular events have been shown to be mediated by cell-signaling pathways, such as the TGF-β/SMAD/CTGF pathway

for collagen synthesis (Quan et al. 2001) and the ROS/MAPK/AP-1/MMP pathway for ECM degradation (Yaar et al. 2007), as well as by the senescence-associated gene pattern that controls cell-cycle progression and cell proliferation (Naylor et al. 2011). Moreover, during the last decade, global gene-expression profiling has been applied in the research field of skin aging, and hundreds of genes have then been found to participate in various cellular activities that regulate skin homeostasis, indicating that the aging process of the dermis is extremely complex (Lener et al. 2006).

On the other hand, wrinkling is thought to be a complex biophysical process resulting from repeated strains on a progressively, structurally and biochemistry altered aging skin with impaired mechanical properties, which is mainly caused by the degradation of ECM quantity and quality in the dermis (Humbert et al. 2011).

Recent studies suggest that microRNAs (miRNAs) may also play a role in the regulation of gene expression in organism aging (Jung et al. 2012; Smith-Vikos et al, 2012). miRNAs are a class of short, endogenous, single-stranded, non-coding RNA molecules. By binding the specific 3'-UTRs sequence of the target mRNAs, they can regulate the expression of multiple genes at the post-transcriptional level through degradation or translational inhibition of the targeted transcripts (Engels et al. 2006). Some recent studies have compared young and aged organs or tissue like heart (Zhang et al. 2012), cornea (Zhao et al. 2013), kidneys (Kwekel et al. 2015) etc., indicating clues about the role of miRNAs in aging regulation at the tissular level, whereas for skin, especially dermis, studies about age-related miRNAs remain limited, and most of them were carried out at the cellular level (Li et al. 2013; Mancini et al. 2012). In this study, we aimed to obtain an overall view of miRNAs expression in human aged dermis vs. young dermis, construct the miRNA-gene-network and reveal the pivotal miRNAs in the regulatory network.

Furthermore, by utilizing a complex of soybean and yeast proteins with proven anti-aging effects that positively modulate the expression of Trf2, protect skin against

UV-induced DNA damage and prevent the signs of cellular senescence, we attempted to nail down the key miRNAs involved in fibroblast aging to further confirm the pivotal miRNAs associated with dermis aging.

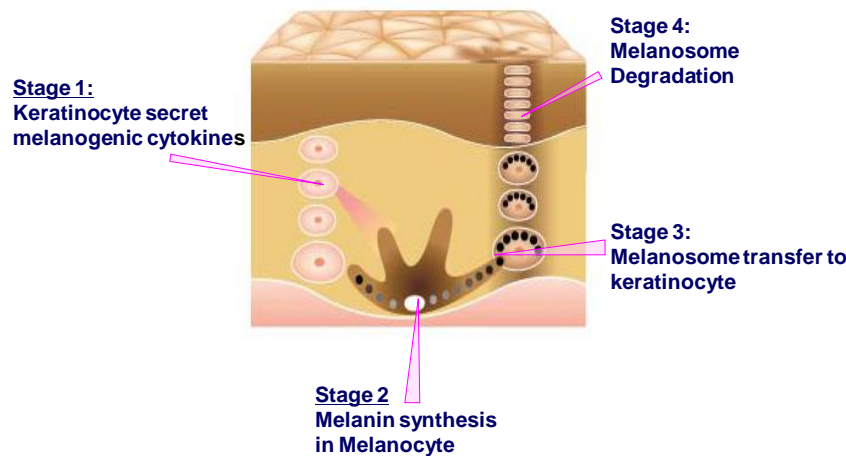● **Mechanism of skin hyperpigmentation:** overall and melanin uptake

Fig. 2.2.1.   Illustration of 4 biological stages related to skin hyperpigmentation

(Xianghong Yan, copyright P&G)

The mechanism of skin hyperpigmentation is one of the major research fields in both dermatology and cosmetic science; especially it has been significantly advanced during recent 30 years. Clinically, the hyperpigmented spot is one of the biggest concerns of skin aging. UVB has been considered major cause of hyperpigmented spots. Overall, in the course of UVB-induced pigmentation, four major steps, cytokines secreted by keratinocyte, activation of melanin synthesis in melanocyte, melanosome transfer to keratinocytes, and decline of melanosome degradation have been reported to be responsible for the hyperpigmentation (Okazaki et al. 1976; Rosdahl et al. 1978; Imokawa et al. 1982; Mishima et al. 1983; Murase et al. 2013).

Especially in the first two steps, several complicated cytokine-receptor signaling cascades such as α-melanocyte stimulating hormone (αMSH) and its receptor,

melanocortin 1 receptor (MC1R), endothelin-1 (ET-1) and its receptor (Endothelin B receptor, ET-B) and stem cell factor (SCF) and its receptor (KIT), have been shown to substantially contribute to UVB-induced melanogenesis (Imokawa et al, 1992, 1995, 1997; Lerner et al, 1961; Bolognia et al, 1989; Chakraborty et al, 1996; Hachiya et al, 2001)

Moreover, the ability of melanosome transfer from melanocytes to keratinocytes has been also shown to determine pigmentation in skin. For example, protease activated receptor-2, a seven transmembrane G-protein-coupled receptor, which is dominantly in charge of phagocytosis in keratinocytes, has been demonstrated to be expressed at a higher level in darker skin compared with lighter skin (Sharlow et al. 2000; Babiarz et al. 2004).

Furthermore, as the fourth step, recently it was proposed that epidermal melanosome degradation mainly driven by autophagy in keratinocyte, an intracellular process whereby the cytosol and organelles are sequestered within double membrane-bound autophagosomes that subsequently deliver their contents to lysosomes/vacuoles for degradation (Seglen et al. 1992; Yoshimori et al. 2004; Levine et al. 2004; Mizushima et al. 2007), also plays a role in determining skin pigmentation (Murase et al. 2013).

Among above four important steps associated with hyperpigmentation, we have paid special attention on melanosome transfer from melanocyte to keratinocyte. In 2002, we revealed the effect of niacinamide on reducing cutaneous pigmentation and suppression of melanosome transfer (Hakozaki et al. 2002), however, the precise mechanism of melanosome transfer needs to be further elucidated.

In the step of melanosome transfer from melanocyte to keratinocyte, melanin-containing melanosomes subsequently migrate to the dendrite tips of the melanocytes by the action of myosin V and dynein (Jordens et al. 2006). It is well known that tyrosinase is a rate-limiting factor in melanin synthesis and that

melanosome transfer is a critical step in melanogenesis (Park et al. 2009; Boissy et al. 2003). Therefore, instead of the tyrosinase inhibitors that have been widely used, several agents are now used with the aim of inhibiting the transfer of melanosomes (Reish et al. 1995; Solano et al. 2006; Kim et al. 2012). Various mechanisms of melanosome transfer have been proposed including direct infusion of melanosomes into keratinocytes through nanotubular filopodia (Singh et al. 2010), uptake of released melanosomes by keratinocytes through phagocytosis (Ando et al. 2010), and partial cytophagocytosis by keratinocytes of melanocyte dendrite tips containing melanosomes (Chakraborty et al. 2003). Recently, a new mechanism of melanosome transfer was reported in which pigment globules containing multiple melanosomes are released into the extracellular space from melanocytes and subsequently ingested by keratinocytes (Ando et al. 2011). Regardless of which mechanism is acting, the result is the cellular uptake of melanin with ultimate formation of perinuclear melanin caps (Tarafder et al. 2014).

Melanin, a natural pigment produced by stimulated melanocytes in the basal layer of the epidermis, converts more than 99.9% of the absorbed UV radiation into heat, and it functions as an excellent photoprotectant that protects the DNA of cells from damage by UV (Byers et al, 2003; Yamaguchi et al, 2006; Takeuchi et al, 2004). Other than its sunscreen effect, in this study we attempted to establish a simplified cell model of melanin uptake and further elucidate the potential effects of melanin on modulating the bioactivity of epidermal keratinocytes.

2.3   Several biofunctional ingredient

- *Opuntia ficus-indica* **extract**

Fig. 2.3.1.   Visual of *Opuntia ficus-indica* (Xianghong Yan, copyright P&G)

Cactus is a member of the succulent plant family cactaceae, which is widely used as food, fodder, dye, and folk medicine. *Opuntia ficus-indica* (OFI), prickly pear, is a cactus species widely distributed in Latin America, South Africa, and the Mediterranean. It is utilized in arid and semiarid zones as a fruit, food, and forage crop (Butera et al. 2002). According to several studies, cactus has high level of important nutrients, such as minerals, carotenoids, fatty acids, and essential oil (Shetty et al. 2012; Hfaiedh et al. 2014). This desert plant can be used as an anti-inflammatory, analgesic, hypoglycemic, and antiviral agent, as the result, it may protect human body against numerous chronic diseases including cancer, cardio- and cerebro-vascular, ocular, and neurological disorders (Hfaiedh et al. 2014; Kuti et al. 2004). One of the most intriguing properties of OFI is its potent antioxidant activity. It is demonstrated that OFI extract (OFIE) inhibits oxidative stress through sodium dichromate and protects against testicular injury *in vivo* (Hfaiedh et al. 2014), and the previous study also showed that it attenuated hepatic steatosis and oxidative stress in obese Zucker (fa/fa) rats (Mora ń-Ramos et al. 2012). Moreover, the hepatoprotective capacity of OFIE has been demonstrated by reduction of oxidative stress in a mouse model of aflatoxin B1 intoxication (Brahmi et al. 2012). However, its antioxidant mechanism remains largely unknown.

- **A biofunctional complex of yeast and soybean**

Fig. 2.3.2.   Visual of the complex of soybean and yeast (originated by Ashland, copyright P&G)

The soybean (US) or soya bean (UK: Glycine max) is a legume species native of East Asia. It is an annual plant that has been used in China for 5,000 years as a food and a component of drugs. Soya contains significant amounts of all the essential amino acids for humans, and it is a perfect source of proteins. Soybean is rich in isoflavones, a potent and well documented anti-oxidant molecule. Isoflavones may be effective in preventing DNA damage in cells, and in the fight against photoaging (Iovine et al. 2014). Soybean hydrolysate is also rich in proteins having chelating and radical scavenging activities, and decreases lipid peroxidation (Zhang et al. 2009).   Moreover soybean is a source of peptides with high potent anti-oxidant activity (Hernández-Ledesma et al. 2009).

Yeast is a tiny plant-like microorganism that exists all around us in soil, on plants and even in the air. The active aerobic metabolism of yeast makes it rich in essential amino acids and small peptides, thus it potentially has beneficial effects to human skin.

Aspartic acid		11,98
Hydroxyproline	1,84	
Methionine	1,84	
Threonine	5,53	
Serine	6,45	
Glutamic acid		15,67
Proline	4,61	
Glycine	5,53	
Alanine	7,37	
Valine	6,45	
Isoleucine	4,61	
Leucine	9,22	
Tyrosine	4,61	
Phenylalanine	5,53	
Lysine	8,29	
Histidine	2,76	
Arginine	4,61	
Tryptophan	1,84	

Essential amino acids are presented in yellow
Results are expressed in percentage of amino acids per 100g of proteins
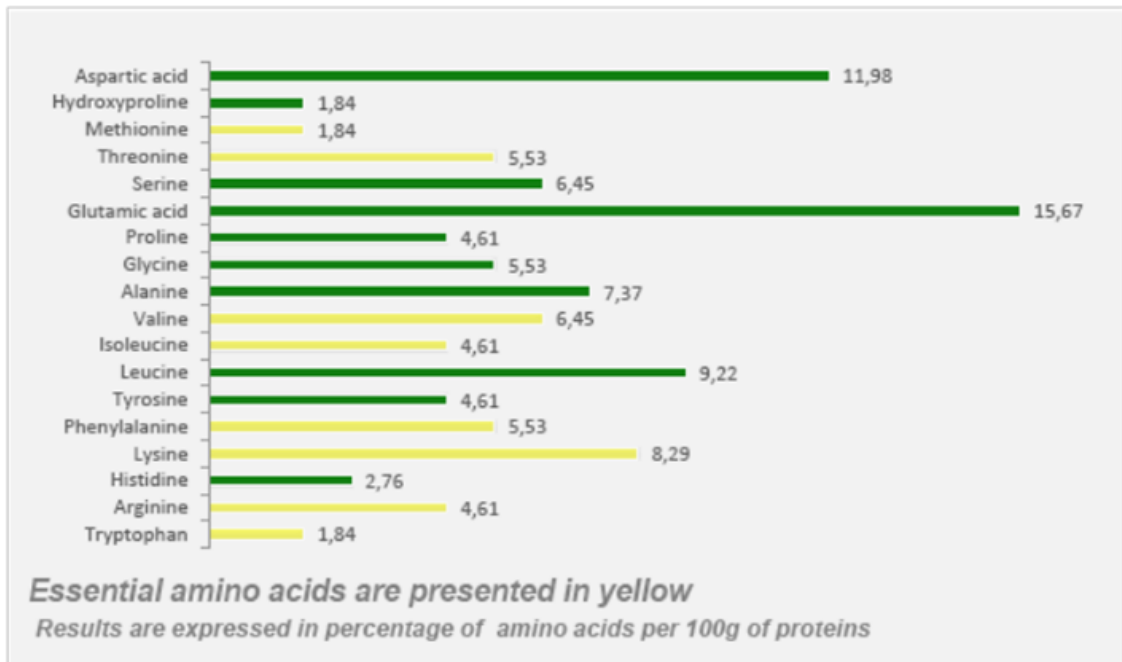
Fig. 2.3.3.   The components of yeast (originated by Ashland, copyright by P&G)

A biofunctional complex of the yeast proteins and soybean is made by hydrolyzed natural yeast (*Saccharomyces cerevisiae*) proteins and hydrolyzed natural soybean, two materials are firstly separately hydrolyzed, and a second hydrolysis was made combining these two extracts. This complex is proven by its ability to modulate the expression of telomere-binding proteins such as TRF2 (De lange. 2005; Imbert et al. 2012; Stewart et al. 2012).
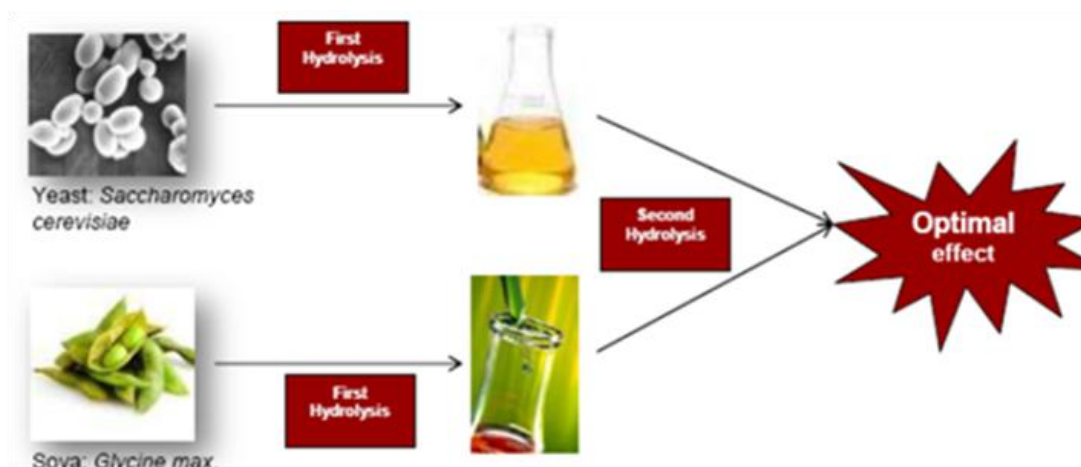

Fig. 2.3.4.   Illustrate of the manufacturing process of the complex of soybean and yeast

(Originated by Ashland, copyright P&G)

34

- **Niacinamide**

Niacinamide (also known as nicotinamide, 3-pyridinecarboxamide) is the physiologically active amide of niacin (vitamin B3). Several reports suggest that niacinamide may have various effects on the skin: it acts as an anti-inflammatory agent in acne (Shalita et al. 1995), as an antioxidant (Bowes et al. 1998), prevents photoimmunosuppression and photocarcinogenesis (Gensler et al. 1997), and increases intercellular lipid synthesis (Tanno et al. 2000). Topical niacinamide has been used to treat cutaneous lesions of dermatitis but has not previously been demonstrated to affect pigmentation. In 1995, it was reported that topical 4% niacinamide was effective in treating acne when used twice daily for 8 weeks (Shalita et al. 1995). In 1997, Tanno et al. reported that niacinamide stimulated biosynthesis of crucial stratum corneum lipids. In a subsequent 4-week human study, the authors reported that topical 2% niacinamide increased ceramide level, cholesterol synthesis and free fatty acid synthesis via an increase in acetyl coenzyme A (Tanno et al. 2000). In 2002, Hakozaki et al. reported the effects of niacinamide on melanogenesis and pigmentation *in vitro* and *in vivo*. In addition, the data suggested that niacinamide is an effective skin lightening agent that works via the suppression of melanosome transfer from melanocytes to keratinocytes (Hakozaki et al. 2002). However, the specific cellular mechanism of niacinamide remains to be further elucidated.

2.4 Purpose of the series of the studies

In order to control the central problems of skin aging prameters such as texture roughness, wrinkles and hyperpigmented spots, revealing the new molecular mechanisms for ameliorating skin aging is the key to development of novel anti-aging strategies. As stated above, skin aging is a structural and compositional remodeling process that is associated with changes in the phenotypes of skin cells. Keratinocytes are the primary cells in the epidermis, and fibroblasts are the primary cells in the

dermis; therefore, keratinocytes and fibroblasts are the major targets, both for investigations on the mechanism of skin aging and for the development of ingredients for modulating skin aging. Numerous studies have shown that AhR, a receptor located in the cytoplasm, might play a crucial role in various functions of keratinocytes; therefore, by targeting AhR, we attempted to reveal how OFIE modulates the function of keratinocytes. Also, over the past ten years, findings on miRNAs have gradually shown that they play important roles in human diseases; in fact, the number of publications on miRNAs has increased markedly in recent years. This drew our interest, and we surmised that miRNAs might also play a major role in skin aging. Thus, by targeting miRNA as a new epigenetic territory, we aimed to elucidate the role of miRNAs in aging of the dermis by two different approaches. Finally, recent studies have shown that keratinocytes might play a primary role in skin pigmentation; consequently, we targeted melanin uptake by keratinocytes and the effects of melanin on the function of keratinocytes.

There are many biofunctional ingredients, which are effective and safe to modulate the aging issues of human skin; consequently, we therefore attempted to evaluate several biofunctional ingredients that potentially modulate skin aging by the newly found mechanisms. In addition, in these studies, a natural complex of soybean and yeast proteins with proven anti-aging efficacy is attempted to serve as the useful tool for nailing down the pivotal miRNA targets in skin aging.

Taken together, all these attempts might permit us to identify important new strategies for modulating skin aging.

3. MATERIAL AND METHOD

3.1 Longitudinal study of skin aging progress

● **Log Angles facial wrinkling longitudinal study**

The baseline survey was conducted at an indoor shopping mall in Redondo Beach, CA, a suburb of Los Angeles, from October 1999 to February 2000. Women who were shopping in the mall were randomly invited to participate in the survey if they were between the ages of 10 and 70 years, in good health and not pregnant. In total, 1437 women were enrolled. Then during February to May of 2008, at the same study location, 122 of these same women participated in a follow up study (mean ±SD age 40.4 ±15.7 years at baseline and 48.8 ±15.7 years at 8 years). They were Caucasian (n = 60), African-American (n = 20), Asian (n = 22) or Latino (n = 20) heritage. None of the subjects had moved away from the Los Angeles area nor reported having had surgical, laser, filler, botulinum toxin or other cosmetic procedures deemed to affect facial wrinkling between their baseline and follow-up studies. The written informed consent was obtained from all study subjects.

Each subject cleansed her face with a commercial facial cleanser and sat quietly for 15 min prior to evaluation. Left oblique view facial images were captured using a Fuji DS330 camera equipped with a close-up lens mounted into a standardized illumination rig fitted with chin and forehead rests. This system was used at both baseline and follow up study 8 years later, thereby minimizing camera and lighting variance between visits; color standards captured at baseline and follow-up were confirmed consistency of lighting conditions. Two images were collected at each visit, one with a neutral relaxed expression (to measure persistent wrinkling) and one with a smiling expression (to measure temporary wrinkling), a total of four images per subject were captured. For image analysis, the perimeter of the region of interest (ROI) on each subject's baseline neutral image was defined, i.e. 'masked', using predefined

facial landmarks (e.g. left and right corners of eye, bridge of nose, and corner of mouth). This mask was then applied to the subject's other three images. Corrections for slight differences in head repositioning were made by registering the subject's mask to facial landmarks without changing the mask shape or size. In this way, the same region of the face was analyzed for all four of the subject's images. Computer-based image analysis was used to identify and quantify facial wrinkles in the ROI automatically, based on shape and pixel contrast. Wrinkles were defined as being > 5 mm in length, having a perimeter∕length ratio < 2.5 and circularity (perimeter^2∕area) > 34. The overall wrinkle severity was expressed as the fraction of the ROI occupied by wrinkles: wrinkle area fraction (WAF) = total wrinkle pixel area∕total ROI pixel area.

Descriptive statistics (mean ±SEM) were used to describe the population. A Pearson Product Moment Correlation was used to analyze the association of baseline temporary wrinkling with 8-year persistent wrinkling as well as the association of the temporary∕persistent wrinkling ratio at baseline with the change in persistent wrinkling over 8 years. Analysis of covariance (ANCOVA) was used to compare persistent wrinkling at baseline with that at 8 years by age group (stratified in decades), with stratum corneum capacitance and cheek lightness as covariates.

● **Akita facial skin longitudinal study**

The baseline evaluation was performed in 1999 on 300 healthy Japanese females who were either in-door workers or house wives, excluding outside workers, and lived in Akita City, Japan. The examination room was maintained at a constant temperature and humidity (room temperature 20 ±2ºC, relative humidity 50 ±5%). In 2010, 11 years later, 108 subjects who had participated in the first evaluation, lived in Akita City until 2010, and agreed to participate in this study were evaluated again under the same measurement conditions. In order to avoid influence from seasonal variations, both the 1999 and 2010 studies were performed in late March. The age of the subjects ranged

from 5 to 64 years old (in 1999). None of the subjects underwent any type of aesthetic treatment such as laser cosmetic procedures during 11 years. The written informed consent was obtained from all subjects.

The subjects washed their faces using the prescribed cleansing foam and then spent 20 min becoming accustomed to the environment of the measurement room at a constant temperature and humidity. Each subject's face was photographed using an image capture system consisting of a high-resolution digital camera with a close-up lens (Fujifilm DS330), fluorescent lighting (5500 K, EFS13UED, Panasonic Corporation), and an anchor to fix the jaw and forehead to take the photos in the same position. The camera was calibrated for each measurement, which made it possible to apply the same measurement conditions, including the position of the face, for both the 1999 study and 2010 study. The ROI (region of interest) of the images was from the outer edge of the eyes to the cheek, and the following characteristic objects were extracted by measuring the contrast in the shape and pixels using an image analysis algorithm. Wrinkles were defined as > 5 mm in length; perimeter/length ratio ≤ 2.5; and circularity (perimeter^2/area) ≥ 34, and detected total wrinkle area fraction (total wrinkle area (pixels)/ROI (pixels)) was quantified. Hyperpigmented spots were defined as ≥ 5 mm^2 in area, color contrast Delta E ≥ 3 compared to its surrounding skin region, and circularity (perimeter^2/area) ≥ 20, and total hyperpigmented area fraction (total hyperpigmented area (pixels)/ROI (pixels)) was also quantified. As the index of skin surface roughness, total texture area fraction (total texture area (pixels)/ROI (pixels)) was quantified as ≤ 3 mm^2 in area, aspect ratio ranging from 0.5 to 2, and color contrast Delta E ≥ 1.5, while pores were defined as ≤ 4 mm^2 in area, color contrast Delta E ≥ 2, and circularity (perimeter^2/area) ≥ 20, that are different from hyperpigmented spots by its size and circularity, for quantification as total pore area fraction (total pore area (pixels)/ROI (pixels)). In addition, the subjective impression of the subject's facial skin from the periorbital region including eye area to the cheek

region were visually evaluated on the color-calibrated monitor, by ten examiners and scored on a 7-point scale from 0 (looks much older than the actual age) to 6 (looks much younger than the actual age) compared to their chronological age. The mean values of the resulting data by the 10 evaluators were analyzed. The measurements from both the 1999 study and the 2010 study, and the difference between them were statistically analyzed.

Pearson's correlation coefficient (r) between all seven skin optical parameters (image analysis data on wrinkles, hyperpigmented spots, texture, and pores, and the skin color [L*a*b*; a color space specified by the International Commission on Illumination with the chromameter) and age was examined at both the 1999 and 2010 studies. Quantitative comparison of those skin parameters from the 1999 and 2010 studies were also made by age group using two-way ANOVA (significance level $p <$ 0.05). Variable importance in projection (VIP) is a score indicating the degree of contribution for each parameter to the dependant variable estimated by PLS (partial least squares) regression statistical analysis. VIP of each skin optical parameter was generated and compared for the selection of key skin parameters contributing on visual evaluation on the degree of subjective impression of younger-looking or older-looking skin on the face, by using the standardized coefficients of skin parameters compared to the visual evaluation data as a dependant variable. The visual evaluation data on the degree of the subjective impression of younger-looking or older-looking skin was compared between the two studies, and the smaller difference and the greater difference between them were respectively defined as "smaller visible skin aging" and "greater visible skin aging" over 11 year of period. To adjust the measurement scale for the selected skin parameters that highly changes in the measurement domain of all the subjects were converted to percentile scores and adjusted to a scale of 0 to -1 (0: no change: 0 percentile, 1: maximal change: 100 percentile). According to the above-described procedures, the scale approached -1 when the changes (aging scores)

were greater, and approached 0 when the aging scores were smaller. The total of the resulting 0 to -1 scale values in these three skin parameters was defined as the Skin Aging Score. Thus, a higher Skin Aging Score indicated greater skin aging while a lower score indicated smaller skin aging. The correlation (Pearson's correlation r) between the Skin Aging Score and the changes in the visual evaluation data on the degree of subjective impression of facial skin aging was studied. A cluster statistical analysis of the Skin Aging Score plotted by the function of age was performed at a confidence level of 80% in the 2010 study, to classify subjects into a low skin aging group, an age-appropriate skin aging group, and a high skin aging group.

3.2　OFIE activates AhR-Nrf2 signaling

● **Reagents and antibodies**

Dimethyl sulfoxide (DMSO) and BaP (benzopyrene) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Interleukin 4 (IL-4) was purchased from R&D Systems (Minneapolis, MN, USA). IL-13 and TNFα were purchased from Peprotech (Rocky Hill, NJ, USA). Anti-AhR rabbit polyclonal IgG antibody (H-211), anti-Nrf2 polyclonal rabbit IgG antibody (H-300), and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). OFIE was provided by Silab (Saint-Viance, France) as an aqueous 3.5% stock solution extracted by water solubilization from flowers of OFI sustainably sourced from Morocco.

● **Cell culture**

Normal human epidermal keratinocytes (NHEKs: Clonetics-BioWhittaker; San Diego, CA, USA) were grown in culture dishes at 37˚C in 5% CO_2. NHEKs were cultured in serum-free keratinocyte growth medium (Lonza, Walkersville, MD, USA) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine. The culture medium was replaced

every 2 days. When it is near confluence (70–90%), cells were disaggregated with 0.25 mg/mL trypsin/0.01% ethylenediaminetetraacetic acid and subcultured. NHEKs of second to fourth passage were used in all experiments. NHEKs (1×10^5 cells) were seeded in 24-well culture plates, allowed to attach for 24 h, and then treated with or without OFIE, DMSO, TNFα, BaP, IL-4, or IL-13. Immunofluorescence and confocal laser scanning microscopic analyses NHEKs (2×10^4 cells) were cultured on slides (Lab-Tek, Rochester, NY, USA) with or without OFIE for 6 h. Slides were then washed in phosphate-buffered saline (PBS), fixed with acetone for 10 min, and blocked using 10% bovine serum albumin (Roche Diagnostics, Basel, Switzerland) in PBS for 30 min. Samples were incubated with primary rabbit anti-AhR (1:50) or anti-Nrf2 (1:50) antibody in Western breeze blocker–diluent (Invitrogen, Carlsbad, CA, USA) overnight at 4˚C. Slides were washed with PBS before incubation with anti-rabbit secondary antibody (Alexa Fluor 546 or 488; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After nuclear staining with 40,6-diamidino-2-phenylindole (DAPI), slides were mounted with UltraCruz mounting medium (Santa Cruz Biotechnology). All samples were analyzed using a D-Eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan).

- **Reverse transcription–polymerase chain reaction and quantitative real-time polymerase chain reaction analyses**

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the Mx3000p real-time system (Stratagene, La Jolla, CA, USA) using SYBR Premix Ex Taq (Takara Bio). Amplification was started at 95˚C for 30 sec as the first step, followed by 40 cycles of qRT-PCR at 95˚C for 5 sec, and at 60˚C for 20 sec. mRNA expression was measured in triplicate and was normalized to ß-actin expression

levels. The primer sequences from SABiosciences (Frederick, MD, USA) are shown in Table 3.2.1.

Table 3.2.1.   Primers for Quantitative Real-time Polymerase Chain Reaction

Gene	Forward primer	Reverse primer	SABiosciences ID
NQO1	5′-GGATTGGACCGAGCTGGAA-3′	5′-AATTGCAGTGAAGATGAAGGCAAC-3′	
NRF2	5′-CTTGGCCTCAGTGATTCTGAAGTG-3′	5′-CCTGAGATGGTGACAAGGGTTGTA-3′	
CYP1A1			PPH01271E
FLG	5′-CATGGCAGCTATGGTAGTGCAGA-3′	5′-ACCAAACGCACTTGCTTTACAGA-3′	
LOR	5′-GGCTGCATCTAGTTCTGCTGTTTA-3′	5′-CAAATTTATTGACTGAGGCACTGG-3′	
β-actin	5′-ATTGCCGACAGGATGCAGA-3′	5′-GAGTACTTGCGCTCAGGAGGA-3′	

● **Detection of ROS production by microscopy**

DCFH-DA (Molecular Probe) is a cell-permeable nonfluorescent probe that is de-esterified intracellularly and oxidized to highly fluorescent 2',7'-dichlorofluorescein in the presence of ROS. After treatment with or without TNFα (10 ng/mL) for 30 min or BaP (1 µM) for 3 h in the presence or absence of 3% OFIE, NHEKs were incubated with DCFH-DA (5 µM) for 30 min at 37˚C, and the fluorescence signal of 2',7'-dichlorofluorescein (ex. 490 nm), the oxidation product of DCFH-DA, was analyzed using a D-Eclipse confocal laser scanning microscope (Nikon).

Transfection with small interfering RNAs (siRNAs) against AhR (s1200) and Nrf2 (s9492), as well as siRNA consisting of a scrambled sequence that would not lead to specific degradation of any cellular message (control siRNA), were purchased from Ambion (Austin, TX, USA). NHEKs cultured in 24-well plates were incubated with mixing with HiPerFect transfection reagent (Qiagen) containing 10 nM siRNA and 3.0 µL of HiPerFect reagent in 0.5 mL of culture medium. After a 48-h incubation period, siRNA-transfected NHEKs were treated with or without OFIE, TNFα, or BaP for 6 h. The transfection of siRNA had no effect on cell viability, as demonstrated by microscopic examination (data not shown).

- **Statistical analysis**

Unpaired Student's t-test (when two groups were analyzed) and one-way analysis of variance (for three or more groups) were used to analyze the results, and a P value < 0.05 was considered statistically significant.

3.3 Study of age-dependent miRNAs in human dermis

- **Tissue collection from donors**

Following the pre-approval from the Independent Ethics Committee of Huashan Hospital affiliated to Fudan University, normal human skin tissue (8x5x3 mm) from sun-protected part of body was obtained from 12 consenting individuals who were undergone Miles surgeries or excisions of benign skin neoplasms. The donors with the age over 60 years old were defined as the aged group, and ones with the age below 10 years old were defined as the young group, respectively; each group was constituted of 6 individuals. Adipose tissue and epidermis were carefully removed by forceps. The remained dermal tissue was stored in the liquid nitrogen at -196°C for subsequent RNA analysis.

- **Primary cultures of human dermal fibroblast and the induction of senescence**

Primary human dermal fibroblasts (HDFs) were obtained from the foreskin of a healthy man (Huashn Hospital, Shanghai, China). Cells were cultured in high glucose DMEM (Gibco) medium supplemented with 10% FBS (Invitrogen) at 37°C in 5% CO_2 humidified air. Serial passaging to induce replicative cell senescence was performed twice weekly at early passage and at decreasing dilution with increasing passage. Cells from either passage 5 or passage 20, referred as young or old cells, respectively, were used in subsequent experiments. The proliferative potential of the culture of different passages was evaluated by cell morphology, survival curve and senescence-associated β-galactosidase staining.

● **RNA extraction**

Total RNA of dermal tissue or dermal fibroblasts was extracted using mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. The quantity and quality was evaluated using a bioanalyzer (Agilent 2100 Bioanalyzer, LabChip Kits).

● **miRNA microarray and data analysis**

miRNA microarray study, including labeling, hybridization, scanning, normalization, and data analysis, was performed by Shanghai Biotechnology Corporation (Shanghai, China) on Agilent Human miRNA Genechip V16.0, which held 1205 human-related miRNAs. Briefly, miRNA molecules were labeled according to the manufacturer's protocol, followed by the hybridization reaction for 20 h at 55˚C. Microarray results were obtained and extracted by Agilent Microarray Scanner and Feature Extraction software 10.7 (Agilent). Raw data were subsequently normalized by Quantile algorithm with Gene Spring Software 11.0 (Agilent). The array output was presented on Excel spreadsheets giving lists of miRNA profile in each young and aged human dermal sample. The lists were sorted based on the most differentially expressed miRNAs between the two sample groups. Filtering criterial set for defining up- or downregulated miRNAs was a fold change ≥ 2 and a p value ≤ 0.01.

● **miRNA target gene prediction, annotation and function analysis**

Target genes regulated by the miRNAs can be predicted theoretically by applying an advanced computational approach. With different algorithms, the prediction of target genes of either upregulated or downregulated miRNAs in this study was performed within the databases of miRDB (Wong et al. 2015) and TargetScan (Grimson et al. 2007). Gene ontology (GO) and pathway enrichment of target genes were retrieved from the database for annotation, visualization, and integrated discovery (DAVID

Bioinformatics Resources, http://david.abcc.ncifcrf.gov/home.jsp). The enrichment was calculated with the modified Fisher's exact test in the DAVID Bioinformatics Resource. Enrichment bar graphs were shown in terms of –log (p-value).

● **miRNA-target gene network**

Target genes associated with organism aging, ECM modulation, cell proliferation, apoptosis, cell cycle, cancer metabolism etc. were selected based on the annotations of enriched GO and pathway terms. The network of selected miRNAs and target genes was depicted by using the software Cytoscape. The regulatory role of each miRNA and its target genes was evaluated and its degree was shown by means of an appropriate color, ranging from pink to yellow.

● **Real-time quantitative reverse transcriptase PCR**

Quantification of miRNA was performed by a two-step reaction process: reverse transcription (RT) and PCR using miScript PCR system (Qiagen, Germany). Each RT reaction was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) for 60 min at 37°C, followed by heat inactivation of RT for 5 min at 95°C. Real-time PCR was performed using LightCycler® 480 Ⅱ Real-time PCR Instrument (Roche, Swiss) with following program: Cycle 1, 95°C for 10min; Cycle 2, 40 cycles of 95°C for 10s, 60°C for 30s. The miRNA-specific primer sequences were designed in the laboratory and synthesized by Generay Biotech (Generay, PRC) based on the miRNA sequences obtained from the miRBase database (Table 3.3.1).

Table 3.3.1.　The miRNA-specific primer sequences

miRNA name	Primer Sequence
U6	CAAGGATGACACGCAAATTCG
hsa-miR-34a	TGGCAGTGTCTTAGCTGGTTGT
hsa-miR-34b-5p	TAGGCAGTGTCATTAGCTGATTG
hsa-miR-29c-3p	TGACCGATTTCTCCTGGTGTTC

hsa-miR-1207-5p	TGGCAGGGAGGCTGGGAGGGG
hsa-miR-1226-5p	GTGAGGGCATGCAGGCCTGGATGGGG
hsa-miR-134	TGTGACTGGTTGACCAGAGGGG
hsa-miR-424	CAGCAGCAATTCATGTTTTGAA
hsa-miR-601	TGGTCTAGGATTGTTGGAGGAG

The expression levels of miRNAs were normalized to U6 and were calculated using the 2-ΔΔCt method. Quantification of mRNA of HDFs was performed with similar methods as described elsewhere (Wang et al. 2014). The mRNA primer sequences were provided in Table 3.3.2.

Table 3.3.2. The mRNA-specific primer sequences

Genes	Primer Sequence	Product Size
p53	5'-CAGCACATGACGGAGGTTGT-3'	125bp
	5'-TCATCCAAATACTCCACACGC-3'	
p16	5'-ATGGAGCCTTCGGCTGACT-3'	108bp
	5'-GTAACTATTCGGTGCGTTGGG-3'	
p21	5'-TGTCCGTCAGAACCCATGC-3'	139bp
	5'-AAAGTCGAAGTTCCATCGCTC-3'	
c-Met	5'-GGTTCACTGCATATTCTCCCC-3'	205bp
	5'-ACCATCTTTCGTTTCCTTTAGCC-3'	
COL1A1	5'-CCCACCAATCACCTGCGTACAGA-3'	214bp
	5'-TTCTTGGTCGGTGGGTGACTCTGA-3'	
Elastin	5'-CCGCTAAGGCAGCCAAGTATGGA-3'	275bp
	5'-AGCTCCAACCCCGTAAGTAGGAAT-3'	
MMP-1	5'-GGGCTGAAAGTGACTGGGAAAC-3'	170bp
	5'-GCTCTTGGCAAATCTGGCGTGT-3'	
GAPDH	5'-GAGTCAACGGATTTGGTCGT-3'	238bp
	5'-TTGATTTTGGAGGGATCTCG-3'	

- **Senescence-associated β-galactosidase staining**

Senescence-associated β-galactosidase staining (SA-β-gal) of cultured HDFs was

conducted with Senescence Cells Histochemical Staining Kit (Sigma) according to the manufacturer's instruction. After overnight incubation in a non-CO_2 enriched atmosphere, plates of cells were observed under a microscope. The numbers of blue-stained cells were counted and their percentages in terms of the total numbers of cells were calculated.

- **Statistical analysis**

Data were given as mean values ±SD. Significant differences were identified applying Student's t-test with a cut off p-value ($p < 0.05$), using the software SPSS.

3.4 Study of specific pattern of miRNAs, modulated in human skin fibroblasts by a biofunctional complex

- **Cell culture**

Neonatal normal human dermal fibroblasts were cultured in Dulbecco's modified Eagle's Medium (DMEM), 1 g/L glucose (Lonza, Rockland, U.S.A.) supplemented with 10% decomplemented fetal calf serum (Lonza), 2 mM of L-glutamine (Lonza) and 0.1 mg/ml Primocin™ (Invivogen, San Diego, U.S.A.). Fibroblasts were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Tested ingredient (Ashland Specialty Ingredients, Wilmington, U.S.A.) was used in our experiments after a 1:100 dilution (1 v/v%).

- **Properties of the tested natural ingredient complex**

Tested ingredient is a complex of hydrolyzed natural yeast (*Saccharomyces cerevisiae*) proteins and hydrolyzed natural soya proteins with a total dry matter weight of 7 g/kg. This ingredient complex was selected by screening several botanical and yeast extracts and their combination, for its ability to modulate the expression of telomere-binding proteins such as TRF2 (Imbert et al. 2012; De lange. 2005; Stewart et al. 2012) in *ex*

vivo human skin, human primary keratinocytes and neonatal human fibroblasts in culture. This ingredient is described in patent FR12 00814.

- **miRNAs of interest**

A list of miRNAs with a described implication in human dermal homeostasis, in fibroblasts senescence and in the regulation of dermal extracellular matrix content was established according to the litterature (Table 4.3.1). Twenty-one miRNAs were hence selected: miR-10b-5p, miR-1185-5p, miR-1204, miR-143-3p, miR-145-5p, miR-146a-5p, miR-155-5p, miR-199a-3p, miR-21-5p, miR-23a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-335-3p, miR-34a-5p, miR-519c-3p, miR-542-5p, miR-638, miR-658, miR-663, miR-7-5p.

- **Target genes**

Bioinformatics was used to obtain predicted and experimentally verified list of target genes for the selected miRNAs in this study. Two types of analyses were conducted.

(i) The first approach used miRWalk database which is a comprehensive database providing the largest available collection of predicted and experimentally verified (validated) miRNA-target interactions (Dweep et al. 2011). Each miRNA that was modulated in our experiments was searched in the "validated targets module" of miRWalk in order to retrieve a list of validated targets that was further processed to keep only genes involved in the extracellular matrix homeostasis and in fibroblast senescence.

(ii) The second approach used Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, U.S.A.) to possibly identify new interactions that could be complementary to the ones identified through miRWalk database. IPA predicts miRNA interactions with target genes, based on the aggregation of data from TarBase (experimentally validated data), miRecords, and TargetScan (predicted miRNA-mRNA interactions) (Vlachos et

al. 2015; Xiao et al. 2009; Lewis et al. 2005). The resulting lists of genes established by these two approaches are represented in Table 4.3.2.

- **qPCR array experiments**

Cells were grown in 100 mm culture dishes and treated or not with 1% tested ingredient in the culture medium for 48 h. Culture medium was then removed and the cells were washed with cold PBS. Total RNA including miRNAs was extracted in each sample using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) and collected in 100 μl of RNase free water. RNA quantity, quality and purity were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, U.S.A.) by measuring A260 nm/A280 nm and A260 nm/A230 nm ratios. For each sample (untreated and treated) 10 ng of total RNA were reverse-transcribed by using the Universal cDNA Synthetis Kit mercury LNATM (Exiqon, Vedbaek, Denmark) on a Mini cycler thermocycler (MJ Research, Waltham, MA, USA). Real-Time qPCR to amplify the 21 selected miRNAs was performed on a StepOnePlus™ thermocycler (Applied Biosystems, Foster City, CA, USA) using the Pick-&-Mix miRNA PCR Panel with custom selection of LNA™ miRNAs primer sets (Exiqon) and SYBR® Green master mix, and Universal RT according to manufacturer's recommendations (Exiqon). The comparative Ct method was used for relative quantification of miRNAs expression. Let-7a-5p was used as the house keeping gene for analysis.

- **Interaction network of miRNAs and genes**

An interaction network was built using Ingenuity Pathway Analysis (Qiagen). This network included the 18 miRNAs that showed a modulation in their expression after treatment with the ingredient, and the 25 target genes described in miRWalk database (Table 4.4.1). Around these miRNAs and genes, an interaction network was developed and allowed to extend it with 8 new components (in yellow, Figure 4.4.2b): nidogen 1

50

(NID1), integrin beta 6 (ITGB6), integrin beta 8 (ITGB8), collagen IV alpha 5 (COL4A5), ADAM metallopeptidase with thrombospondin type 1 motif 6 (ADAMTS6), reversion-inducing-cysteine-rich protein with kazal motifs (RECK), chymase 1 (CMA1), and fibroblast growth factor 13 (FGF13).

3.5 Study of melanin uptake by keratinocyte by a newly established cell model

● Cell culture

Human epidermal keratinocytes were isolated from skin by enzymatic digestion method as described previously (Hsieh et al. 2012). Skin samples were obtained from donors with informed consents, under the approval of the Institutional Review Board of Chang Gung Memorial Hospital in Tao-Yuan, Taiwan. Briefly, skin was rinsed in 10 % iodine for 30 sec and cleaned with 1×PBS, then incubated in dispase (type II, 2.5 mg/ml in 1×PBS) for 2 h at 37 ˚C to dissociate the epidermis from dermis. The epidermal layer was peeled off carefully and cut into small pieces and incubated in 0.25% trypsin for 30 min at 37˚C to release single cell. Cells were centrifuged and the cell pellet was resuspended in keratinocyte serum-free medium (KSFM, GIBCO®) containing EGF and bovine pituitary extract and maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Keratinocytes between passages 3 and 5 were used for all the experiments. In the present study, epidermal keratinocytes at 70-80% confluence were treated with melanin (0-100 μg/ml) for 24 h or indicated time and used for the analysis. All experiments were performed in triplicate.

● Measurement of melanin uptake

The content of melanin uptake in keratinocytes was determined by spectrophotometric method. Cells were washed thoroughly by 1×PBS, homogenize d with 1N NaOH and heated at 95˚C for 30 min to dissolve intracellular melanin. The melanin content was determined by measuring the absorbance at 495 nm using a spectrophotometer.

- **Examination of melanin uptake in cultured keratinocytes and solar lentigo tissue**

Keratinocytes cultured on coverslips were fixed in 1×PBS containing 4% paraformaldehyde for 15 min and stained with modified Wright-Giemsa stain (Sigma) for the microscopic examination of melanin uptake in cells. HE-stained tissue sections of solar lentigo was obtained from the tissue bank in Chang Gung Memorial Hospital (Tao-Yuan, Taiwan) and pathology was diagnosed and identified by Dr. Wen-Rou Wong who is a skin specialist in Chang Gung Memorial Hospital. Solar lentigo is a small pigmented spot with a clearly defined edge on the skin associated with aging and exposure to ultraviolet radiation from the sun. Histologically, the hallmark of solar lentigo is an increase in basal melanin.

- **MTT assay**

Epidermal keratinocytes grown in 24-well culture plate with or without melanin treatment for 24 h were washed once with 1×PBS, followed by addition of 0.5 ml KSFM containing 0.05 mg/ml 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). After incubation at 37°C for 1 h, the medium was removed and formazan crystals in the cells were dissolved in 1 ml DMSO for OD reading at 570 nm using a spectrophotometer.

- **Immunofluorescent staining**

Keratinocytes cultured on coverslips were fixed in 4% paraformaldehyde in 1×PBS for 15 min and then in methanol at -20°C for 10 min. Cells were blocked in 1×PBS containing 1% BSA and 1% goat serum for 30 min. Rabbit antibody against Ki67 (Lab Vision) was added to blocking solution in a ratio of 1:50 and incubated at 4°C overnight. After washing with 1×PBS, FITC -conjugated goat anti-rabbit IgG (BioFX) was added (1:2000 in 1×PBS) for 30 min. After washing with 1×PBS, cell nuclei were

counter-stained by propidium iodide for 10 min at 37°C. Coverslips were mounted with fluorescent specific medium for the examination under fluorescent microscope.

- **Immunohistochemistry**

Rehydrated tissue section was pretreated in 10 mM citrate buffer, pH6.0, at 95°C for 20 min followed by cooling at room temperature for 20 min. Tissue section was blocked in 1×PBS containing 1% BSA and 1% goat serum for 30 min. Rabbit antibody against Ki67 (Lab Vision) was added to blocking solution in a ratio of 1:50 and incubated at 4°C overnight. After washing with 1×PBS, section was incubated with biotinylated goat anti-rabbit IgG (Thermo Scientific) for 15 min. After washing with 1×PBS, section was incubated with streptavidin-conjugated peroxidase (Thermo Scientific) for 10 min. In order to differentiate from the brown color of melanin in lentigo tissue, positive signals were developed by reacting peroxidase with ImmPACTTM SG substrate (VECTOR) for 15 min and Ki67 expression was developed into blue color. Cell nuclei were counterstained by nuclear fast red (VECTOR) for 10 min and mounted with VectaMountTM AQ mounting medium (VECTOR).

- **Flow cytometry**

Keratinocytes were harvested by trypsinization. After washing with 1×PBS and centrifugation, the cell pellet was resuspended in 1 ml ice-cold 70% ethanol and incubated overnight on a rocker at 4°C. Cells were collected, washed and incubated in a solution of 0.5% Triton X-100 and 0.05% RNase at 37°C for 1 h. Cell nuclei were then stained by propidium iodide (PI) solution (50 mg/ml PI in 1×PBS) and incubated at 4°C for 20 min. Cell cycle distribution was quantified with a FACS Calibur system (Becton-Dickinson). Percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined and analyzed using CellQuest Pro software (Becton Dickinson).

RNA isolation and RT/real-time PCR

Total RNA was extracted from keratinocytes by acid guanidinium thiocyanate–phenol–chloroform extraction method, and complementary DNA (cDNA) was synthesized using 1 μg total RNA in a 20 μl RT reaction mix containing 0.5 μg/μl of random primers, 0.1 mM dNTP, 0.1 M DTT and 5×first strand buffer. Real -time PCR was performed using an SYBR Green I technology and MxPro- Mx3000P QPCR machine (Stratagene), and a master mix was prepared with Smart Quant Green Master Mix with dUTP & ROX Kit (Protech). Relative gene expressions between experimental groups were determined using MxPro software (Stratagene) and GAPDH was used as an internal control. All real-time PCRs were performed in triplicate, and changes in gene expressions were reported as multiples of increases relative to the controls. The following primers were used:

GAPDH:5'-GAGGGGCCATCCACAGTCTT-3'   (forward)   and 5'-TTCATTGACCTCAACTACAT-3' (reverse).

DKK1: 5'-CGTTGTTACTGTGGAGAA-3' (forward) and 5'-GTGTGAAGCCTAGAAGAAT-3' (reverse).

Western blot analysis

Epidermal keratinocytes were rinsed with cold 1×PBS, scraped and solubilized in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), followed by sonication and centrifugation at 12,000 g for 20 min at 4˚C. The protein concentration in the supernatant was determined by Bradford protein assay kit (Bio-Rad). Cell lysates containing equal amounts of protein were separated by 10% SDS-PAGE, and transferred onto PVDF membrane (Millipore). The membrane was incubated in blocking solution (1% BSA, 1% goat serum in 1×PBS) for 1 h, followed by incubation

with primary antibody diluted in blocking solution. After washing, the membrane was incubated in 1×PBS containing secondary antibody conjugated with horseradish peroxidase for 1 h. The membrane was washed and the positive signals were developed with enhanced chemiluminescence reagent (Amershan Pharmacia Biotech). The following primary antibodies were used: anti-CDK1 antibody (MS-110-P1, Thermo), anti-CDK2 antibody (MS-459-P0, Thermo), anti-cyclin A antibody (ab38, Abcam), anti-cyclin B antibody (MS-338, Thermo), anti-cyclin E antibody (14-6714, eBioscience), anti-DKK1 (AF1096, R&D systems) and anti-tubulin-α antibody (MS-581-P, Thermo). Tubulin was used as the sample loading control.

4. RESULTS

4.1 Longitudinal study of skin aging progress

● **Los Angles facial wrinkling longitudinal study**

For the 122 women in the study, persistent wrinkling, i.e. wrinkling measured with a relaxed expression, increased by an average of 39% over the 8-year period. Temporary wrinkling, i.e. wrinkling measured with a smiling expression, increased by an average of 22% over the 8-year period (Table 4.1.1).

Table 4.1.1.   Wrinkle area fraction (WAF) group means in 2000 and 2008

All subjects	n	WAF 2000 (mean ± SEM)	WAF 2008 (mean ± SEM)
Persistent wrinkling	122	0·045 ± 0·04	0·063 ± 0·05
Temporary wrinkling	122	0·078 ± 0·05	0·095 ± 0·06
Persistent wrinkling by subgroups			
Baseline age group (years)			
10s	16	0·014 ± 0·008	0·017 ± 0·010
20s	14	0·016 ± 0·009	0·025 ± 0·011
30s	24	0·036 ± 0·007	0·050 ± 0·009
40s	30	0·047 ± 0·006	0·082 ± 0·008*
50s	20	0·074 ± 0·007	0·091 ± 0·009
60s	17	0·075 ± 0·008	0·090 ± 0·010
Change in menstrual status			
No change	94	0·047 ± 0·004	0·060 ± 0·005
Entered menopause	28	0·037 ± 0·003	0·072 ± 0·009*
Baseline cheek hydration			
> 50	59	0·040 ± 0·004	0·049 ± 0·006
< 50	63	0·050 ± 0·004	0·076 ± 0·005*
Baseline cheek pH			
< 5·3	41	0·055 ± 0·005	0·063 ± 0·007
> 5·3	81	0·040 ± 0·004	0·062 ± 0·005*
< 5·6	87	0·044 ± 0·004	0·058 ± 0·005
> 5·6	35	0·048 ± 0·006	0·075 ± 0·007
Baseline cheek skin lightness			
L* > 55	101	0·047 ± 0·003	0·068 ± 0·004*
L* < 55	20	0·035 ± 0·008	0·036 ± 0·010
Ethnicity			
African-American	20	0·036 ± 0·007	0·036 ± 0·009*
Caucasian	60	0·059 ± 0·004	0·081 ± 0·005
East Asian	22	0·029 ± 0·007	0·047 ± 0·009
Latino	20	0·032 ± 0·007	0·053 ± 0·009

*Change from 2000 to 2008 is significantly different (P < 0·05) compared with the corresponding subgroups' change from 2000 to 2008.

To track the progression of individual lines and wrinkles on each person, we inspected each subject's pattern of wrinkling around the eyes and on the cheek with and without facial expression at baseline and compared it with the pattern at 8 years later. We consistently found that the subjects unique pattern of persistent facial wrinkling observed with a neutral expression at 8 years later was predicted by the pattern of temporary wrinkling observed with a smiling expression at baseline (Fig. 4.1.1).
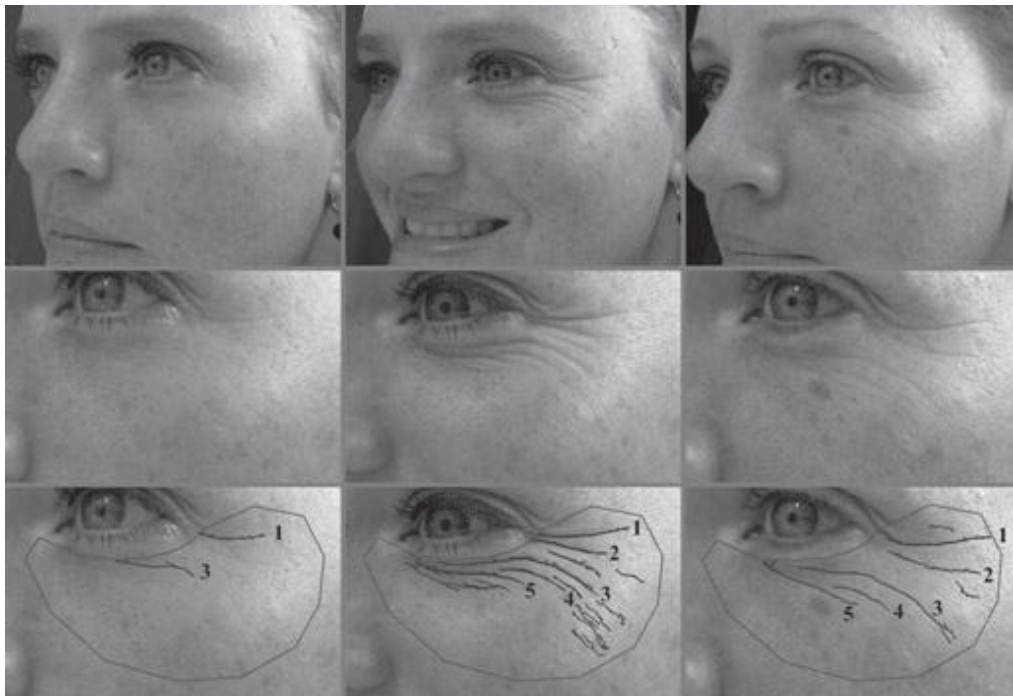

Fig. 4.1.1.   Progression of temporary into persistent wrinkling. At baseline at age 28 years (left column), only a few shallow wrinkles are evident in this subject's neutral image, which are better observed in the zoomed images without (middle row) and with (bottom row) wrinkle image analysis overlays. The baseline expression image (middle column) shows substantial temporary wrinkles around the eye, which is not evident in the baseline neutral image. Eight years later at age 36 years (right column), the pattern of persistent wrinkles (individually numbered) in the neutral image can be traced back to the pattern of temporary wrinkles in the baseline expression image.

Quantitatively, there was a strong correlation ($r = 0.77$, $P < 0.001$) between each subject's severity of temporary wrinkling at baseline and subsequent persistent wrinkling at 8 years later (Fig. 4.1.2). We were interested in knowing if having high severity of smiling-induced temporary wrinkles relative to persistent wrinkles at baseline increased the likelihood of having a greater increase in persistent wrinkles over the 8-year period. For subjects over 25 years of age (those who showed wrinkling), the increase in persistent wrinkling from baseline to 8 years later tended to be greater for those subjects with higher baseline ratios of temporary to persistent wrinkling ($r = 0.50$, $P < 0.001$).
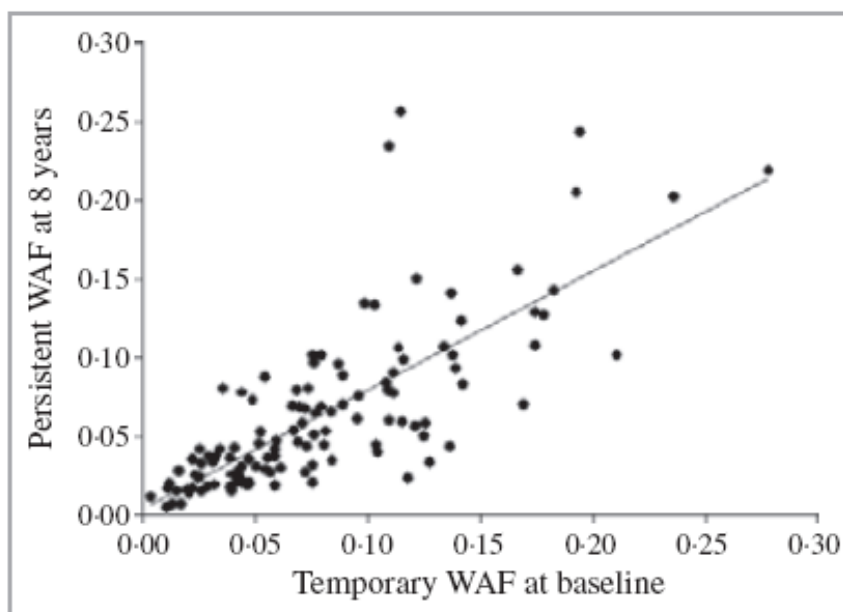
Fig. 4.1.2.   Correlation between baseline temporary and 8-year persistent wrinkling. Each point represents a subject in the study ($r = 0.77$, $P < 0.001$). WAF, wrinkle area fraction.

● **Akita facial skin longitudinal study**

VIP scores of each skin parameters were obtained after the regression analysis compared to the subjective impression on facial skin aging visual grading data. VIP scores of hyperpigmented spots, wrinkles and textures were significantly higher than

VIP scores of the other skin optical parameters therefore, these three skin parameters were used for the subsequent statistical analysis as the primary visual skin aging index. The Skin Ageing Score obtained by adding the relative changes in these three parameters was highly correlated with the changes in the visual evaluation of the degree of subjective impression of younger-looking or older-looking skin (r = 0.5873, p < 0.001) (Fig. 4.1.3).
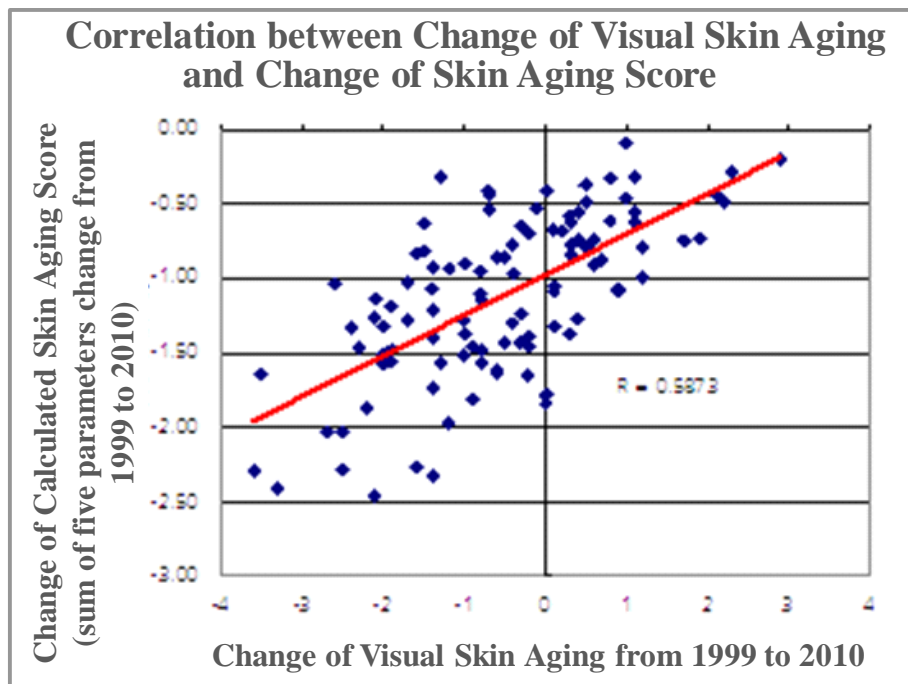

Fig. 4.1.3.   Correlation between the visual appearance of skin aging and the calculated skin-aging

score.

When the Skin Aging Score was plotted against age at the time of the 2010 study, the change in score was greater as the age advanced. According to the results of the cluster analysis of Skin Aging Score against age, the subjects were classified into three groups: mild skin aging group (30 subjects with mean age (mean ±SD) of 49.7 ± 19.5 years, mean Skin Aging Score: -0.5781), age-appropriate skin aging group (53 subjects with mean ±SD of 45.98 ±16.48 years, mean Skin Aging Score: -0.9999),

and severe skin aging group (25 subjects with mean $\pm$SD of 48.0 $\pm$15.8 years, mean Skin Aging Score: -1.8572) (Fig. 4.1.4).
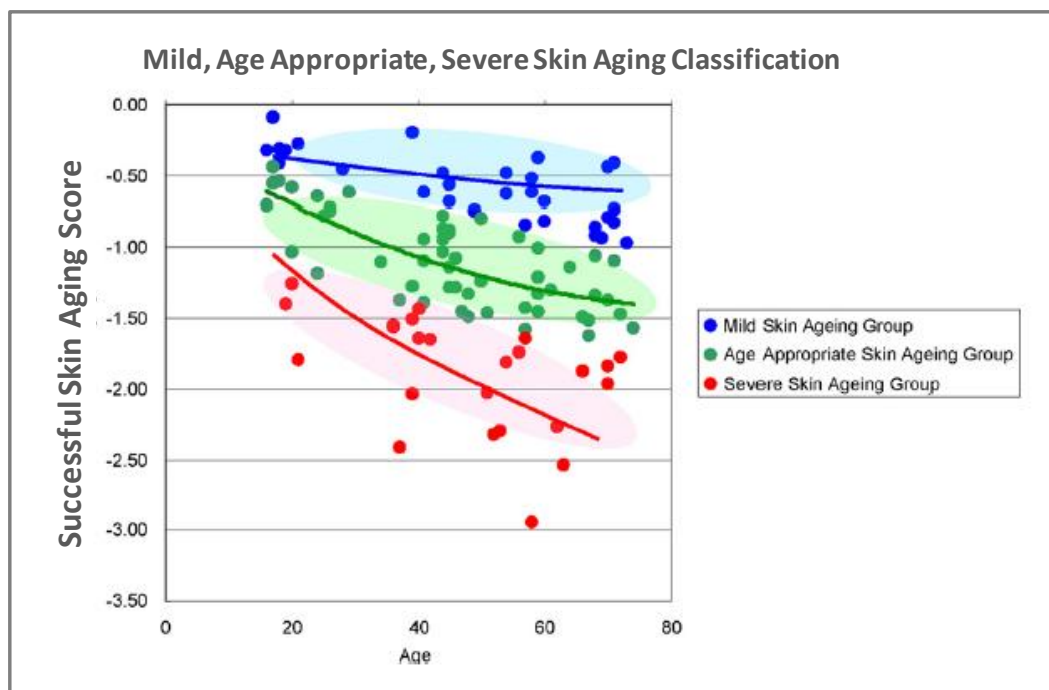

Fig. 4.1.4.   A skin-aging score calculated from the factors 'hyperpigmented spots', 'wrinkles', and 'texture' was shown to be suitable for classifying subjects into mild, age-appropriate, and severe skin-aging groups.

4.2   OFIE activates AhR-Nrf2 signaling

● **Upregulation of Nrf2 and NQO1 by OFIE**

At first, we examined the cytotoxic effect of graded concentrations of 3%, 7%, 10%, and 15% OFIE on keratinocytes, and assessed by trypan blue dye exclusion test. Since 3% OFIE did not affect the viability of NHEKs, we used concentrations of OFIE of less than 3% throughout the following experiments. Our result showed that concentrations of less than 3%, OFIE significantly upregulated Nrf2 expression in a dose-dependent manner (Fig. 4.2.1A). In addition, OFIE activated Nrf2 by inducing its cytoplasmic-to-nuclear translocation, as visualized in the immunofluorescence study

(Fig. 4.2.1B). In addition, same as the previous report that the activation of Nrf2 induces the subsequent transcription of antioxidant enzymes such as NQO1, in our experiment, OFIE upregulated NQO1 expression in a dose-dependent manner (Fig. 4.2.1C). To confirm the Nrf2 dependence of the NQO1 expression, we knocked down Nrf2 in keratinocytes by transfecting them with Nrf2 siRNA. This transfection inhibited Nrf2 mRNA expression by 54.3% ± 5.5% in NHEKs. As shown in Figure 4.2.1D, the knockdown of Nrf2 significantly abrogated the NQO1 upregulation, indicating that the Nrf2-NQO1 pathway was activated by OFIE.
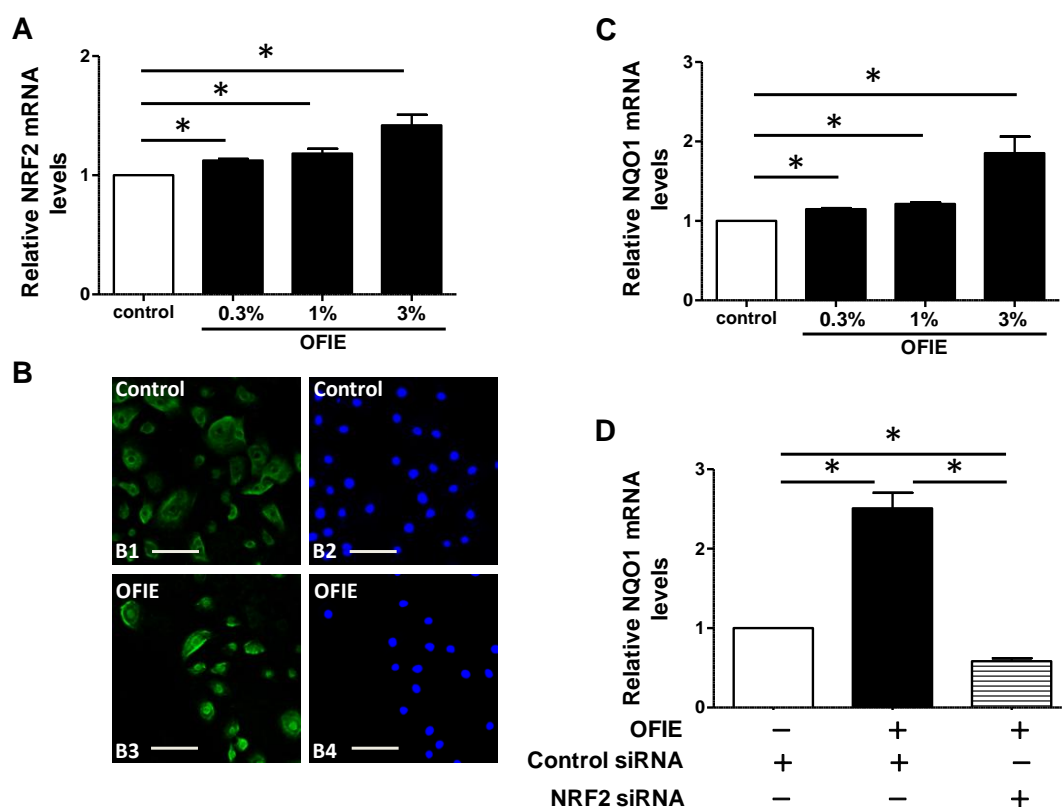

Fig. 4.2.1.   (A) OFIE upregulated the Nrf2 expression of NHEKs in a dose dependent manner. (B) Nrf2 was mainly localized in the cytoplasm of control NHEKs (B1, Nrf2 [green]; B2, nuclear staining by DAPI [blue]). OFIE induced the nuclear translocation of Nrf2 (B3, Nrf2 [green]; B4, nuclear staining by DAPI [blue]). Scale bar=50 μm. (C) OFIE dose-dependently upregulated the NQO1 expression in NHEKs. (D) The upregulating capacity of OFIE on NQO1 expression was inhibited in the NHEKs transfected with Nrf2 siRNA. *P < 0.05. DAPI, 4,6-diamidino-2-phenylindole; NHEK, normal human

epidermal keratinocyte; NQO1, NAD(P)H:quinine oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; OFIE, Opuntia ficus-indica extract; siRNA, small interfering RNA.

- **Antioxidant activity of OFIE was Nrf2 dependent.**

As the *in vivo* administration of OFIE produced antioxidant activity, we next examined whether in vitro treatment with OFIE inhibited ROS generation in keratinocytes stimulated by TNFα or BaP. Control (Fig. 4.2.2A1, A2) or OFIE-treated keratinocytes (Fig. 4.4.2B1, B2) did not show ROS production. Same as what reported previously, both TNFα (Fig. 4.2.2C1, 4.2.2C2) and BaP (Fig. 4.2.2E1, 4.2.2E2) induced robust ROS generation in the treated keratinocytes, while this was inhibited in the simultaneous presence of OFIE (Fig. 4.2.2D1, D2, and 4.2.2F1, F2). To verify that the antioxidant action of OFIE was Nrf2 dependent, NHEKs transfected with Nrf2 siRNA were treated with TNFα or BaP in the simultaneous presence of OFIE (Fig. 4.2.3A, B). The inhibitory action of OFIE was canceled in the Nrf2 knockdown keratinocytes challenge with either TNFα +OFIE or BaP +OFIE (Fig. 4.2.3A, B).
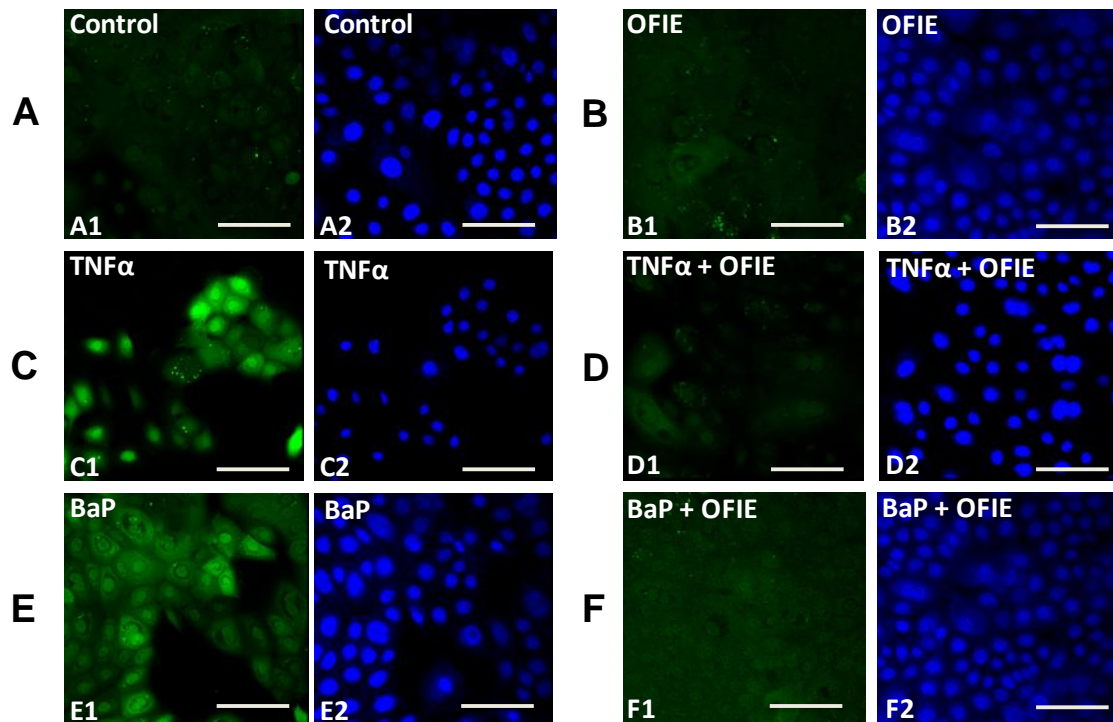
Fig.4.2.2. ROS production of NHEK was visualized by fluorescence analysis. (A) DMS control (A1, ROS; A2, DAPI), (B) OFIE (3%) (B1, ROS; B2, DAPI), (C) TNFα (10 ng/mL) (C1, ROS; C2, DAPI), (D) TNFα (10 ng/mL) + OFIE (3%) (D1, ROS; D2, DAPI), (E) BaP (1 lM) (E1, ROS; E2, DAPI), (F) BaP (1 μM) + OFIE (3%) (F1, ROS; F2, DAPI). Scale bar = 50 μm. BaP, benzo[a]pyrene; ROS, reactive oxygen species; TNFα, tumor necrosis factor α.
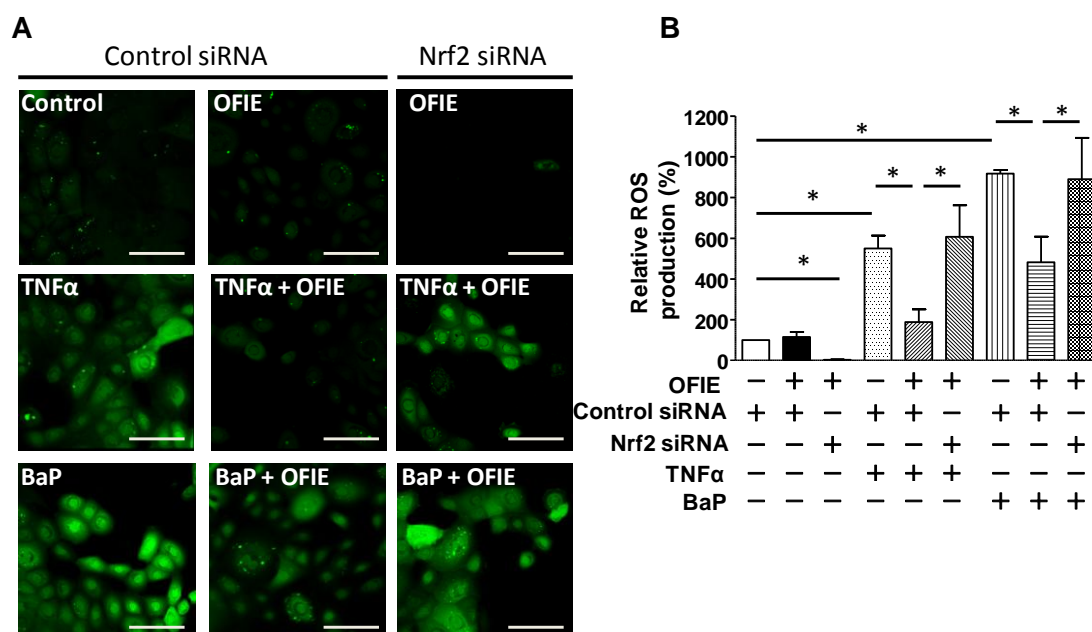
Fig. 4.2.3. (A) ROS production was visualized by fluorescence analysis in NHEKs transfected with control siRNA or Nrf2 siRNA. Inhibitory action of OFIE on the ROS production by TNFα or BaP was canceled in the NHEKs transfected with Nrf2 siRNA. Scale bar = 50 μm. (B) Relative ROS production of Figure 3A was quantified by fluorescence intensity. *P <0 .05.

● **OFIE activated the AhR-Nrf2 pathway**

Previous studies have demonstrated that the upregulation of Nrf2 was mediated by AhR-dependent and AhR independent pathways (Tsuji et al. 2012; Wu et al. 2014). Thus, we next examined whether OFIE activates AhR, namely the cytoplasmic-to-nuclear translocation of AhR, and subsequent CYP1A1 induction, which is a specific marker for AhR activation (Ikuta et al. 2009). AhR mainly localized in the cytoplasm of control keratinocytes (Fig. 4.2.4A1, A2), while OFIE appeared to induce the nuclear translocation of AhR (Fig. 4.2.4A3, A4). The activation of AhR by OFIE occurred in parallel with dose-dependent upregulation of CYP1A1 (Fig. 4.2.4B). To confirm the AhR dependence of the Nrf2-NQO1 pathway, the expression of Nrf2 and NQO1 was assessed in the NHEKs transfected with control siRNA or AhR siRNA. Transfection of AhR siRNA inhibited the AhR mRNA expression by 91.9% ± 2.1% in

NHEKs. The upregulated expression of Nrf2 and NQO1 by OFIE was inhibited in the AhR knockdown keratinocytes (Fig. 4.2.4C, D), confirming the AhR dependence of Nrf2-NQO1 induction by OFIE.
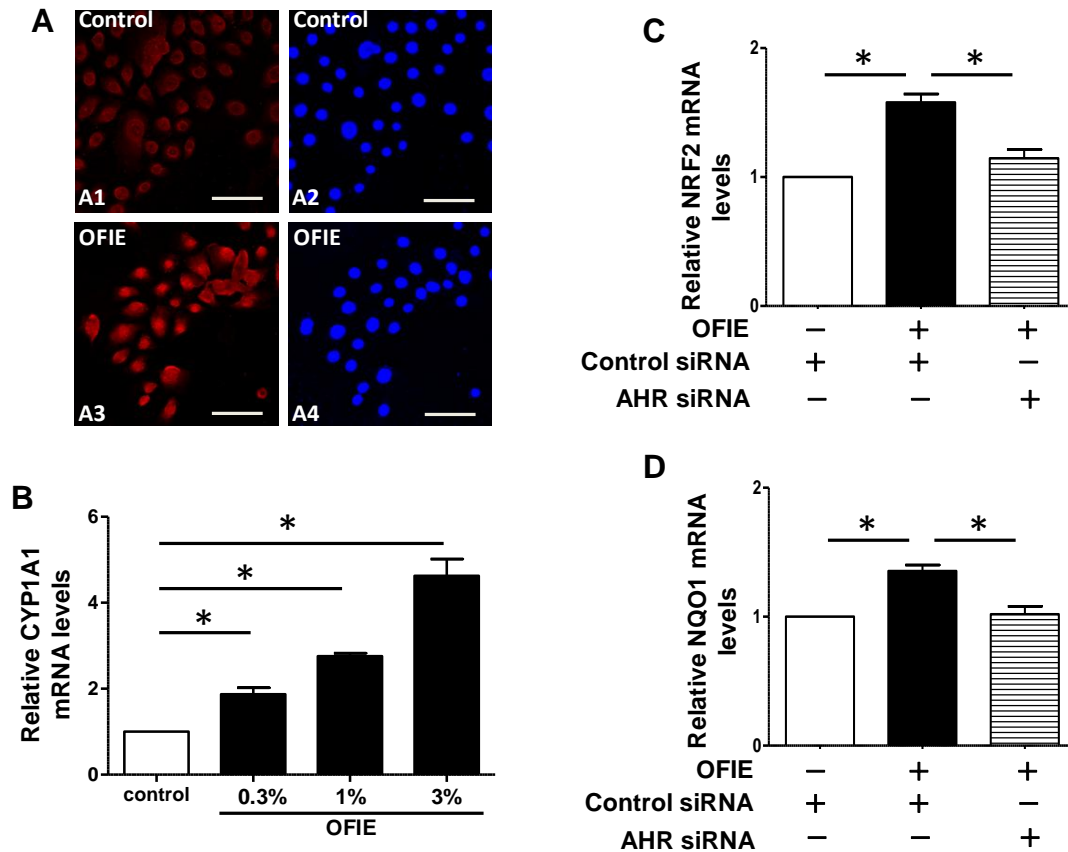

Fig. 4.2.4.   (A) AhR was localized mainly in the cytoplasm of control keratinocytes (A1, AhR; A2, DAPI). OFIE induced the cytoplasmic-to-nuclear translocation of AhR (A3, AhR; A4, DAPI). (B) OFIE dose-dependently upregulated the CYP1A1 expression of NHEKs. (C) The enhancing capacity of OFIE on Nrf2 expression was canceled in the NHEKs transfected with AhR siRNA. (D) The enhancing capacity of OFIE on NQO1 expression was canceled in the NHEKs transfected with AhR siRNA. Scale bar = 50 µm. *P < 0 .05. AhR, aryl hydrocarbon receptor.

● **AhR-dependent upregulation of epidermal barrier proteins by OFIE**

In addition to the induction of the CYP1A1 gene expression, another hallmark of AhR activation is the upregulation of the expression of epidermal barrier proteins such as

65

FLG and LOR. In accordance with these previous findings, OFIE dose-dependently upregulated the expression of FLG (Fig. 4.2.5A) and LOR (Fig. 4.2.5B). The enhancing effects of OFIE on FLG or LOR expression were again canceled in the AhR knockdown keratinocytes, as shown in Figure 4.2.5C, D.
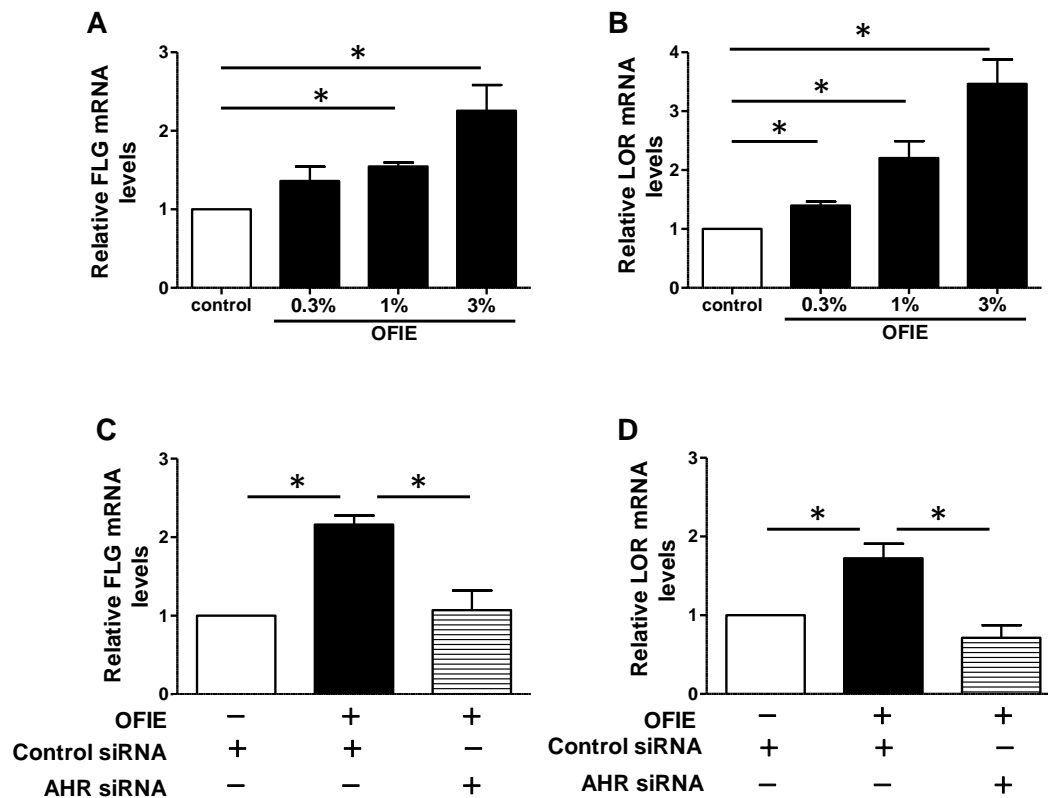

Fig. 4.2.5.   OFIE upregulate barrier proteins by AhR-dependant manner (A) OFIE upregulated the FLG expression in NHEKs in a dosedependent manner. (B) OFIE upregulated the LOR expression in NHEKs in a dose-dependent manner. (C) The enhancing effect of OFIE on the FLG expression was canceled in the AhR knockdown NHEKs. (D) The enhancing effect of OFIE on the LOR expression was canceled in the AhR knockdown NHEKs. $*P < 0.05$.

● **OFIE prevented Th2 cytokine-induced downregulation of epidermal barrier proteins**

It is well known that the downregulation of epidermal barrier proteins is one of the distinct phenomena of Th2-mediated inflammatory skin diseases such as atopic

dermatitis. We next examined whether OFIE is capable of preventing the Th2-mediated downregulation of epidermal barrier proteins. IL-4 or IL-13 significantly inhibited the expression of FLG (Fig. 4.2.6A) and LOR (Fig. 4.2.6B). The simultaneous presence of OFIE potently prevented the IL-4- and IL-13-induced inhibition of FLG (Fig. 4.2.6A) and LOR expression (Fig. 4.2.6B).
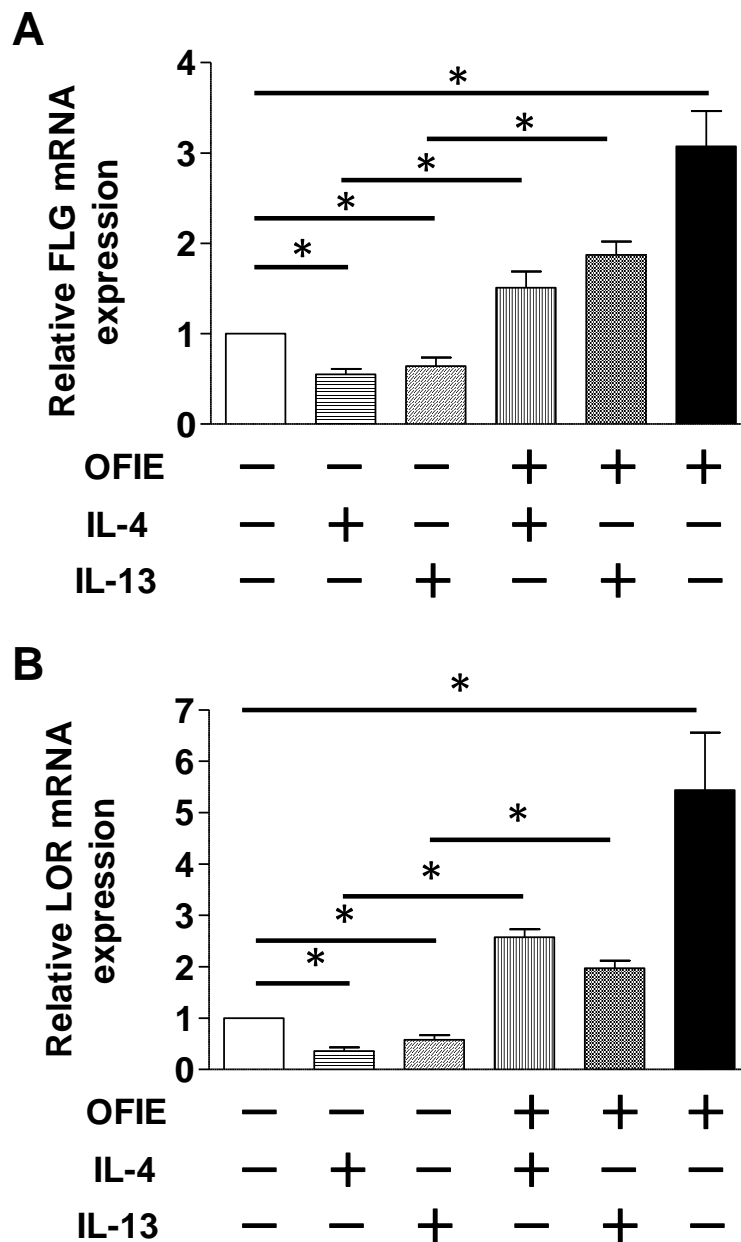

Fig. 4.2.6. IL-4 or IL-13 significantly downregulated the FLG expression. (A) The simultaneous presence of OFIE prevented the inhibitory action of IL-4 or IL-13. (B) IL-4 or IL-13 significantly

downregulated the LOR expression. The simultaneous presence of OFIE prevented the inhibitor action of IL-4 or IL-13. *P < 0.05. IL, interleukin.

4.3   Study of age-dependent miRNAs in human dermis

- **Differential expression of miRNAs in human aged dermis**

To identify differentially expressed miRNAs in aged dermis, the 12 dermal samples were categorized into young group (below 10yrs old) and old group (over 60yrs old). miRNA profiles of the two groups were compared using Agilent Human miRNA array. With filtering criterial of a two fold change and a p-value≤0.01, the expression of a total of 40 miRNAs was found to be significantly altered in aged dermis (Fig. 4.3.1a). Of these, 25 miRNAs were upregulated, whereas 15 were downregulated (Table 4.3.1). For most of the differentially expressed miRNAs, a 2- to 10-fold change was observed in aged dermis (Fig. 4.3.1b). Subsequently miR-34b-5p, miR-34a, miR-29c-5p were selected for validation by RT-PCR. The result confirmed the upregulation of these miRNAs in aged dermis (Fig. 4.3.1c) in a manner consistent with the alteration demonstrated by means of the miRNA array.

Upregulated miRNAs	Downregulated miRNAs
miR-548q, miR-34b-5p (miR-34b*), miR-1226-5p (miR-1226*), miR-601, miR-557, miR-3937, miR-623, miR-29c-5p (miR-29c*), miR-3127, miR-3619, miR-4270, miR-1202, miR-1207-5p, miR-3656, miR-572, miR-2861, miR-638, miR-29b, miR-29a, miR-134, miR-762, miR-4281, miR-34a, miR-29c-3p (miR-29c), miR-3665	miR-424, miR-1973, miR-154, miR-455-3p, miR-487b, miR-1260, miR-181a, miR-4286, miR-1280, miR-1260b, miR-145b-5p, miR-1274b, miR-720, miR-127-3p, miR-3651

Table 4.3.1.   Differentially expressed miRNAs in human aged dermis
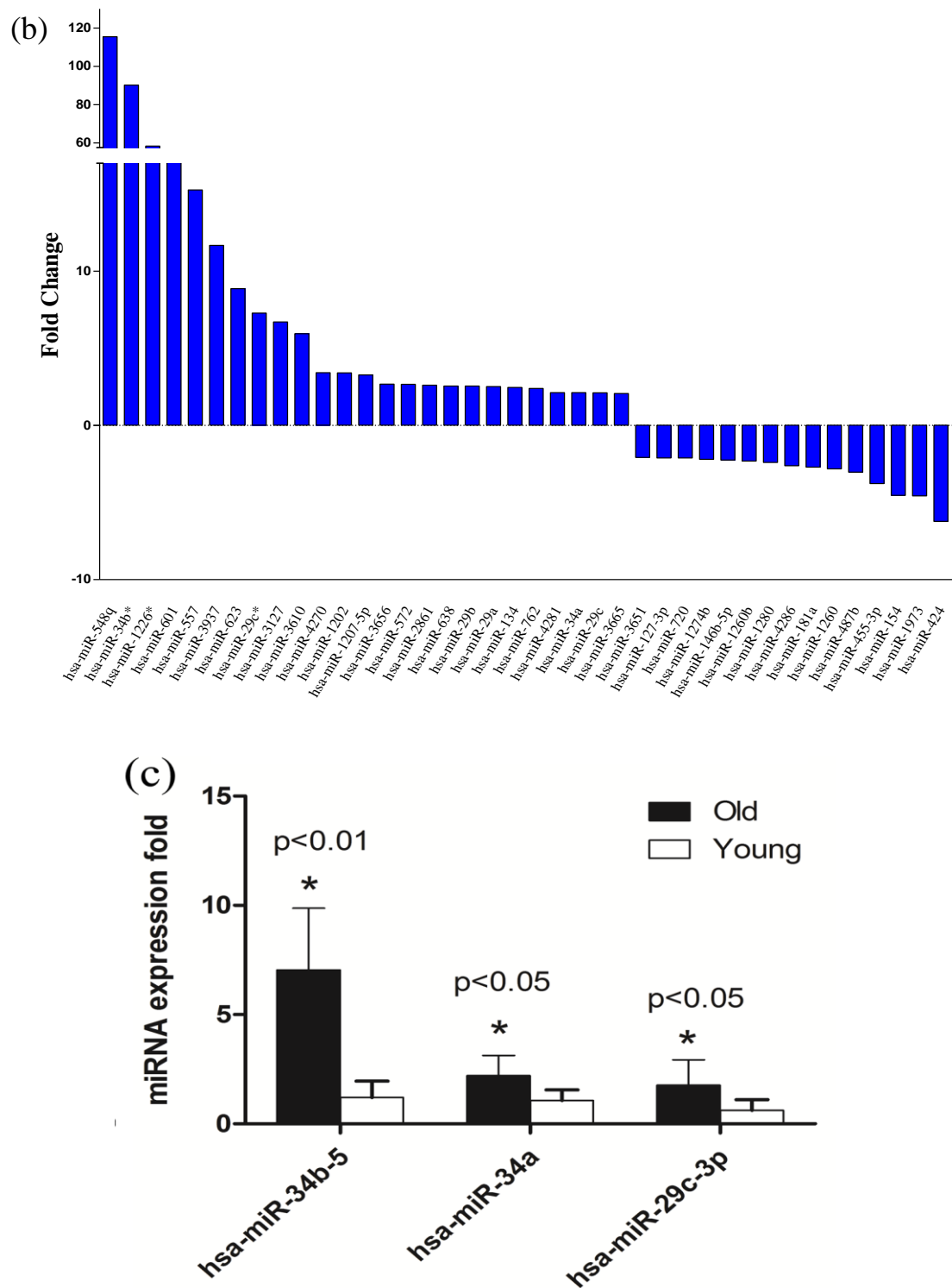
(a)
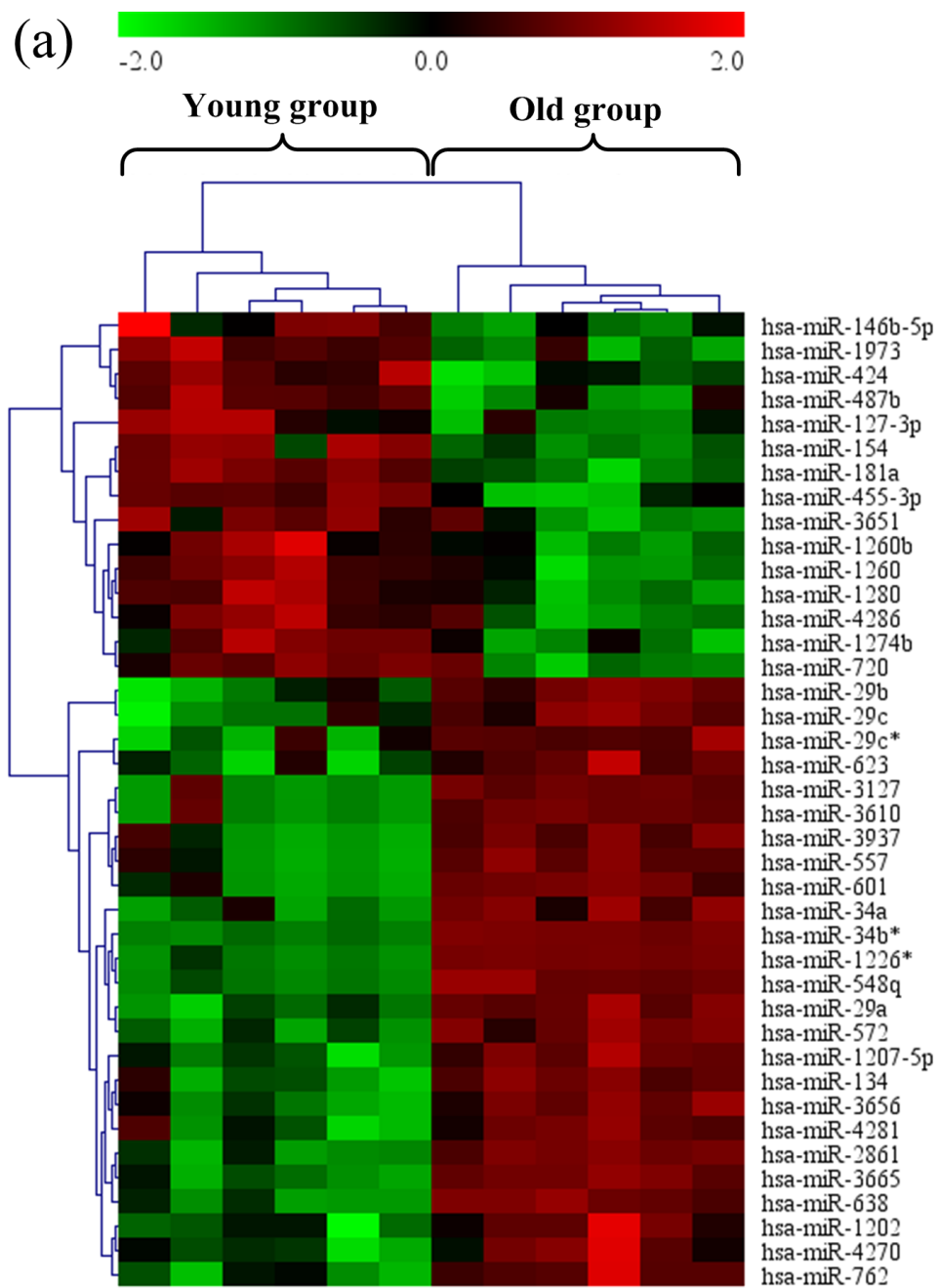
Fig. 4.3.1. Differential expression of miRNAs in aged human dermis. (a) Hierarchical clustering heat map of miRNA from young and aged human dermis. High expression of miRNAs is shown in red, and low expression is shown in green. (b) Bar graph shows significantly altered levels of expression of 40

miRNAs in aged dermis tissue compared with young dermis. (c) Validation of miRNA in dermis. The relative expression of the selected miRNAs was determined by quantitative RT-PCR. Significant upregulation was seen in these miRNAs and was consistent with the alteration demonstrated by means of the miRNA array. Data are presented as mean ±SD (U6; n = 6 for each group. p < 0.05).

- **miRNA target gene prediction, annotation and function analysis reveals multiple processes involving dermis aging**

To analyze the potential role of these differentially expressed miRNAs in dermis aging, prediction of the target genes was performed within the databases of miRDB and TargetScan. The results showed a total of 1731 target genes for upregulated miRNAs and 844 for downregulated ones, indicating that a complex network of miRNAs and their target genes is involved in skin aging.

Further bioinformatic analyses, including GO enrichment and pathway enrichment, were then conducted based on the large scale predicted target genes. GO project provides ontology of defined terms representing gene product properties, which covered three domains: biology process (BP), molecular function (MF) and cellular component (CC). Each domain contains numerous GO terms describing events and functions referring different clusters of genes. With a p ≤ 0.05 and False discovery rate (FDR) ≤ 0.1, GO enrichment results were retrieved by DAVID tool referring predicted target genes of upregulated miRNAs or downregulated miRNAs respectively. A total of 28 significantly enriched GO terms were found referring to the upregulated miRNA profile, including 16 pertaining to BP, 5 to MF and 7 to CC. Fig. 4.3.2a showed some of the most significantly enriched terms for each domain; these mainly involved events such as cell adhesion, positive regulation of transcription, positive regulation of gene metabolism, and metabolism of collagen, synapse, and cytoplasmic vesicles etc. Based on the negative impact on target genes by the upstream miRNAs, it might be presumed that the overexpression of the 25 miRNAs in aged dermis might inhibit these

71

biomolecular events mentioned above, thereby contributing to the aging process. In the meantime, with respect to the downregulated miRNA profile, 27 significantly enriched GO terms were discovered; these consisted of 21 terms in BP domain, 4 in MF and 3 in CC. The most significantly enriched terms shown in Fig. 4.3.2b revealed events such as posttranscriptional regulation of gene expression, negative regulation of transcription, negative regulation of gene metabolism, protein kinase activity and metabolism involving nucleoplasm, extrinsic component of membrane. Accordingly, decreased expression of the 15 miRNAs in aged dermis might be involved in the aging process, by relieving their negative impact on the events mentioned above.
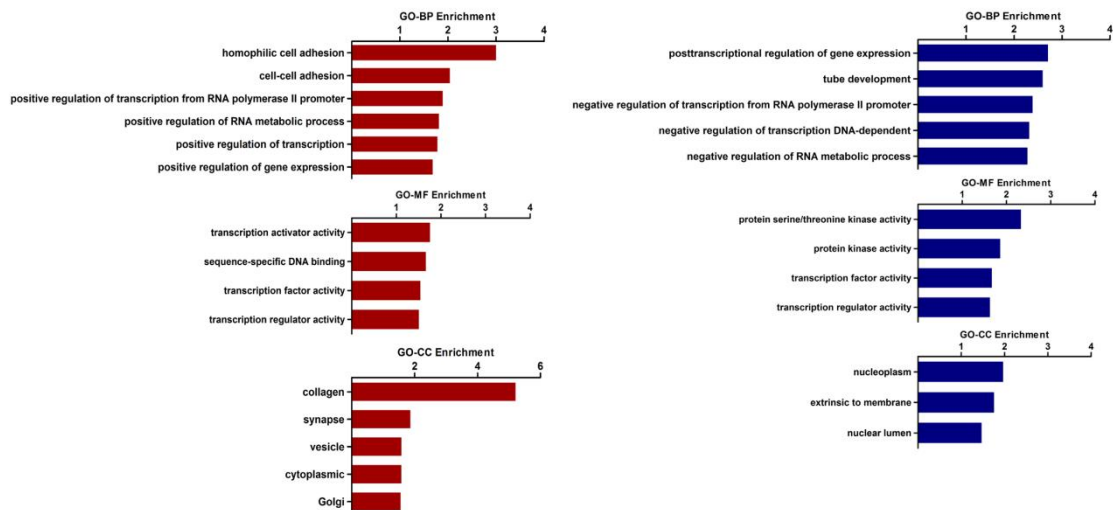

Fig. 4.3.2. Gene ontology enrichment analysis based on the large scale predicted target genes visualized with bar graphs, represented by (a) Most significantly enriched GO terms referring to the upregulated miRNA profile, illustrated by BP, MF, CC domain, respectively, and (b) the most significantly enriched GO terms referring to the downregulated miRNA profile. $p \leq 0.05$ and FDR ≤ 0.1. BP: biology process. MF: molecular function. CC: cellular component.

With similar methods, a pathway-enrichment analysis was performed within DAVID resources. With a $p \leq 0.05$ and FDR ≤ 0.1, 6 terms were significantly enriched referring to the target genes of differentially expressed miRNA profile (Table 4.3.2 and
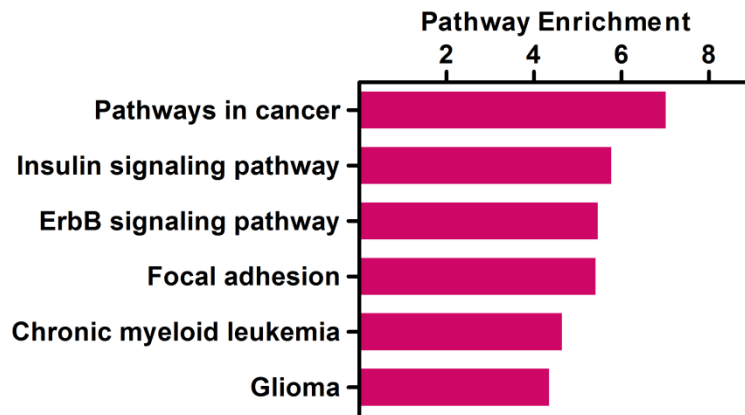
Fig. 4.3.3).

Fig. 4.3.3. Pathway enrichment analysis based on the large scale predicted target genes visualized by bar graphs. Significantly enriched terms referring to the whole differentially expressed miRNA profile in aged dermis. $p \leq 0.05$ and FDR ≤ 0.1

Table 4.3.2. List of significantly enriched pathway terms and the involved target genes referring to the whole differentially expressed miRNA profile in aged dermis.

Category	Terms	Gene number
KEGG Pathway	hsa05200: Pathways in cancer	75
KEGG Pathway	hsa04910: Insulin signaling pathway	38
KEGG Pathway	hsa04012: ErbB signaling pathway	28
KEGG Pathway	hsa04510: Focal adhesion	49
KEGG Pathway	hsa05220: Chronic myeloid leukemia	24
KEGG Pathway	hsa05214: Glioma	21

● **Construction of miRNA-gene-network**

In order to get further insight into the miRNA biology during dermis aging, target genes that associated with organism aging, ECM modulation, cell proliferation, apoptosis, cell cycle, cancer metabolism etc. were selected based on the annotations of enriched GO and pathway terms (Table 4.3.3).

Table 4.3.3. Target genes of the differentially expressed miRNAs that may participate in skin aging

Upregulated miRNA	Target genes
hsa-miR-29 family	HBEGF,IGF1,CAV2,CCNJ,COL11A1,COL15A1,COL1A2,COL22A1,COL2A1,COL3A1,COL4A1,COL4A2,COL4A4,COL4A5,COL4A6,COL5A2,COL5A3,COL6A2,COL7A1,EIF4E2,FRAT2,GNG12,LAMA2,PDGFC,PTEN,VEGFA,COL6A3
hsa-miR-34a	AREG,CCNE2,FGF23,FOSL1,HSPA1A,HSPA1B,PDGFRA,RRAS,E2F5,MAP2K1,IGSF1,E2F3,COL12A1,RALGDS
hsa-miR-34b-5p	ARNT2,CBL,CCNG1,CDH6,CDK17,CDK19,CREB1,IGFBP1,MAPK10,MET,MYC,MYCBP2,NCOA4,PIAS1,PIAS2,PIK3C2A,PRKAA2,SPP1,TFDP2,TGFA,THBS1,RRAS2,CDK4
hsa-miR-1207-5p	CAMK2A,PRKACA,PRKCA,RAPGEF2,STAG1,TLN1,VASP,FGF9,CBX6
hsa-miR-1202	ACTG1,CDC6,ETS1,RALBP1,TAOK3
hsa-miR-1226*	CDCA7,FYN,GADD45A,JAK1,MAX,SKP2,CBX5
hsa-miR-134	MAP3K7IP1,PDCD7,BDNF
hsa-miR-2861	COL11A2,KLK3,MAP3K12,MYLK2,PPP1CA,CACNA1A,SOS1,E2F2
hsa-miR-3127	FZD4,PIK3C2B,PRC1
hsa-miR-3665	CCBE1,TNN
hsa-miR-3937	TPM3
hsa-miR-4270	HDAC5,PTCH2,WNT2B
hsa-miR-4281	CALM1,CDK5R2,CTBP2,MYCBP2
hsa-miR-548q	AKT1
hsa-miR-557	CDKN1C,CTNNA2,TP53I11,RUNX1,STAT5B,FLT1,RPS6KB1,CCNT2,E2F7,LEF1,CBX4
hsa-miR-601	EIF4EBP3,PIK3R3,PRKACB,NRG3
hsa-miR-623	MAPK8IP2,MEF2C
hsa-miR-762	CDC42SE1,GNAI2,ITGA5,PIK3R5,VTN,MAPK8IP3
hsa-miR-424	AKT3,CCNE1,CDC37L1,CDC42SE2,CDCA4,CHEK1,COL24A1,ELK4,FGF13,FGF18,FGF2,HSPG2,IKBKB,LRP6,MKNK1,MYLK,PP

	M1A,PRKAR2A,PTPRR,RAF1,RPS6KA6,SMAD7,WNT3A,WNT4, PIK3R1,CBX2,CBX4
hsa-miR-181a	CCNK,CDC73,COL16A1,HSP90B1,IGF2BP2,IGSF11,RAD21,CBL B,CBX4,CBX7
hsa-miR-154	CAMK2G,CCND2,DOCK1,TAOK1
hsa-miR-455-3p	CSNK1A1,HDAC2,PAK2,SMAD2,PRKAB2,FZD10
hsa-miR-1260	CSNK2B,ELK1,FGF12,PTPN11,WNT10B,SUFU,CDC42BPA,RPS6 KA3,TAOK2
hsa-miR-146b-5p	ABL2,ERBB4,NRAS,SMAD4,TRAF6
hsa-miR-1973	SHC4
hsa-miR-3651	KRAS
hsa-miR-4286	MKNK2,PRKAG1

Subsequently, the miRNA-Gene-Network was constructed. Fig. 4.3.4 demonstrated differentially expressed miRNAs in aged dermis and their interaction with the selected target genes, which revealed the current best understanding of the essential roles of miR-29 family, miR-34 family and miR-424 in regulating dermis aging. In addition, target genes like TAOK2, IGSF1, CDC42BPA, HBEGF, IGF1, VEGFA, COL6A3, E2F5, PRKAB2 were found to be the key participants in the regulatory network.
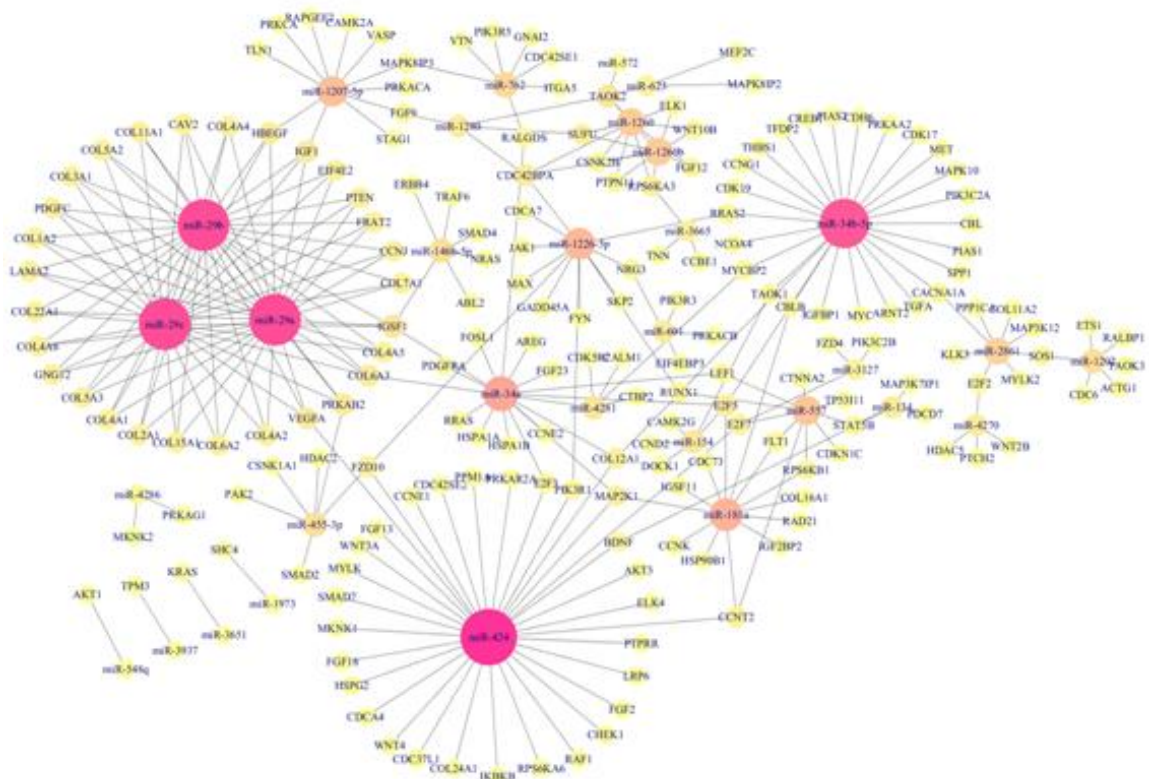
Fig. 4.3.4. miRNA-Gene-Network. Regulatory roles of miRNAs and mRNAs were evaluated by degrees in the network, depicted as varied colors. The essential roles of miR-29 family, miR-34 family and miR-424 in network were revealed, shown as pink circles.

- **Alteration of miRNAs expression associated with cultured replicative senescent fibroblasts**

Dermal fibroblasts are one of the most important cell components in dermis. Their decreased proliferation capacity and the impaired function during cell senescence can result in skin aging (Wang et al. 2009). To observe the consistency between aged tissue and senescent cells in terms of their miRNA expression, replicative senescence of human dermal fibroblasts were induced, and the changes in miRNA expression between early and late passages were evaluated. As the growth curve comparison shown in Fig. 4.3.5a, the proliferation capacity of cells in their late passage decreased significantly compared with those in early passage. Furthermore, fibroblasts in their

late passage displayed a much more flattened and hypertrophic morphology (Fig. 4.3.5c-f), and the SA-β-gal staining revealed a larger proportion (19.9%, P < 0.01) of blue-green stained cells in late passage cells (Fig. 4.3.5g). All of these results indicated senescence events had occurred in these cells. In addition, the classic molecular markers p53, p21 and p16 (Salama et al. 2014) were also applied for cell senescence verification by real time RT-PCR. Interestingly, the expression of p16 was significantly upregulated whereas p53 and its downstream p21 maintained unchanged (Fig. 4.3.5b).
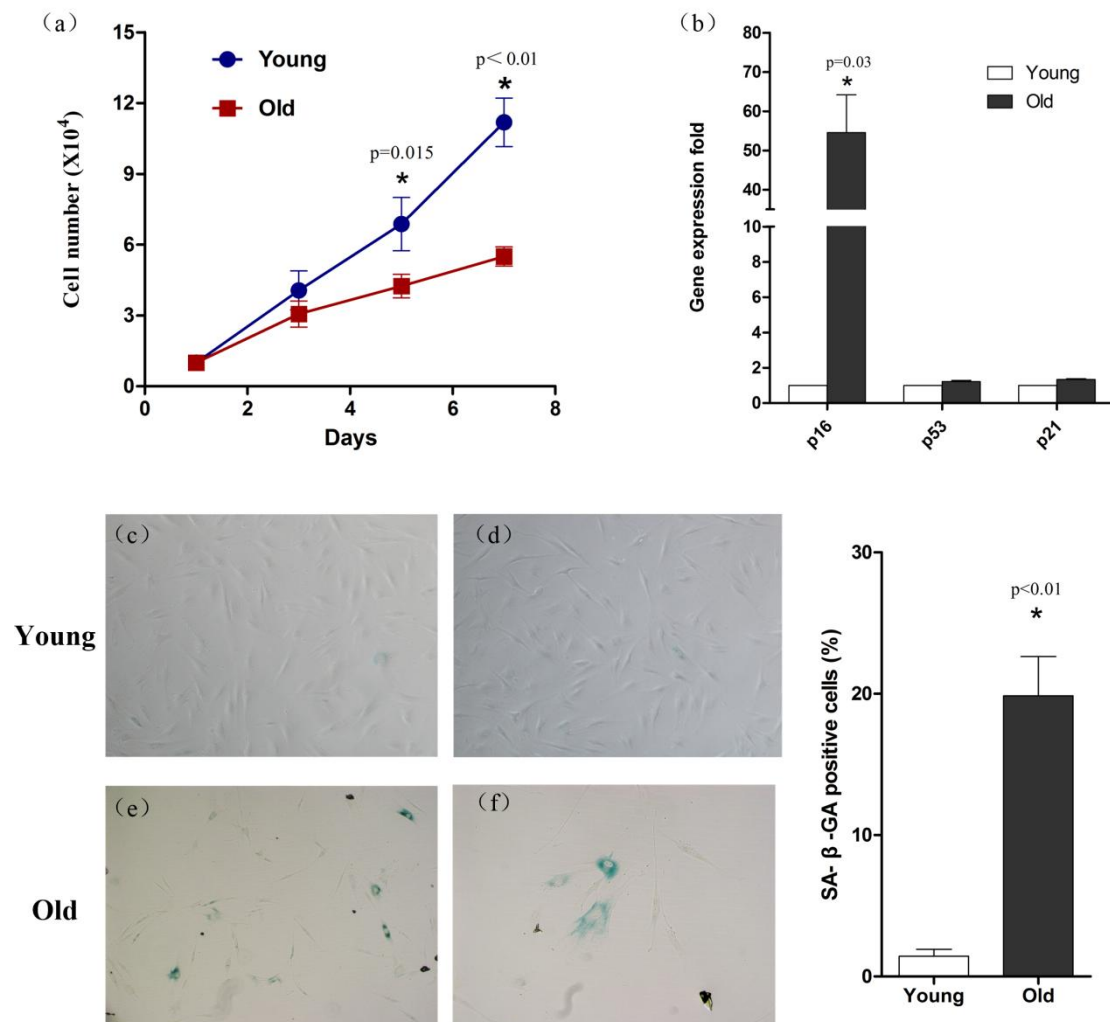

Fig. 4.3.5.   Induction of senescence of human dermal fibroblasts. (a) Comparison of growth curve of dermal fibroblasts between early- (young) and late-passage (old). (b) Relative expression levels of senescence markers p53, p21 and p16 by quantitative RT-PCR. Significant upregulation of p16 was

discovered (p = 0.03). (c-f) SA-β-gal staining of senescent fibroblast in early- (young) and late- passage (old) cells. The green-blue cells were referred as senescent ones. (c-d) Senescent fibroblast was rarely seen among early-passage (x 200). (e) Positively stained fibroblasts were scattered in the plate (x 200). (f) Senescent fibroblasts displayed a much more flattened and hypertrophic morphology (x 400). (g) Significant differences were observed in the proportion of stained cells between early- (young) and late-passage (old) cells (p < 0.01).

Considering both the miRNA-gene-network in this study and previous reported studies, several miRNAs were selected to evaluate their alteration in expression between early and late passages by real time RT-PCR. These miRNAs included miR-1226-5p, miR-601, miR-1207-5p, miR-34b-5p, miR-34a, miR-29c-3p, miR-181, miR-154 and miR-424. As shown in Fig. 4.3.6, when compared with cells in their early passage, the selected miRNAs were all upregulated in senescent HDFs, and the miR-34 and miR-29 families showed particularly marked changes. Notably, while most of the selected miRNAs displayed similar alteration tendency with the results from miRNA array, miR-424, showed contradictory results between tissue and cultured cells.
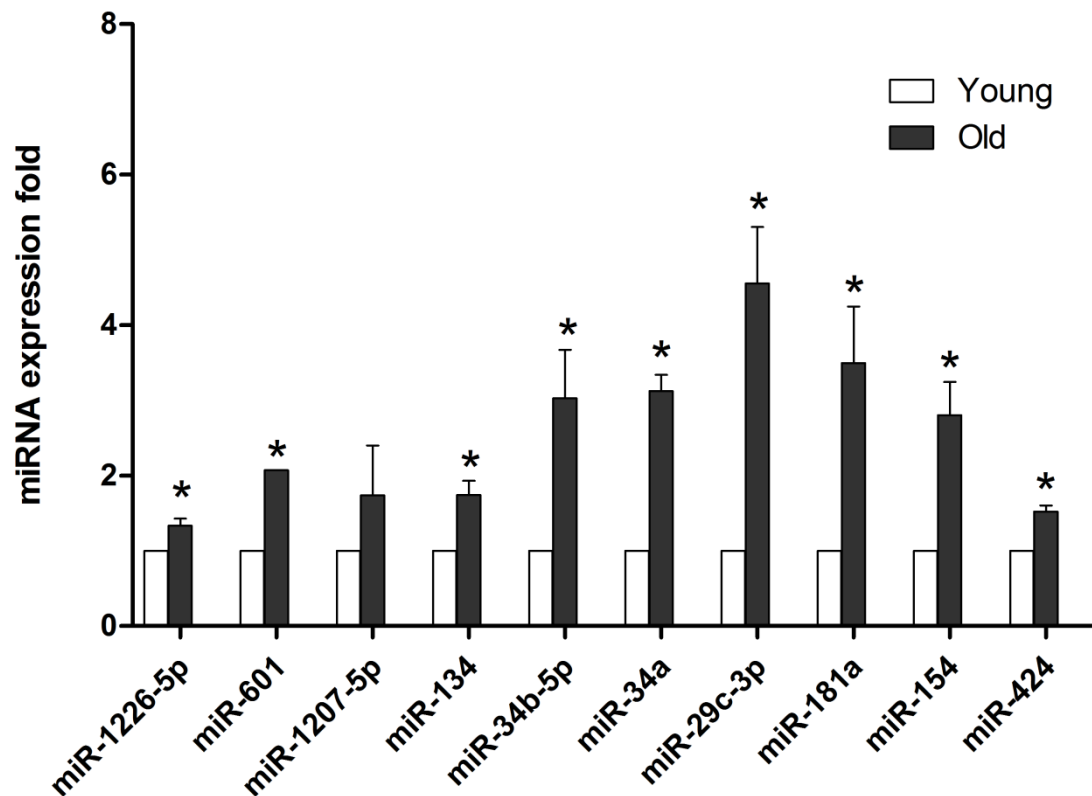
Fig. 4.3.6. Alteration in the expression of miRNAs between young and senescent fibroblasts as determined by quantitative RT-PCR. Selected miRNAs in senescent cells all showed upregulated expression, among which the miR-34 family and miR-29 family displayed more marked changes ($p < 0.05$).

4.4 Study of specific pattern of miRNAs modulated in human skin fibroblasts by a biofunctional complex

● **List of studied miRNAs.**

The purpose of the present study was to identify miRNAs involved in fibroblast senecence and the modulation of dermal extracellular structure, and to identify potential target genes modulated by bioinformatic prediction. A list of miRNAs of interest was obtained following an overview of the scientific literature and database searches. Modulation of the expression level of the miRNAs of this list was assessed

by miRNA qPCR array, in cultured normal human fibroblast treated or not with the natural ingredient complex.

Twenty-one miRNAs of interest and relative to fibroblasts physiology were selected according to the literature (Table 4.4.1). Among the selected miRNAs involved in fibroblast senescence, some have been described with decreased expression levels during senescence (miR-143-3p, miR-145-5p, miR-155-5p, miR-21-5p, miR-23a-3p) (Bonifacio et al. 2010) and miR-7-5p (Marasa et al. 2010). Other miRNAs are known with an increased expression levels during the senescence process (miR-10b-5p, miR-146a-5p, miR-199a-3p, miR-26a-5p, miR-34a-5p, miR-542-5p (Bonifacio et al. 2010), miR-1185-5p, miR-1204, miR-519c-3p, miR-658 (Marasa et al, 2010), miR-29a-3p, miR-30a-5p (Martinez et al. 2011), and miR-638, miR-663 (Maes et al. 2009). miR-29-3p is also involved in fibroblasts extracellular matrix synthesis and senescence (Suh et al. 2012). miR-335-3p was described with a role in proliferation, migration and differentiation (Tomé et al. 2011). These miRNAs are listed in Table 4.4.1.

Table 4.4.1.   List of studied miRNAs.

microRNAs	Modulation	Bibliographic references
miR-143-3p, -145-5p, -155-5p, -21-5p, -23a-3p	down	Bonifacio *et al.* (2010) Plos one 6 (5) 20509.
miR-10b-5p, -146a-5p, -199a-3p, -26a-5p, -34a-5p, -542-5p	up	
miR-7-5p	down	Marasa *et al.* (2010) Aging 2: 333-43.
miR-1185-5p, -1204, -519c-3p, -658	up	
miR-29a-3p, -30a-5p	up	Martinez *et al.* (2011) Proc. Natl. Acad. Sci. USA 108: 522-27.
miR-638, -663	up	Maes *et al.* (2009) J. Cell Physiol. 22: 109-19.
miR-29a-3p	_	Suh *et al.* (2012) Genome Biol. 13: R121.
miR-335-3p	_	Tomé *et al.* (2011) Cell Death Differ. 18: 985-95.

miRNAs implicated in fibroblast senescence were selected according to the scientific literature.

● **Relative expression of each miRNAs and potential target genes.**

A miRNA qPCR array including the 21 miRNAs of interest was used to test the modulations of these miRNAs after ingredient application on cultured normal human fibroblasts for 48h. Three miRNAs (miR-1204, miR-519c-3p, miR-658) were not detected by qPCR and were thus considered as non expressed by fibroblasts in our experimental conditions, reducing the list of miRNAs from 21 to 18. Amplification of these 18 miRNAs (Table 4.4.2, column 1) showed RQ values between 0.573 (for miR-335-3p) and 0.919 (for miR-155-5p) (Table 4.4.2, column 2). Out of these 18 miRNAs, we selected 14 miRNAs with a RQ lower or equal to 0.85 (Table 4.4.2, column 2, in bold): miR-10b-5p, miR-1185-5p, miR-143-3p, miR-145-5p, miR-199a-3p, miR-23a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-335-3p, miR-34a-5p, miR-638, miR-663, miR-7-5p.

miRWalk database allowed the identification of validated target genes involved in senescence and extracellular matrix homeostasis, in correlation with these miRNAs of interest (Table 4.4.2, column 3). Ingenuity Pathway Analysis was used to identify new potentially modulated target genes (Table 4.4.2, column 5). The genes identified in common with the 2 methods are also mentioned in Table 4.4.2, column 4.

Interestingly, among the target genes, family members of collagens (COL1A1, COL2A1, COL2A2, COL3A1, COL4A1, COL 4A2, COL5A3, COL11A2, COL15A1), growth factors and their receptor (EGF, EGFR, VEGFA), transcription factors (SOX2, SOX6, SOX9, FOXO3) or metalloproteinases and their inhibitor (MMP3, MMP9, MMP 14, TIMP2) were identified. These genes are well known to be closely related to extracellular matrix homeostasis. Other target genes, such as SIRT1 (Sirtuin 1), SIRT2 (Sirtuin 2), BIRC5 (Survivin), AURKB, SHH, and LIN28A may also be involved in fibroblast aging process.

Table 4.4.2.　Relative expression of each miRNAs and potential target genes.

microRNAs	RQ	miRWalk Targets	Common Targets	IPA Targets
miR-10b-5p	0.85	SOX2, EGFR	/	/
miR-1185-5p	0.823	/	/	COL15A1, LIN28A
miR-143-3p	0.76	SOX2, MMP3, FOXO3, VEGFA	/	COL1A1, COL5A3, SOX6
miR-145-5p	0.775	EGFR, MMP3, MMP9, VEGFA, COL1A1, SHH	SOX2, SOX9, EGF	FOXO3
(miR-146a-5p	0.897	EGFR, MMP14, COL1A1, COL2A1	/	MMP9, LIN28A)
(miR-155-5p	0.919	SOX2, SOX6, MMP9, MMP14, VEGFA, AURKB	EGFR, FOXO3	SIRT1)
miR-199a-3p	0.749	SOX9	/	/
(miR-21-5p	0.896	SOX9, EGFR, FOXO3, VEGFA, MMP14, COL1A1, -2A1, -11A2, LIN28A, SIRT1	SOX2	SOX6, COL4A1)
miR-23a-3p	0.717	/	/	SOX6, COL4A1, COL11A2
miR-26a-5p	0.577	COL1A1, COL4A2, COL5A3	/	SOX6, MMP14
miR-29a-3p	0.831	SOX2, SOX9, SHH	FOXO3, COL1A1, -3A1, -4A1, -4A2, -5A3, -15A1	VEGFA, COL2A2, SIRT1
miR-30a-5p	0.83	SOX2, EGFR, AURKB, COL11A2, SHH	/	SOX9, FOXO3, TIMP2, LIN28A, SIRT1
miR-335-3p	0.573	/	/	/
miR-34a-5p	0.811	COL2A1, COL11A2	SOX2, VEGFA, SIRT1	BIRC5, LIN28A
(miR-542-5p	0.899	BIRC	/	/)
miR-638	0.794	VEGFA, COL11A2	/	SOX2
miR-663	0.845	VEGFA	/	MMP14, COL1A1
miR-7-5p	0.812	EGF, TIMP2, VEGFA, SIRT2	EGFR	SOX6, COL2A1

The relative quantification (RQ) was experimentally measured for each of the 18 miRNAs (column 1), in fibroblasts treated with the ingredient versus untreated fibroblasts (column 2). Four miRNAs (miR-146a-5p, miR-155-5p, miR-21-5p, miR-542-5p) showed RQ value higher than 0.85 and were not considered in the study (miRNAs indicated in brackets). RQ values equal or below 0.85 are highlighted in bold, and correspond to 14 miRNAs (miR-10b-5p, miR-1185-5p, miR-143-3p, miR-145-5p, miR-199a-3p, miR-23a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-335-3p, miR-34a-5p, miR-638, miR-663, miR-7-5p). Target genes identified with miRWalk and selected for their potential implication in extra-cellular matrix homeostasis and fibroblast senescence are mentioned in column 3. miR-1185-5p, miR-23a-3p, miR-335-3p showed no validated target genes in miRWalk, reducing to 11 the number of miRNAs of interest (miR-10b-5p, miR-143-3p, miR-145-5p, miR-199a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-638, miR-663, miR-7-5p) (column 1, underlined). Ingenuity Pathway

Analysis was also used to complementarily identify potential target genes (column 5). The target genes identified in common by miRWalk and Ingenuity Pathway Analysis are listed in column 4.

- **Predictive results on gene expression**

However, miR-1185-5p, miR-23a-3p, miR-335-3p showed no validated target genes in miRWalk, reducing to 11 the list of miRNAs of interest (miR-10b-5p, miR-143-3p, miR-145-5p, miR-199a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-638, miR-663, miR-7-5p) (Table 4.4.2, column 1, underlined). The hypothetical effect of the downregulation of these specific miRNAs on the expression of the potential target gene identified with miRWalk database is represented in Fig. 4.4.1. A tendency to a decreased miRNA level observed in our experiments may, as a consequence, induce the upregulation of the potential target genes listed for each miRNAs. Among the miRNAs, miR-145-5p and miR29a-3p target a large amount of genes of interest (respectively 9 and 10 genes). Some miRNAs (miR-145-5p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34-5p and miR-638) can target collagens (COL1A1, COL2A1, COL2A2, COL3A1, COL4A1, COL 4A2, COL5A3, COL11A2, COL15A1). Interestingly, miR-26a-5p and miR-29a-3p seem to have a high potential to modulate extracellular matrix composition since they target respectively 3 and 6 collagen family members (Table 4.4.2). To be noticed among the 11 miRNAs studied, miR-26a-5p showed the higher downregulation following ingredient treatment.

SIRT1, a gene involved in the regulation of cellular senescence, is a miRWalk-validated potential target for miR-34a-5p. Additionally, Ingenuity Pathway Analysis predicted that miR-29a-3p and miR-30a-5p may also target SIRT1 and upregulate its expression, in response to downregulation (Fig. 4.4.1).

Genes other than collagens and SIRT1 were targeted by several miRNAs (eg. SOX2 and VEGFA by 6 miRNAs; SOX9, EGF and EGFR by 3 miRNAs) suggesting a potential importance of these genes in the control of fibroblast homeostasis,

extracellular matrix secretion and dermal aging. On the other hand, miR199a-3p and miR-663 are predicted to potentially target only one gene (SOX9 and VEGF1, respectively). The downregulation of all these miRNAs by the tested ingredient may have a positive impact on extracellular matrix secretion by fibroblasts.
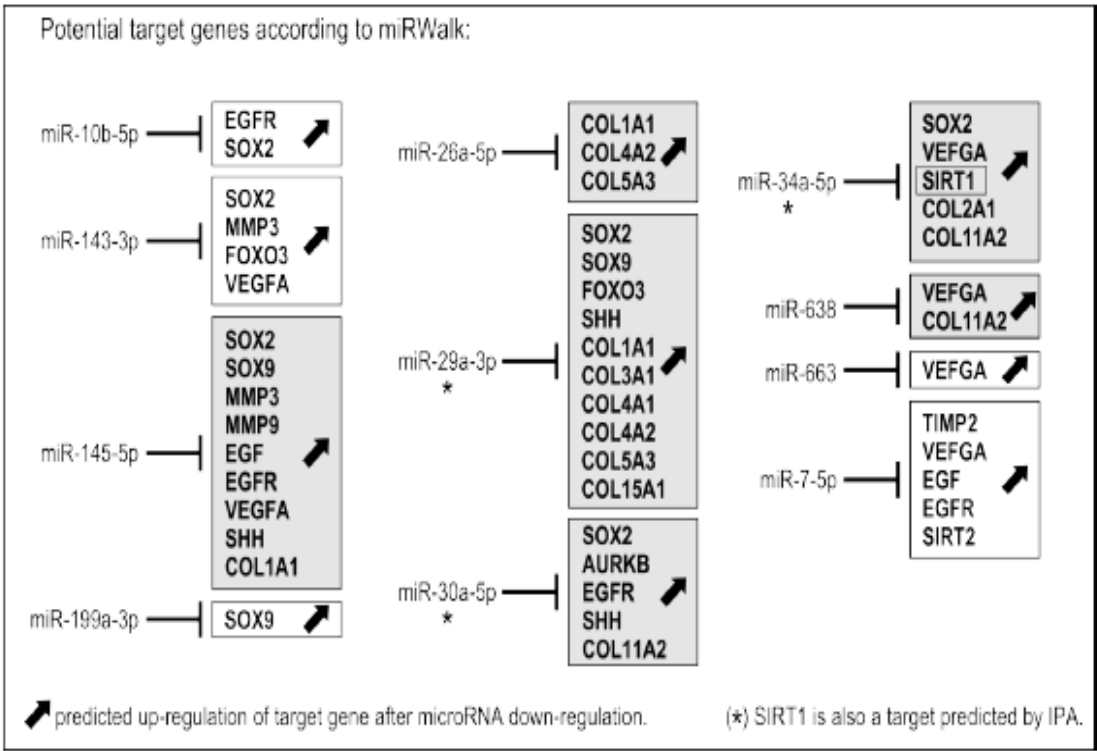

Fig. 4.4.1.   Predictive results on gene expression based on miRWalk validated target genes.

Fibroblasts treated with tested ingredient expressed 14 miRNAs with a RQ ≤ 0.85 (miR-10b-5p, miR-143-3p, miR-145-5p, miR-1185-5p, miR-199a-3p, miR-23a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-335-3p, miR-638, miR-663, miR-7-5p). As miR-1185-5p, miR-23a-3p, miR-335-3p showed no validated target genes in miRWalk, the list of miRNAs of interest was reduced to 11. Predicted upregulation of potential target genes identified in the miRWalk database is indicated for each of the 11 miRNAs (Table 4.4.2). Grey boxes indicate predicted upregulation of collagens as a possible consequence of the downregulation of

miR-145-5p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, and miR-638. SIRT1 (boxed) is a miRWalk-validated potential target for miR-34a-5p only. However, Ingenuity Pathway Analysis indicates possible upregulation of SIRT1 in response to miR-29a-3p and miR-30a-5p downregulation (*; Fig. 4.4.1).

- **New genes closely linked with miRNAs modulated by ingredient treatment.**

Bioinformatics was used to build an overview of the potential interactions between the 18 modulated miRNAs and their potential target genes identified using miRWalk database. By using Ingenuity Pathway Analysis we developed an interaction network (Fig. 4.4.2a). This approach allowed to further identify 8 new potential target genes closely related to the molecules already included in the network (ADAMTS6, CMA1, COL4A5, FGF13, ITGB6, ITGB8, NID1, RECK), with a potential role in extracellular matrix homeostasis. This interaction network offers the possibility to visualize all the partners and their inter-connections at once. However, 3 miRNAs (miR-10b-5p, miR-335-3p, miR-542-5p) could not be connected to the network. The network components were grouped according to their biological activity (eg. collagen genes, metalloproteinases, transcription factors such as SOXs, FOXO3 and sirtuins which are relaed to senescence regulation, BIRK5 and AURKB components of the chromosomal passenger complex). miRNAs were placed at the periphery of the interaction network and predicted relationships with protein coding genes were established. New relationships that could be established by the introduction of 8 new components in the network were highlighted in blue. The resulting network without the relationships with miRNAs was depicted in Fig. 4.4.2b to help visualization. This simplified network focuses on the interactions between the 35 miRWalk potential target genes and the 8 newly identified targets by using Ingenuity Pathway Analysis. The addition of new molecules to the network widens the range of target that may be studied and validated in further in vitro studies.
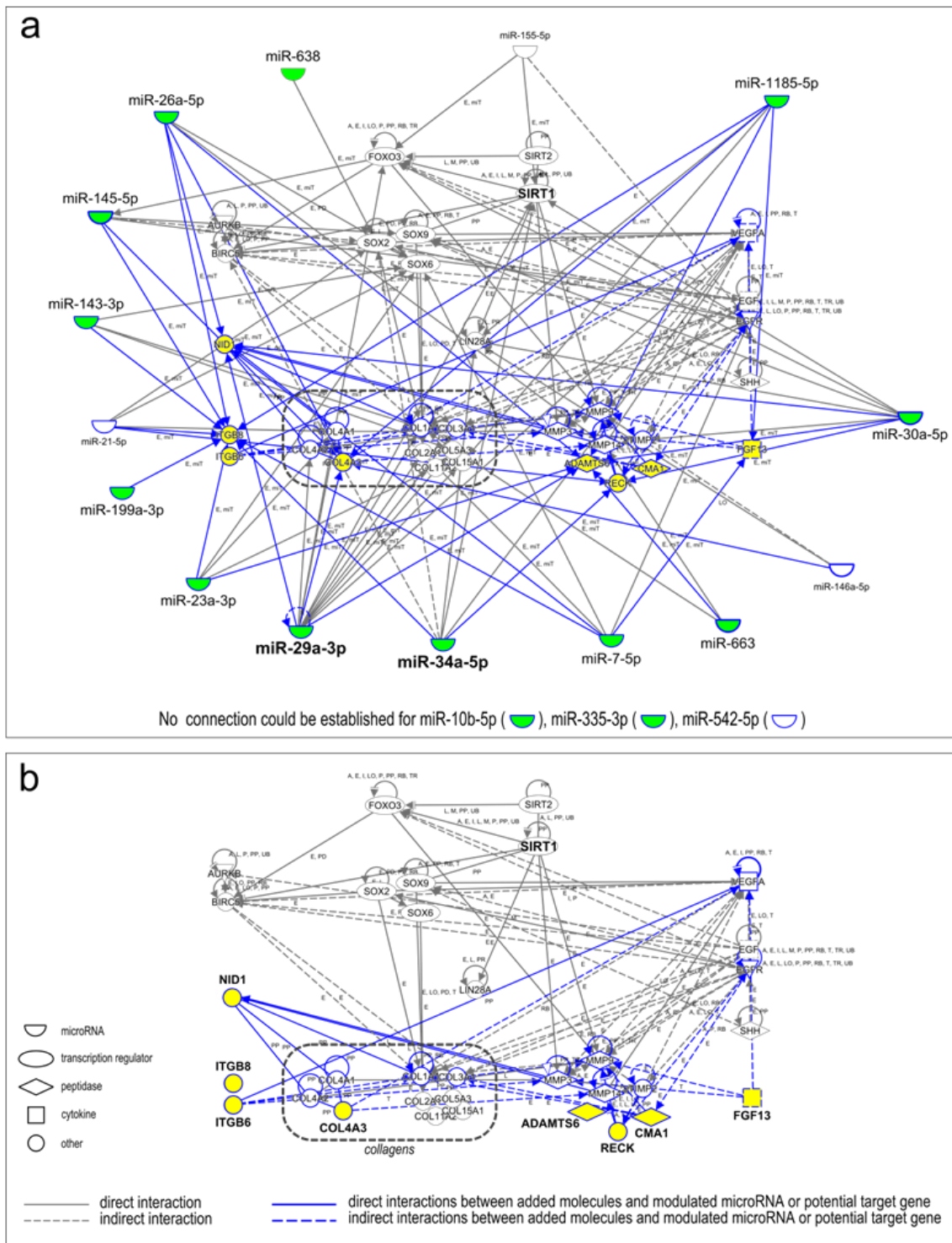
Fig. 4.4.2. Identification of new genes closely linked with miRNAs modulated ingredient treatment. (a) Interaction network including (i) 18 miRNAs experimentally modulated by tested ingredient (Table 4.4.2), (ii) their target genes described in miRWalk database (Fig. 4.4.1), and (iii) 8

new components (highlighted in yellow) identified by building an interaction network with the use Ingenuity Pathway Analysis around previously identified miRNAs and genes. Blue lines highlight new relationships that could be established by the introduction of 8 new components in the network. Green color indicates the 14 downregulated miRNAs with RQ values equal or below 0.85, after ingredient complex application on fibroblasts. 3 miRNAs could not be linked to the network (miR-10b-5p, miR-335-3p, miR-542-5p). (b) Partial interaction network focusing on the relationships between (ii) the target genes described in miRWalk database and (iii) the 8 new components with potential interest.

4.4   Study of melanin uptake by keratinocyte by a newly established cell model

● **Direct uptake of melanin in human epidermal keratinocytes**

In order to study the direct effect of melanin on the cellular function of epidermal kerationcytes, we incubated keratinocytes with melanin in culture medium for 24 h and the uptake of melanin was measured by spectrophotometric method. The uptake of melanin by keratinocytes occurred soon after the addition of melanin for 1 h and the extent of uptake significantly increased in a dose- and time-dependent manner (Fig. 4.5.1a and 1b, respectively). The uptaken melanin was revealed to be accumulated surrounding the nucleus (Fig. 4.5.1c), which was similarly observed in human lentigo tissue (Fig. 4.5.1d) as indicated by yellow arrows.
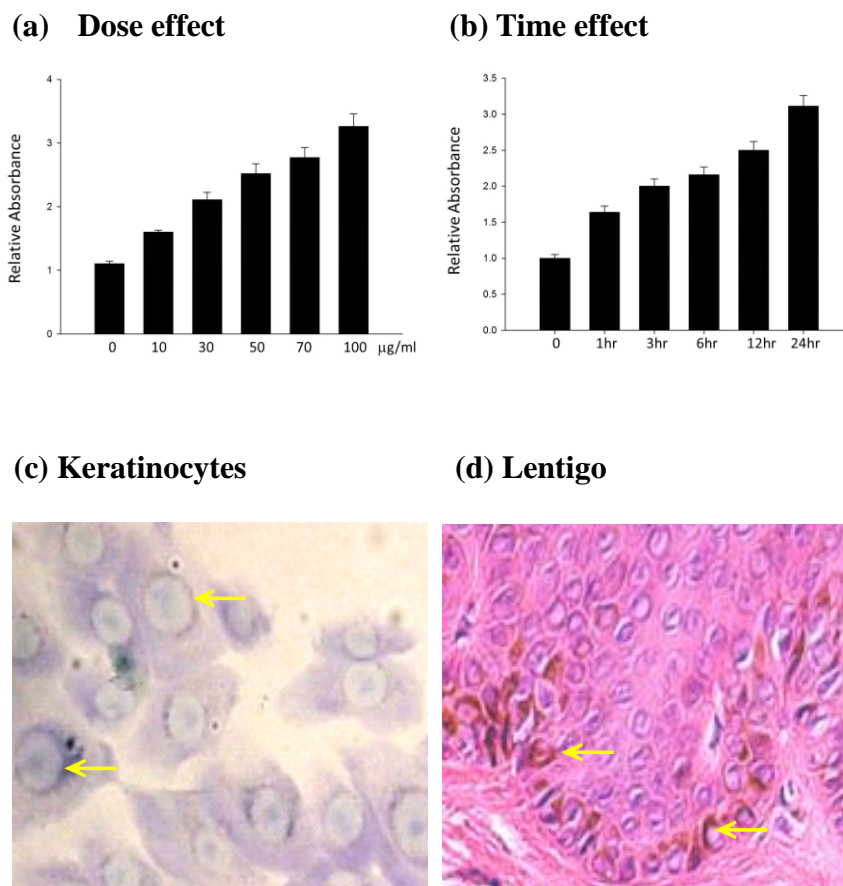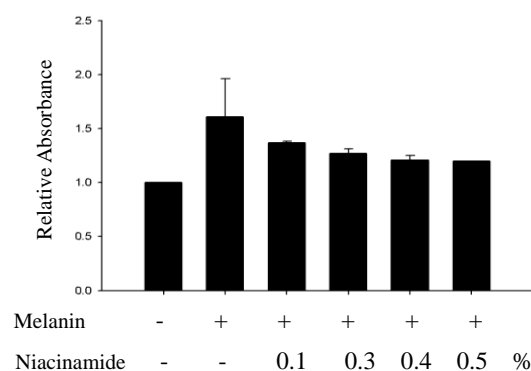
(a) Dose effect

(b) Time effect

(c) Keratinocytes

(d) Lentigo

Fig. 4.5.1. Melanin uptake in human epidermal keratinocytes was dose- and time-dependent. (a) Melanin at the concentration from 0 to 100 μg/ml as indicated was added to 70-80% confluent human epidermal keratinocytes for 24 h and the melanin uptake was measured by spectrophotometric method. (b) Melanin at the concentration of 100 μg/ml was added to 70-80% confluent human epidermal keratinocytes and the melanin uptake was measured by spectrophotometric method at 0, 1, 3, 6, 12 and 24 h. The perinuclear accumulation of melanin was observed in (c) cultured human epidermal keratoinocytes and in (d) human lentigo tissue.

● **Niacinamide and trypsin inhibitor suppressed melanin uptake in epidermal keratinocytes**

To understand the possible mechanism for epidermal keratinocytes to directly uptake

melanin, we tested two well-known inhibitors for the transfer and uptake of melanosome into epidermal keratinocytes on this simplified cell model. Epidermal keratinocytes were pretreated with niacinamide or trypsin inhibitors for 3 h and then 50 µg/ml melanin was added for 24 h. The amount of melanin uptaken by epidermal keratinocytes was demonstrated to be dose-dependently suppressed by niacinamide or trypsin inhibitor (Fig. 4.5.2a and 4.5.2b, respectively).

(a)   Niacinamide

| Melanin | - | + | + | + | + | + | |
| Niacinamide | - | - | 0.1 | 0.3 | 0.4 | 0.5 | % |

(b)   Trypsin inhibitor

| Melanin | - | + | + | + | + | + | |
| Trypsin inhibitor | - | - | 1 | 2 | 5 | 10 | µg/ml |

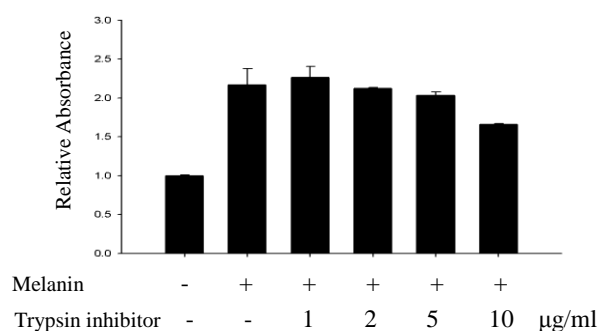Fig. 4.5.2.   Niacinamide and trypsin inhibitor reduced the melanin uptake in epidermal keratinocytes. Human epidermal keratinocytes at 70-80% confluency were pretreated with (a) niacinamide (0-0.5% as indicated) and (b) trypsin inhibitor (0-10 µg/ml as indicated) for 3 h and then melanin at the concentration of 50 µg/ml was added for 24 h. Melanin uptake was measured by spectrophotometric method.

- **Melanin inhibited cell proliferation and Ki-67 expression in epidermal keratinocytes**

The uptake of melanin appeared to reduce the cell density of epidermal kertinocytes from microscopic observation. Epidermal keratinocytes with different concentration of melanin treatment for 24 h were analyzed by MTT assay. Results in Fig. 4.5.3a demonstrated the dose-dependent effect of melanin on decreasing the cell number of epidermal keratinocytes. To further prove the inhibitory effect of melanin on the cell proliferation of epidermal keratinocytes, the expression of Ki-67, a cell proliferating marker, was fluorescently stained in epidermal keratinocytes. As clearly shown in Fig. 4.5.3b, the number of cells with positively stained Ki-67 expression (green fluorescent signal) in the nucleus was significantly decreased after melanin treatment. The dose-dependent effect of melanin on suppressing the Ki-67 expression in keratinocytes was calculated and confirmed in Fig. 4.5.3c. Similar correlation was also found in human solar lentigo tissue as shown in Fig. 4.5.4. Yellow arrow pointed out the cell with perinuclear accumulation of melanin (brown color) in which Ki67 expression was observed to be reduced. On the other hand, cell with positive Ki67 signal (blue color) as pointed out by red arrow was found to contain less melanin accumulation in the cells.

(a) MTT assay

(b) Ki67 expression

(c) Ki67 expression ratio
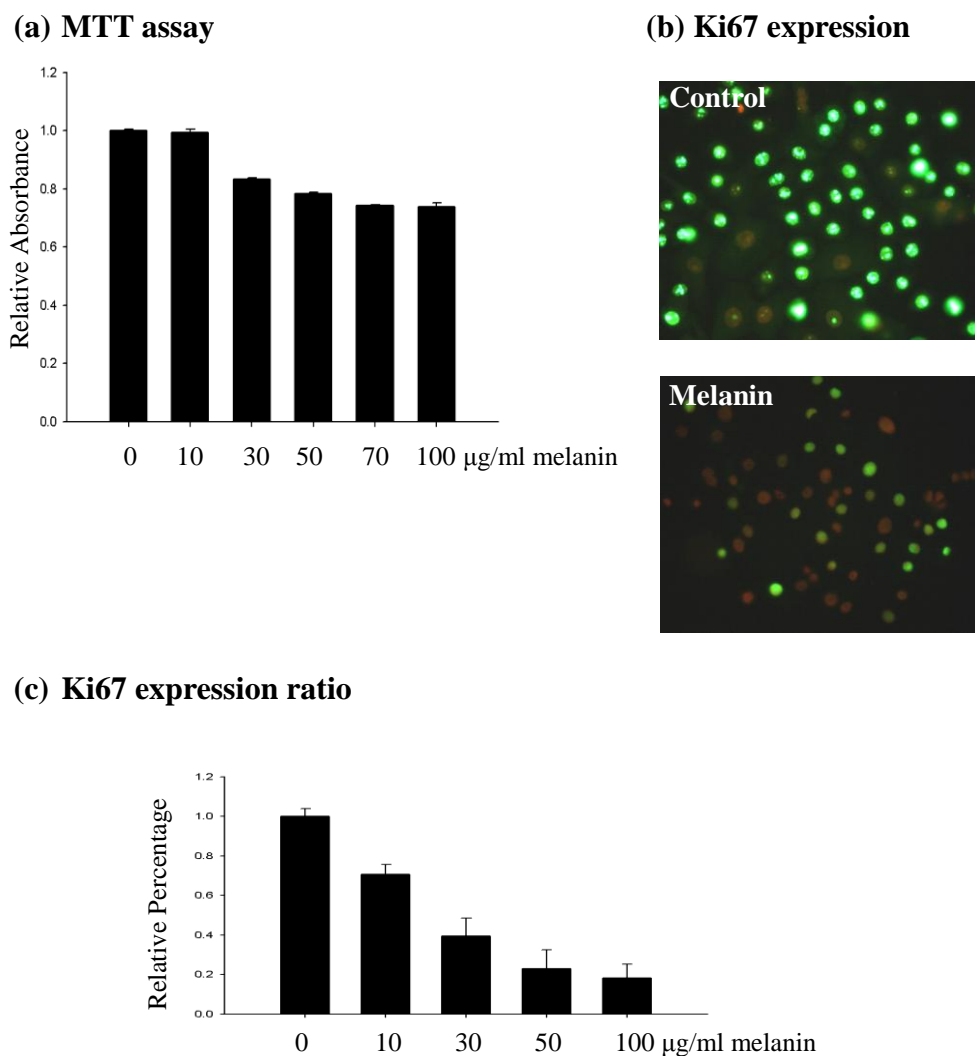
Fig. 4.5.3.　Melanin inhibited the proliferation of human epidermal keratinocytes. (a) Melanin at the concentration from 0 to 100 μg/ml as indicated was added to 70-80% confluent human epidermal keratinocytes for 24 h and MTT assay was performed. (b) Representative photo for the fluoresecently labeled Ki-67 expression in epidermal keratoinocytes without (upper panel) or with (lower panel) 50 μg/ml melanin treatment for 24 h. (c) The dose-dependent effect of melanin on the number of Ki-67 positive epidermal keratonicytes was quantified.

Fig. 4.5.4.　Melanin inhibited the in vivo Ki-67 expression in keratinocytes. The Ki-67 expression in human lentigo tissue was revealed by immuno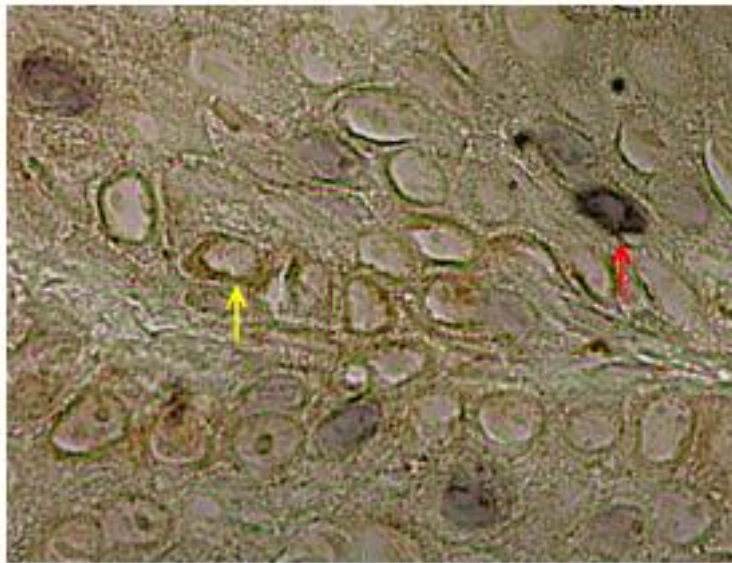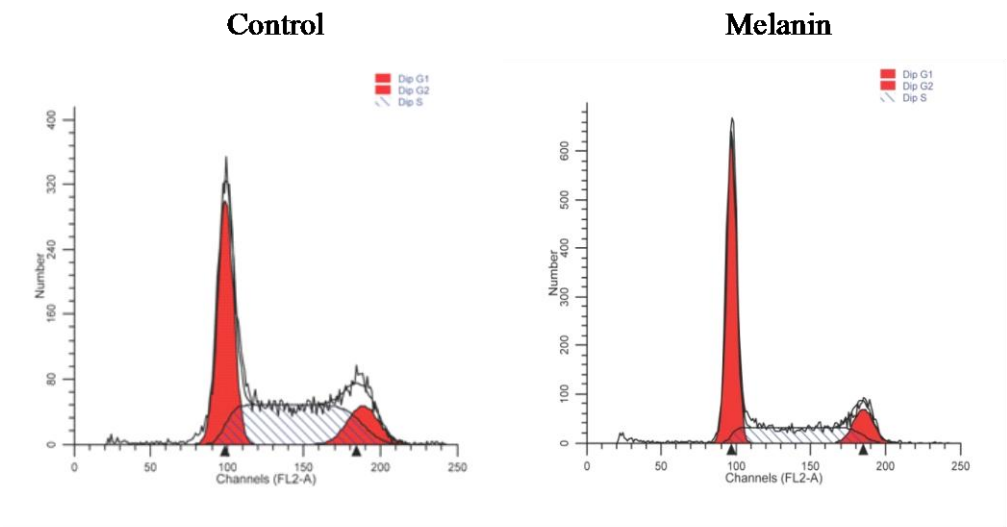staining the protein and developed into blue color. Yellow arrow indicated the cell with melanin uptake and red arrow indicated the cell without melanin uptake. Representative photo was shown from five different lentigo tissues with similar results.

● **Melanin arrested the cell cycle progression of epidermal keratinocytes**

The inhibitory effect of melanin on the proliferation of epidermal keratinocytes was further confirmed by the cell cycle distribution analysis using flow cytometry. Results in Fig. 4.5.5a clearly revealed that the melanin treatment resulted in the increase of G1phase-cells from 41.84% to 57.57% and the decrease of S phase-cells from 45.52% to 30.09%. Clearly, the cell cycle progression of epidermal keratinocytes was delayed and partially arrested at G1 phase by melanin. The expressions of cell cycle-dependent genes including CDK1, CDK2, cyclin E, cyclin A and cyclin B were dose-dependently suppressed in epidermal keratinocytes by the treatment with melanin for 24 h (Fig. 4.4.5b).

(a)

Control   Melanin

	Control	Melanin
G1	41.84%	57.57%
S	45.52%	30.09%
G2/M	12.64%	12.33%

(b)

CDK 1

CDK 2

cyclin A

cyclin B

cyclin E

tubulin

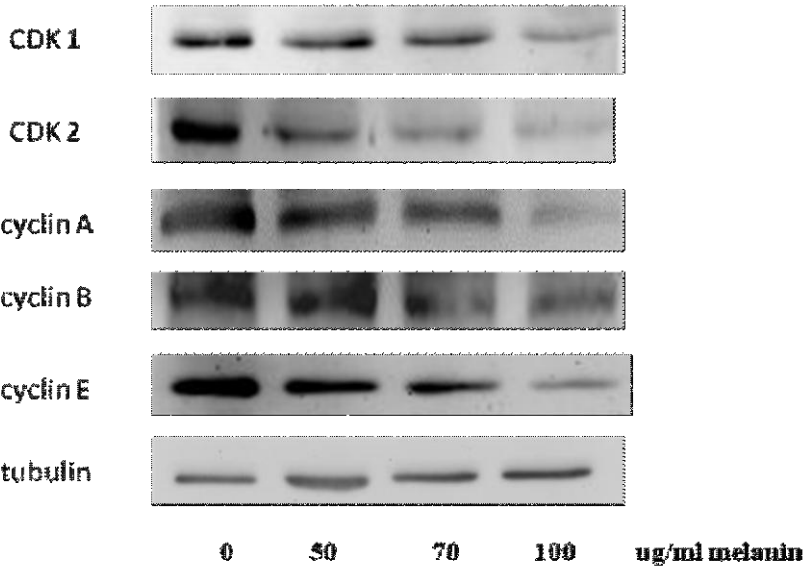0   50   70   100   ug/ml melanin

7

Fig. 4.5.5. Melanin inhibited cell cycle progression and the gene expressions of cell cycle-dependent genes. (a) Epidermal keratinocytes were treated with 50 μg/ml melanin for 24 h and processed for the analysis of cell cycle distribution by flow cytometry. (b) Protein lysates were prepared from cells treated with different concentrations of melanin and western blot analysis was performed.

- **Microarray study revealed the downregulation of DKK1 gene expression in human epidermal kerationocytes by melanin**

To further explore the potential effect of melanin on the biological functions of epidermal keratinocytes, we isolated RNA isolated from this simplified cell model and microarray analysis was performed by Phalanx Biotech Group (Hsinchu, Taiwan, R.O.C.) using The Human Whole Genome OneArray® v6 containing 32,679 DNA oligonucleotide probes to reveal the gene expression profile changed by melanin. Results shown in Fig. 4.5.6a revealed 800 genes with more than two-fold changes in melanin-treated keratinocytes. Among them, DKK1 was found to be remarkably suppressed in epidermal keratinocytes by melanin. In Fig. 4.5.6b and Fig. 4.5.6c, the mRNA and protein expression levels of DKK1 in epidermal keratinocytes were further confirmed to be decreased in a dose-dependent manner.

(a)
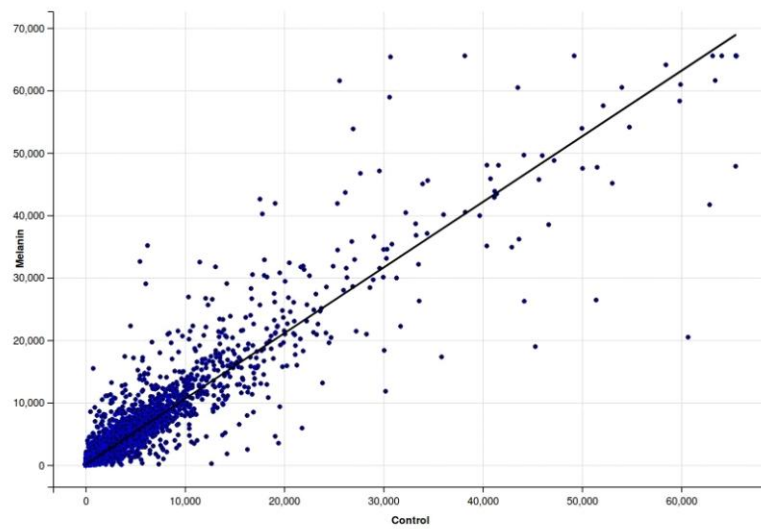
(a) RT/real time PCR
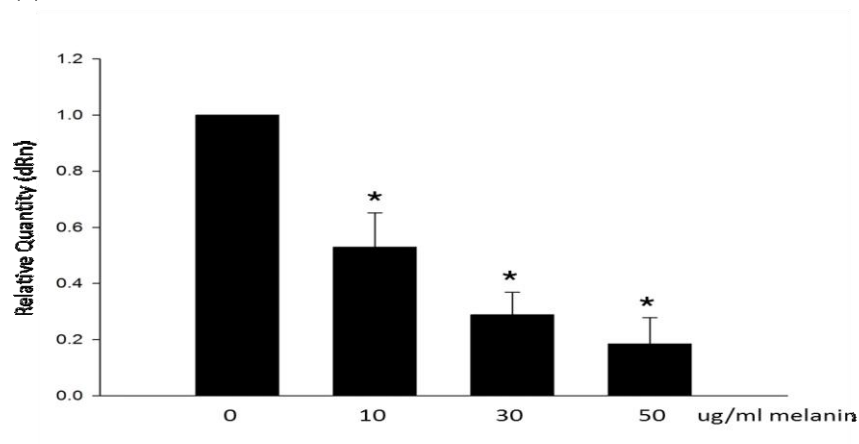
(b) Western blot analysis
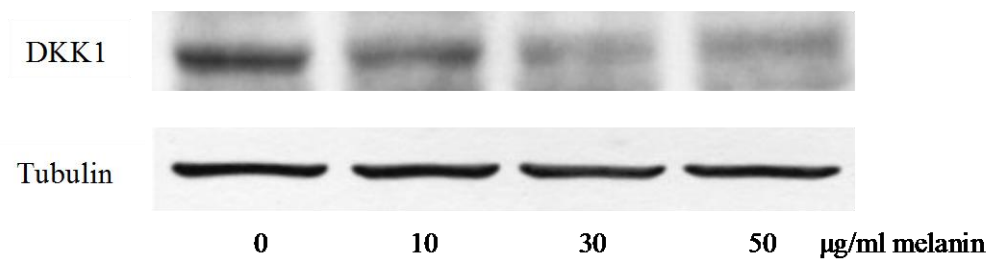
Fig. 4.5.6.   Microarray analysis revealed the inhibition of DKK1 gene expression in keratinocytes by

melanin. (a) Full microarray data of expressed gene probes plotted as melanin-treated cells mean signal vs. control mean signal. Melanin at the concentration from 0 to 50 μg/ml as indicated was added to 70-80% confluent human epidermal keratinocytes for 24 h and the mRNA level was measured by (b) RT/real-time PCR and (c) western blot analysis, respectively.

5. DISCUSSION

5.1 Longitudinal study of skin aging progress

In order to understand the skin aging progression and parameters which impact women's impression of facial skin aging, two studies were conducted by following up of the changes in facial skin of the same subjects over a long time span of 8 years and 11 years, which enabled the exclusion of variability caused by individual differences such as different living environments, genetic background, and to precisely determine the changes. The results of Los Angles facial wrinkling longitudinal study classified the skin conditions susceptible to wrinkles, and clinically proved that long-term repetition of expression lines eventually settled into permanent wrinkles. The results of Akita facial skin longitudinal study by age group indicated that hyperpigmented spots, wrinkles and texture roughness are highly correlated to visible aging of the facial skin. The results suggested that those three parameters are the most important skin aging parameters to determine the score of the appearance aging, suggesting they are the center problems of skin aging concern and major clinical targets of the skin care technologies for anti-aging. In addition, according to the skin aging over time and its variability between the age groups, the subjects were classified into the severe skin aging group, moderate skin aging group and the mild skin aging group, suggesting skin aging progress hugely varies by individual. There are many limitations to these two longitudinal studies, in the future, in order to understand the factors which impact the skin aging progress, it is necessary to investigate the correlation between the skin aging progress and environmental exposure, nutritional state, genetic background.

5.2 Antioxidant *Opuntia ficus-indica* extract activates AhR-Nrf2 signaling and upregulates filaggrin and loricrin expression in human keratinocytes

Keratinocytes are the major constituents of the epidermal barrier, expressing the sensor

molecules for oxidation/antioxidation responses such as the AhR-Nrf2 system (Furue et al, 2014). Although OFIE was shown to exhibit potent antioxidant activity *in vivo* (Hfaiedh et al. 2014; Mora ń-Ramos et al. 2012; Brahmi et al. 2012), its mechanism remains largely unknown. In this study, we investigated the *in vitro* antioxidant mechanism of OFIE using human keratinocytes. Since Nrf2 plays a pivotal role in orchestrating the antioxidant response (Ma et al. 2013), we first examined whether OFIE could induce Nrf2 activation and inhibit the ROS production of keratinocytes. As expected, OFIE induced the cytoplasmic-to-nuclear translocation of Nrf2 with upregulation of Nrf2 and NQO1 expression. The activation of Nrf2 potently inhibited the ROS generation in keratinocytes treated with TNFα or BaP. These events were Nrf2 dependent because all of these findings were significantly inhibited in Nrf2 knockdown cells. The inhibitory action of OFIE on TNFα signaling may be beneficial in therapeutic interventions for various skin diseases, such as psoriasis and photoaging, in which TNFα plays a critical role (Kerdel et al. 2014; Feng et al. 2014). Various ligands are known to activate the Nrf2-NQO1 battery. Ketoconazole activates AhR and then upregulates Nrf2-NQO1 expression (Tsuji et al. 2012). However, the Nrf2 upregulation by Z-ligustilide has been shown to be AhR independent. The present study demonstrated that OFIE is a potent activator of the AhR-Nrf2-NQO1 pathway because the upregulation of Nrf2-NQO1 expression was inhibited in AhR knockdown keratinocytes. Concordantly, OFIE induced the cytoplasmic-to-nuclear translocation of AhR with enhancement of CYP1A1 expression, a specific marker enzyme for AhR activation. Besides its pivotal role in the regulation of oxidation/antioxidation responses, the ligation of AhR increases the expression of various epidermal barrier proteins encoded by genes at human chromosome 1q21 called the epidermal differentiation complex (Furue et al. 2014; van den Bogaard et al. 2013; Howell et al. 2009; Kennedy et al. 2013). In keeping with this notion, the present study demonstrated that OFIE enhanced FLG and LOR expression in an AhR-dependent

manner in NHEKs. The capacity of OFIE to enhance FLG and LOR expression may be useful for preventing epidermal barrier disruption induced by ultraviolet B irradiation. The present study has also demonstrated that both IL-4 and IL-13 decreased FLG and LOR expression and that the inhibitory action of Th2 cytokines was restored by OFIE, suggesting that OFIE may be beneficial for damaged skin even in atopic dermatitis. In conclusion, the antioxidant OFIE was shown to be a potent AhR-Nrf2 activator and could restore the impaired expression of skin barrier proteins. These findings may facilitate the use of OFIE in clinical and cosmetic dermatology.

5.3   miRNA-34 and miRNA-29 families may play major roles in dermis aging

The present study, for the first time, compared the miRNA profile in aged human dermis with that in young human dermis, and discovered abundant differentially expressed miRNAs during aging, including miR-34 family and miR-29 family, which were the essential participants in the miRNA-gene regulatory network that is associated with skin aging.

MiR-34 family, which maintained highly conserved across various organisms, was firstly known to be tumor suppressor genes that participate in inhibition of cell cycle and stimulation of apoptosis (He et al. 2007). However, recent study has also revealed their role in affecting lifespan of *C. elegans* (Yang et al. 2015). In the meantime, accumulation of miR-34 family with age is also observed in organs or tissues, including hearts (Seeger et al. 2015), liver (Li et al. 2011), and brains (Liu et al. 2012). Accordingly, the discovery that upregulation of miR-34 family in aged dermis in our study is in consistence with the results found in other fields mentioned above. Several mechanisms of miR-34 family in regulating aging or cell senescence have been elucidated so far, and the signaling pathway being mostly investigated is the feedback loop of miR-34, SIRT1 and p53 (Yamakuchi et al. 2009). The transcription of miR-34 family can be directly activated through p53, one of the most common proteins that

participate in inhibition of tumor development and cell senescence, and SIRT1, as a target gene of miR-34 family, is consequently inhibited. Since many compelling evidence has revealed the pro-longevity role of SIRT1 in mammals due to its protection from cellular oxidative stress, DNA damage and metabolic disturbance (Lopez-Otin et al. 2013), inhibition of this gene leads to cell senescence and organism aging. Even so, it is worth noting that, in our study no significant difference of p53, p21 or SIRT1 (Data not shown) expression was found between early- and late-passage HDFs, indicating alternative pathways independent of p53 may be involved for miR-34 family in regulating skin aging. Indeed, it was reported that overexpression of miR-34a was identified during the p16-mediated cellular senescence in human mammary epithelia cells (Overhoff et al. 2014), and miR-34a could also modulate oncogene-induced senescence through its integration with ETS family transcription factor (Christoffersen et al. 2010). Overall, these evidences together with our findings suggest that further studies are required to elucidate the pathway of miR-34 family during skin aging.

Published data also provides a range of evidence that miR-29 family can be associated to organism aging. It has been suggested that miR-29 family plays a pivotal role in the regulation of cell proliferation, viability and senescence by controlling multiple overlapping pathways (Ugalde et al. 2011). While some study points out that the transcriptional activation of miR-29 family is triggered in response to DNA damage and occurs in a p53-dependent manner in progeroid mice (Ugalde et al. 2011), others demonstrate that miR-29 can negatively regulate B-Myb expression and participate in p16/Rb-driven cellular senescence (Martinez et al. 2011). This may explain the increased expression of p16 in our senescent HDFs when miR-29 was upregulated. Meanwhile, it is important to note that most of the predicted target genes of miR-29 family encode varied types of collagens, which may account for ECM modulation during skin aging. In fact, for the fibrotic remodeling process during the formation of

aortic aneurysms, increased expression of miR-29b and decreased collagen gene expression augmented aneurysm growth, whereas inhibition of this miRNA slowed down the progression (Milewicz et al. 2012). On the other hand, downregulation of miR-29 family has been reported in many fibrosis diseases (He et al. 2013), especially in the skin tissues of systemic sclerosis, and the process is believed to be regulated by TGF-β/SMAD signaling pathways (Maurer et al. 2010). Taken together, given that our current study discovered the increased expression of miR-29 family in aged human dermis, the role of these miRNAs in modulating ECM degradation during aging is of great importance and needs to be further investigated.

In summary, all the evidence discussed above strongly suggested that miR-34 and miR-29 families play major roles in aging. However, there have been no reports that these two miRNAs are associated with aging of the human dermis. A novel finding from our study is that miR-34 and miR-29 families are significantly upregulated in the dermis tissue of aged subjects. In addition, we confirmed these miRNAs are upregulated in replicative senescent fibroblasts. Furthermore, by means of bioinformatic interaction analysis, we found that miR-34 and miR-29 families influence numerous mRNAs that are important to dermal skin structure integration. These new findings are consistent with evidence obtained from other models in previous studies, and may provide as a new strategy for modulating the progress of skin aging.

As the next step of our researches, we intend to elucidate the molecular pathways through which miR-34a and miR-29a miRNAs affect the aging of the dermis.

5.4 Modulation of a specific pattern of miRNAs, including miR-29a, miR-30a and miR-34a, in cultured human skin fibroblasts, in response to the application of a biofunctional ingredient that protects against cellular

senescence *in vitro*.

Following the above study on microRNA and dermis aging, this part of the study is intended to nail dwon and confirm the provitol microRNAs associated with fibroblast senesense by employing a natural derived complex.

The present study, for the first time, investigated the modulation of a series of selected miRNAs by a specific complex of ingredients with proven ability to preserve human skin cells from senescence *in vitro*. Results pointed out the regulation of a signature of eleven miRNAs including miR-29a-3p and miR-34a-5p, which are key players in the miRNA-gene regulatory network associated with aging (Jung et al. 2012; Smith-Vikos et al. 2012). The observed downregulation of miR-34a-5p by the complex in our study is in consistence with the results found in other fields as well as in our own previous study. In addition, in the present study, the observed downregulation of miR-34a-5p by the complex predicts the possibility of a subsequent upregulation of SIRT1 (Fig. 4.4.1). Interestingly, aside from miR-34a-5p and according to predictions (Table 4.4.2), miR-29a-3p and miR-30a-5p may also regulate SIRT1.

In our study the observed downregulation of miR-29a-3p by the complex is in consistence with the predicted positive modulation of the expression of collagens in the treated fibroblasts (Fig. 4.4.1). Interestingly, several miRNAs appear as possible modulators of collagens (miR-145-5p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, and miR-638), reinforcing the possibility of an expected positive modulation on collagen synthesis.

In the study, we also found that microRNAs in fibroblast modulated not only extracellular matrix such as various collagens but also integrin which was the structural protein of basement membrane between the epidermis and dermis (Fig. 4.4.2). Importantly, integrin is also suggested to regulate epidermal stem cell niche (Morgner et al. 2015), which is associated with keratincoyte proliferation and differenciation. Hence, the result indicated that fibroblast could modulate the proliferation and

differentiation of keratinocyte via basement membrane. This result implies that microRNA may also play a role in the interaction between the different types of cells in skin aging.

Taken together, our findings suggest that a pattern of miRNAs, including miR-29a-3p miR-34a-5p, was modulated after the application of 1% of the ingredient complex for 48 hours on human skin fibroblasts in culture, and may contribute to the already described activity of the ingredient complex *in vitro*. Indeed, these miRNAs (miR-10b-5p, miR-143-3p, miR-145-5p, miR-199a-3p, miR-26a-5p, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-638, miR-663, miR-7-5p) may play a role in the observed protection against cellular senescence in vitro, and from UV-induced DNA damage (Imbert et al. 2012; Bergeron et al. 2012), by the possible regulation of collagens and SIRT1 levels of expression. However, further validation needs to be obtained in order to understand the molecular pathway.

5.5 Melanin uptake reduces cell proliferation of human epidermal keratinocytes

The process of melanosome transfer has been investigated for decades either by co-culturing epidermal keratinocytes with melanocytes or incubating epidermal keratinocytes in the presence of isolated melanosomes mostly from melanoma cells. Although several mechanisms of melanosome transfer are likely to occur in the skin, recently, a new mechanism of melanosome transfer has been reported that involves the release of melanosome-containing globules and then uptake by keratinocytes (Ando et al. 2011). However, it has been found that the removal of melanosomal membranes due to the detergent treatment during the purification process did not block the transfer into keratinocytes, suggesting that the melanosomal membrane is not critical for the ingestion by keratinocytes.

In the present study, we demonstrated that keratinocytes could uptake melanin directly into cytoplasm. Melanin alone in cytoplasm could also be accumulated to

perinuclear site that did not require the components from melanosome. The direct uptake of melanin alone could also be inhibited by trypsin inhibitor and niacinamide, indicating the involvement of protease-activated receptor-2 (PAR-2) in this process, which was similar to previous studies using co-cultured cell model or incubation with isolated melanosomes.

Compared to the previous studies using co-cultured cell model or incubation with isolated melanosomes, this simplified cell model for direct uptake of melanin can be used to investigate the pure effect of melanin on the biological activity of epidermal keratinocyte without being interfered by some unknown factors in the melanosomes particularly those isolated from cancerous melanoma cells instead of normal cells. This cell model was apparently much easier and could avoid the experimental complication due to the different preparation of melanosomes and therefore data with higher reproducibility could be obtained.

Melanin plays an important role in protecting human skin from the UV irradiation. Nuclear accumulation forms a melanin cap above cell nuclei and functions to protect DNA from UV-induced damage. The uptake of melanin in keratinocytes resulted in the inhibition of cell proliferation not only in cultured epidermal keratinocytes, but also being observed in human solar lentigo tissue. The inhibition of cell proliferation is consistent to the result of delay of cell cycle, which may allow the cells to repair the potential damage of DNA and prevent the change of DNA sequence that could be fixed after the replication of DNA in keratinocytes. A recent study reported an inhibited cell proliferation of SV40T-transformed human epidermal keratinocytes (SV-HEKs) after the treatment with isolated melanosomes also in a PAR-2 dependent manner (Choi et al. 2013). It is very likely that the inhibition of cell proliferation was caused by the melanin that got inside the cells through the uptake of melanosomes. In addition, based on the results of our microarray results, melanin was indeed found to downregulate the expressions of many cell cycle-dependent genes in

epidermal keratinocytes that contributed to the inhibition of cell proliferation.

Dickkopf 1 (DKK1), an inhibitor of Wnt signaling, not only functions during the embryonic development (Denicol et al. 2013), but also regulates joint remodeling (Zhang R et al.) and bone formation (Daoussis et al, 2011). DKK1 is therefore believed to play a role in the pathogenesis of rheumatoid arthritis (Miao et al. 2013) and multiple myeloma (Kocemba et al. 2012). Recently, the levels of DKK1 in palmoplantar dermal fibroblasts were found to be higher than those observed in non-palmoplantar dermal fibroblasts (Yamaguchi et al. 2004). In a paracrine signaling way, DKK1 secreted from fibroblasts can suppress melanocyte function through the regulation of microphthalmia-associated transcription factor (MITF) and beta-catenin (Yamaguchi et al. 2007). In addition, DKK1 can also induce the expression of keratin 9 and alpha-Kelch-like ECT2-interacting protein (alphaKLEIP) but decrease the expression of beta-catenin, glycogen synthase kinase 3beta, protein kinase C, and proteinase-activated receptor-2 (PAR-2) in epidermal keratinocytes, which is consistent with the expression pattern in human palmoplantar skin (Yamaguchi et al. 2008). More interestingly, treatment with exogeneous DKK1 could result in the hypopigmentation and thickening of a reconstructed skin model, elucidating why human palmoplantar skin is thicker and paler than non-palmoplantar skin (Yamaguchi et al. 2009). Therefore, DKK1 can potentially be used to reduce skin pigmentation or to repair certain damaged skins. Our study, for the first time, demonstrated that keratinocytes can also produce DKK1 by themselves and the DKK1 gene expression in epidermal keratinocytes could be downregulated by the uptake of melanin. Also, the decrease of DKK1 by melanin uptake was correlated well with the inhibited proliferation of epidermal keratinocytes. In other words, the uptake of melanin in epidermal keratinocytes might play a crucial role in the process of pigmentation that is likely mediated by the suppression of DKK1 gene expression. These interesting results warrant further extensive investigation.

In conclusion, our findings demonstrated a cell model that was potentially useful to study the effects of melanin uptake in epidermal keratinocytes; in addition, our findings demonstrated that after melanin uptake, keratinocyte cell cycle is delayed, which is consistent to the observation that in the solar lentigo area, the cell proliferation is inhibited. The discovery that DKK1 gene expression in this cell model was downregulated by melanin uptake further confirmed the physiological meaning of this cell model, which could be used to quickly screen drugs that might potentially result in hyper- or hypo-pigmentation. This simplified cell model could also be useful for exploring the molecular mechanism of melanin uptake and how melanin impacts keratinocyte biological activities, in addition, except DKK1, target proteins of skin pigmentation could be identified and help us to further understand the skin pigmentation issue.

5.6 Overall Discussion

Overall, this thesis covered a longitudinal study of skin-aging progression using an epidemiologic approach and an analysis of the cellular mechanisms of skin aging using molecular biology and bioinformatic approaches. Clinically, we found that texture, wrinkles, and hyperpigmented spots were the three most important skin-aging parameters. These three parameters correspond well to the three major cell types in skin: epidermal keratinocytes, dermal fibroblasts, and melanocytes, respectively. With the aim of solving the issues related to these key aging parameters, we used a molecular biology approach to target these major skin components. First, we targeted keratinocytes and investigated AhR-Nrf2 signaling, which plays a major role in skin anti-oxidation and barrier function in the epidermis. Then, we targeted fibroblasts in the dermis and searched for important miRNAs that control the dermal extracellular matrix and skin aging. Finally, moving beyond single cells, we investigated the interaction between keratinocytes and melanocytes. In particular, we investigated

melanin uptake by keratinocytes from melanocytes and the biological function of the internalized melanin in keratinocytes. Together, the findings from the series of studies provided novel and valuable insights into the mechanism of skin aging and suggested new skin anti-aging strategies.

Although keratinocytes, fibroblasts, and melanocytes are the major cell types in the skin and their biological alterations are critical for the skin aging process, the interactions between different types of cells are also important. During the course of this study, the pratical researches with basic biological views concerning about the skin aging including keratinocyte, fibroblast, and melanocyte also opened the door to understand the importance of cell-cell interactions within the skin. In a series of studies, melanin uptake was shown to involve interactions between melanocytes and keratinocytes and to be associated with both cells. The results of the microRNA study indicated that fibroblasts might modulate the proliferation and differentiation of keratinocytes through microRNAs in the basement membrane, which further implies the importance of the interactions between the different types of cells in skin aging. In recent years, it has been revealed that some skin diseases such as melasma are associated with melanin from the epidermis deposited in the dermis (Nordlund et al. 2006). Therefore, there is a need to further investigate the interactions between melanocytes and fibroblasts. Furthermore, in addition to keratinocytes, fibroblasts, and melanocytes, there are immune cells in the skin such as Langerhans cells in the epidermis and microphages in the dermis. There is mounting evidence that indicates that aging is driven by pro-inflammatory cytokines (Zhuang et al. 2014), which can be associated with keratinocytes, immune cells or the interactions among various cells in the skin. Therefore, in order to elucidate the complete picture of the skin-aging mechanism, the interactions between immune cells and various primary skin cells should be included in the scope of the investigation. This way, it may help us elucidate bigger picture of skin aging mechanism more completely, and it may bring us

breakthrough approaches or strategies to skin anti-aging.

6. CONCLUSION

Skin is the largest organ in the human body and fulfills many important functions. Skin aging involves structural and compositional remodeling associated with phenotypic changes in skin cells that can manifest as texture roughness and pores, wrinkling and sagging, or hyperpigmentation. Since ancient times, people, especially women, have paid much attention to the beauty of their skin because it defines a person's appearance and even social status. Recently, due to longer life spans, skin aging has become a major concern because it affects not only a person's image but also a person's quality of life. Hence, modulating the progression of skin aging is becoming an important and emerging need.

In a series of studies using longitudinal and epidemiologic approaches, we found that the three skin parameters of texture roughness, wrinkles, and hyperpigmented spots could be the central parameters of skin aging. This finding helps clarify the major targets of dermatologic procedures and skin care technologies. Then, using a cellular molecular biology approach, we found that first, the novel AhR-Nrf2 signaling in keratinocytes could orchestrate both skin barrier and anti-oxidation activities, which are the two most important functions in the epidermis. In addition, we showed that OPIE, a natural ingredient, can improve skin barrier function and inhibit oxidation via AhR-Nrf2 signaling. These results indicate that targeting AhR-Nrf2 signaling can be used as a skin anti-aging strategy. This strategy would first improve and maintain skin texture. In addition, because oxidation is a primary cause of skin aging and not only alters keratinocyte differentiation and thus texture roughness but also induces excessive melanogenesis, it causes hyperpigmented spots. Therefore, targeting AhR-Nrf2 signaling might also help improve or prevent hyperpigmented spots.

Second, our findings demonstrated for the first time that miRNA34 and miRNA29 could play pivotal roles in dermis aging, and the results from the bioinformatic analysis showed that these microRNAs could regulate multiple types of

extracellular matrix in the dermis. In addition, our results showed that a biofunctional complex of yeast and soybean with anti-aging efficacy could modulate these microRNAs effectively. The results indicate that as a new skin anti-aging strategy, targeting those two miRNAs might allow the control of the structural remodeling of the dermis holistically, which might make it possible to prevent and repair facial wrinkles effectively.

Finally, using a newly established simplified cell model, we found that niacinamide and a trypsin inhibitor suppressed melanin uptake in epidermal keratinocytes. In addition, we found that melanin arrested the cell cycle progression of keratinocytes and their proliferation; moreover, it downregulated Dickkopf 1 (DKK1) gene expression in keratinocytes. Melanin uptake involves the interaction between melanocytes and keratinocytes, and our findings indicate that by also controlling melanin uptake by keratinocytes from melanocytes, we might be able to control the development of hyperpigmented spots and maintain healthy conditions for keratinocytes, thus also influencing skin texture.

Overall, the results of this series of studies first identified three key parameters that define the appearance of skin aging, and then, with a molecular approach, we identified the mechanisms associated with these skin aging parameters. Our new findings will potentially be useful for the development of new strategies for modulating skin aging, thereby addressing peoples' concerns regarding skin aging and improving their quality of life.

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Publication list

Original articles

Main papers

1. <u>Xianghong YAN</u>, Ta-Min WANG, Yung-Ching MING, Yuan-Ming YEH, Tzu-Ya CHEN, and Jong-Hwei Su PANG.

   Melanin uptake reduces cell proliferation of human epidermal keratinocytes.

   Journal of Cosmetics, Dermatological Science and Application, 5, 300-310, 2015.

2. Tong LI*, <u>Xianghong YAN</u>*, Min JIANG, and Leihong XIANG.

   * These authors contributed equally to this work

   The comparison of miRNA profile of the dermis between young and old human.

   Journal of Dermatological Science, in press.

3. <u>Xianghong YAN</u>, Catherine SERRE, Laurine BERGERON, Ludivine MUR, Valère BUSUTTIL, Jean-Marie BOTTO, and Nouha DOMLOGE.

   Modulation of a specific pattern of miRNAs, including miR-29a, miR-30a and miR-34a, in cultured human skin fibroblasts, in response to the application of a biofunctional ingredient that protects against cellular senescence *in vitro*.

   Journal of Cosmetics, Dermatological Science and Application, in press

4. Takeshi NAKAHARA, Chikage MITOMA, Akiko Hashimoto-HACHIYA, Masakazu TAKAHARA, Gaku TSUJI, Hiroshi UCHI, <u>Xianghong YAN</u>, Junichi HACHISUKA, Takahito CHIBA, Hitokazu ESAKI, Makiko Kido-NAKAHARA, and Masutaka FURUE.

   Antioxidant *Opuntia ficus-indica* extract activates AhR-Nrf2 signaling and upregulates filaggrin and loricrin expression in human keratinocytes.

   Journal of Medicinal Food **18** (10), 1143–1149, 2015.

5. Kukizo MIYAMOTO, Yasuko INOUE, Kesyin HSUEH, Zhiwu LIANG,

Xianghong YAN, Takashi YOSHII, and Masutaka FURUE.

Characterization of comprehensive appearances of skin aging: An 11-year longitudinal study on facial skin aging in Japanese females at Akita.

Journal of Dermatological Science **64**, 229–236, 2011.

6. Greg G HILLEBRAND, Zhiwu LIANG, Xianghong YAN, and Takashi YOSHII.

New wrinkles on wrinkling: an 8-year longitudinal study on the progression of expression lines into persistent wrinkles.

British Journal of Dermatology **162**, 1233–1241, 2010.

Related paper

1. 竹井賢二郎, 高原正和, 八谷顕子, 井上健示, 厳向紅, 辻学, 中原剛士, 構寧, 古江増隆

Galactomyces Ferment Filtrate (SK-II<ピテラ>)は表皮細胞の紫外線誘発酸化ストレスを抑制する

−Aryl Hydrocarbon Receptor を介した抗酸化作用について−

Aesthetic Dermatology **24**, 342-350, 2014.

Presentations

International Meetings

1. Xianghong YAN.

    How advanced researches drive technology development and business success?

    2015 Personal Care Summit, Shanghai, China (June, 2015).

2. Xianghong YAN.

    How advanced research drive technology development and business success?

    32th Colloid Interface Technology Symposium, Tokyo, Japan (January, 2015).

3. Xianghong YAN.

    New functional ingredients of anti-aging.

    SCC (Society of Cosmetics Chemists) Cosmetics Innovation Summit 2014, Hong

    Kong (November 2014).

4. Xianghong YAN, Tong Li, Min JIANG, and Leihong XIANG.

    Age-dependency of miRNAs in human skin.

    3rd Eastern Asian Derm Congress, Jeju Island, Korea (September 2014).

5. Xianghong YAN.

    Anti-aging by skin care: A historic review.

    5th Global Chinese Dermatologist Summit, Hong Kong (July 2013).

6. Xianghong YAN and Takashi YOSHII.

    Skin aging and anti-aging,

    Cosmetics Design Ingredient Day, Shanghai, China, (May 2013).

7. Xianghong YAN and Takashi YOSHII.

    SK-II skin care: Whitening and anti-aging.

    9th Sustainable Personal Care Technology Summit, Shanghai, China (June 2012).

8. Xianghong YAN and Takashi YOSHII.

SK-II anti-aging: A historic review and future prospect.

Asia Cosmetics Innovation Summit 2012, Shanghai, China (May 2012).

9. Xianghong YAN, Greg HILLEBRAND, Zhiwu LIANG, and Takashi YOSHII.

SK-II skin longitudinal study.

1st Eastern Asia Dermatology, Fukuoka, Japan (October, 2010).

10. Xianghong YAN and Takashi YOSHII.

Fusion of natural ingredients and advanced science,

Cosmetics Leaders Asia 2010, Shanghai, China (September 2010).

11. Xianghong YAN, Greg HILLEBRAND, Zhiwu LIANG, and Takashi YOSHII.

Progression of facial wrinkling, An 8-year longitudinal study.

Chinese Society of Dermatology 15th Annual Congress, Tianjin, China, (June 2009).

12. Xianghong YAN and Takashi YOSHII.

Pitera: SK-II Equity.

10th Japan-China Joint Dermatology Meeting, Hangzhou, China (October 2008).

Domestic　Meetings

１．伊達朗，厳向紅，吉井隆

"ヒト皮膚細胞内 sirt-1 誘導の皮膚老化に対する影響"

2007 年 6 月 22 日，札幌，第 30 回日本基礎老化学会

２．松原晃，箱崎智洋，松下理沙，伊達朗，厳向紅，渡邊めぐみ，湯山恵津子，安井裕之

"新型分光測光機 Tri-Ring Spectrophotometer によるヒト皮膚内部の光透過性に関する研究"

2009 年 8 月 1 日，新潟，第 27 回日本美容皮膚科学会学術大会

Membership of the academic societies:

Japanese Cosmetics Science Society

Japanese Society of Anti-Aging Medicine

The Society of Cosmetics Chemists of Japan.

Language:

- Chinese: Native Language

- English: Fluent (TOEIC 900)

- Japanese: Fluent (Passed 1st grade exam in 1996)