J. Soc. Cosmet. Sci. Korea
Vol. 44, No. 1, March 2018, 73-79

pISSN 1226-2587
eISSN 2288-9507
http://dx.doi.org/10.15230/SCSK.2018.44.1.73

바이오 안테나인 일차 섬모 조절을 통한 피부 미백 기술

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(2018년 3월 10일 접수, 2018년 3월 23일 수정, 2018년 3월 24일 채택)

Primary Cilia, A Novel Bio-target to Regulate Skin Pigmentation

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(Received March 10, 2018; Revised March 23, 2018; Accepted March 24, 2018)

요 약: 일차 섬모(primary cilia)는 세포에서 안테나처럼 돌출되어 나온 기관인데, 외부 자극에 반응할 수 있는 각종 수용체와 채널, 신호 전달 인자들을 가지고 있다. 피부는 자외선, 온도, 습도, 중력, 장력 등 외부 환경에 반응하여 멜라닌이나 콜라겐을 만들고 피부 장벽을 형성한다. 피부에서는 일차 섬모가 없으면 헤어의 생성이나 각질의 분화가 억제된다는 보고가 있다. 또한 피부 색소 생성과 관련하여서는 일차 섬모가 sonic hedge-hog-smoothened-GLI2 신호 전달에 의해 활성화되면 멜라닌 생성이 억제된다는 것이 알려져 있다. 피부가 자외선을 받으면 멜라닌 생성 호르몬의 양이 증가하고 멜라닌 생성 호르몬은 멜라닌 생성 세포 내 cAMP의 양을 증가시켜 멜라닌 생성 효소의 발현을 높인다. 이에 멜라닌 생성 호르몬과 세포 내 cAMP의 양을 증가시키는 물질을 처리하여 멜라닌 생성을 높였을 때 일차 섬모의 변화를 확인한 결과 일차 섬모가 감소하는 것을 확인하였다. 또한 기존 미백 원료인 유용성 감초 추출물(an ethanol extract of *Glycyrrhiza glabra* (EGG) root)과 Melasolv (3,4,5-trimethoxy cinnamate thymol ester (TCTE))가 일차 섬모의 발현을 증가시키고 멜라닌 생성 효소인 tyrosinase의 발현을 억제함을 확인할 수 있었다. 따라서 일차 섬모를 조절할 수 있다면 피부 색소 침착을 효과적으로 조절할 수 있을 것이다.

Abstract: The primary cilium protrudes from the cell body like a bio-antenna that has many receptors, channels and signaling molecules to sense and response to external stimuli. The external environment such as ultraviolet irradiation, temperature, humidity, gravity and shear stress always influences skin. Skin responds to external stimuli and differentiates by making melanin, collagen and horny layer. Ciliogenesis participates in developmental processes of skin, such as keratinocyte differentiation and hair formation. And it was reported that skin pigmentation was inhibited when ciliogenesis was induced by sonic hedgehog-smoothened-GLI2 signaling. When skin is exposed to ultraviolet irradiation, alpha-melanocyte stimulating hormones (α-MSH) increase melanin synthesis through activation of the cAMP pathway in melanocytes. We observed that α-MSH and cAMP production inducers inhibited ciliogenesis of melanocytes. Therefore, we thought that regulation of ciliogenesis is potential candidate target for the development of agents to treat undesirable hyperpigmentation of skin. As a result, we found out that an ethanol extract of *Glycyrrhiza glabra* (EGG) root and 3,4,5-trimethoxy cinnamate thymol ester (TCTE, Melasolv) significantly inhibit melanin synthesis of normal human melanocyte by inducing primary cilium formation. This study proposed new theory to regulate skin pigmentation and cosmetic components for skin whitening.

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Keywords: *Primary cilia, melanogenesis, Smo-GLI2 signaling, skin whitening*

1. Introduction

The primary cilium is a major cellular sensory organelle that functions as antennae for sensing extracellular information in many cell types (Kleene and Van Houten, 2014). Interactions of cells and external stimuli including chemical, mechanical, and paracrine signals are mediated by the primary cilium (Satir et al., 2010). Cilia are microtubule-based cellular organelles that are maintained by an intraflagellar transport (IFT) mechanism. IFT is required for the elongation of the cilia, and is a major intracellular transport system that carries non-membrane-bound particles and building materials from the cell body to the growing cilia (Follit et al., 2006). The cilia membrane includes a number of receptor proteins, ion-conducting channels, and signal transduction components (Satir et al., 2010). Primary cilium formation is activated by sonic hedgehog (SHH)-smoothened (Smo) signaling pathway (Ishikawa and Marshall, 2011). In addition, as a signaling hub, the Wnt signaling pathway regulates primary cilium formation (Gerdes and Katsanis, 2008). Cells without cilia enhance Wnt signaling, whereas cells with multiple cilia reduce its responses (Lancaster et al., 2011).

The pigmentation of human skin is influenced by external stimuli such as UVR, microenvironmental stiffness, inflammation, and hormones (Choi et al., 2014). A complex process is involved in the synthesis and distribution of melanin, a pigment for skin pigmentation. Synthesis of melanin is regulated mainly by alpha-melanocyte stimulating hormones (α-MSH), which bind to the melanocortin 1 receptor (MC-1R) and increase melanin synthesis through activation of cyclic AMP pathway and the expression of melanogenic enzymes, such as tyrosinase, TRP-1, and TRP-2 (Bertolotto et al., 1998).

It was previously reported that melanin concentrating hormone receptor 1 (MC1R) is also located in primary cilia of the central nervous system (Hamamoto et al., 2016). And the induction of primary cilium formation by Smo-GLI2 signaling suppresses melanin production by reducing the expression of melanogenic enzymes. This Effect of primary cilia on pigmentation is also confirmed in a human skin model (Choi et al., 2016). Therefore, activators of ciliogenesis in melanocytes can be potential candidate or lead compound for development of an agent to treat undesirable hyperpigmentation of skin.

In this study, we found that an ethanol extract of *Glycyrrhiza glabra* (EGG) root and 3,4,5-trimethoxy cinnamate thymol ester (TCTE, Melasolv) significantly inhibit melanin synthesis of normal human melanocyte by inducing primary cilium formation. Our data suggest a new skin whitening mechanism that can be used to cosmetics for a person with undesirable hyperpigmentation of skin.

2. Materials and Methods

2.1. Cell Culture

Normal human epidermal melanocytes (NHEMs) from the neonatal foreskin of moderately or darkly pigmented donors were purchased from Cascade Biologics (Portland, OR). The Melan-A melanocyte cell line, which is an immortalized mouse melanocyte cell line derived from a C57BL/6 J (black, a/a) mouse, was kindly provided by Dr. Dorothy C. Bennett (St. George’s Hospital Medical School, London, UK). Normal human melanocytes were maintained in M-254 medium with Human Melanocyte Growth Supplements (HMGS) (Cascade Biologics, Inc., Mansfield, UK). The Melan-A cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 0.2 μM phorbol 12-myristate 13-acetate.

2.2. Melanin Assay

Melanin contents in melanocytes were determined as described previously (Choi et al., 2005). Cells were harvested with trypsin/EDTA, centrifuged for 5 min at 1000 × g, washed with PBS twice, and then final cell

pellets in tubes were photographed. Cell pellets were dissolved with 1 N NaOH. The homogenized cell extracts were transferred into 96-well plate in triplicate and relative melanin content was determined by measuring absorbance at 405 nm using an ELISA plate reader.

2.3. Western Blot Analysis

All lysates were prepared in 2 × Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% [v/v] glycerol, 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, and 0.01% [w/v] bromophenol blue) (Bio-Rad, Hercules, CA). All cellular proteins were quantified using Bradford solution (Bio-Rad) according to the manufacturer's instructions. The samples were then separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). After blocking with 4% (w/v) skim milk in TBST (25 mM Tris, 140 mM NaCl, and 0.05% [v/v] Tween® 20), the membranes were incubated overnight with specific primary antibodies. Anti-polyglutamylated-tubulin antibody (AG-20B-0020, 1:1000) was purchased from Adipogene (San Diego, CA); anti-tyrosinase antibody (05-647; 1 : 1000) was purchased from Upstate Biotechnology (Lake Placid, NY, USA); anti-TRP1 antibody and anti-GAPDH antibody (sc25778; 1 : 2000) were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies and the signals were detected with SuperSignal West Dura HRP Detection Kit (Pierce, Rockford, IL).

2.4. Immunofluorescence Analysis by Confocal Microscopy

NHEMs and Melan-a cells cultured on Lab-Tek™ glass chamber slides (Thermo Scientific, Waltham, MA) were washed with cold PBS, fixed for 20 min with 4% (w/v) paraformaldehyde, washed twice with PBS, and incubated in PBS containing 0.1% (v/v) Triton™ X-100 (PBS-T) for 3 min. The cells were further washed 3 times with PBS-T and then incubated with anti-polyglutamylated-tubulin antibody (1 : 100 dilution, Adipogene, AG-20B-0020), anti-acetylated-tubulin antibody (1 : 1000 dilution, Sigma-Aldrich), and anti-ARL13B antibody (1 : 200 dilution, Proteintech) diluted in PBS-T containing 5% (v/v) normal goat serum for 1 h at room temperature. After washing with PBS, the cells were incubated with appropriate conjugated secondary antibodies and then mounted on glass slides to observe fluorescence using a confocal laser scanning microscope (model LSM510; Carl Zeiss Microimaging Inc., Thornwood, NY).

2.5. Statistical Analysis

Data were obtained from at least 3 independent experiments and are presented as mean ± S.E. Statistical evaluation of the results was performed using 1-way ANOVA (SPSS software, version 21).

3. Results and Discussion

3.1. Melanogenic Inducers Inhibit Primary Cilium Formation in Melan-A Mouse Melanocyte Cell Line

UV radiation stimulates human skin keratinocytes and melanocytes to secrete α-MSH. Alpha-MSH induces melanogenesis by increasing melanogenic proteins such as tyrosinase, TRP1 and TRP2 through cyclic AMP pathway. Forskolin is an adenylyl cyclase activator and IBMX (3-isobutyl-1-methylxanthine) is a cyclic AMP phosphodiesterase inhibitor. Therefore, these compounds activate cyclic AMP pathway and increase melanin synthesis in melanocytes. Melan-A cells were treated with α-MSH, forskolin or IBMX for 4 days. These melanogenic inducers significantly increase melanin synthesis and protein expression of tyrosinase and TRP1 (Figure 1C). And we observed primary cilia of the cells by confocal microscopy. The cilium is a tubulin-based cellular structure. Tubulin undergoes multiple post-translational modifications (PTMs). Among the PTMs of tubulin, polyglutamylation is thought to be associated with an active status of the tubulin in primary cilia (Wloga and Gaertig, 2010). Therefore, we analyzed the expression level of polyglutamylated tubulin protein to assess cilia formation. Melanogenic inducers inhibit the level of polyglutamylated tubulin and cilium assembly (Figure 1B). Figure 1A is a representative picture of primary cilia on melanocytes culture with or without α-MSH.

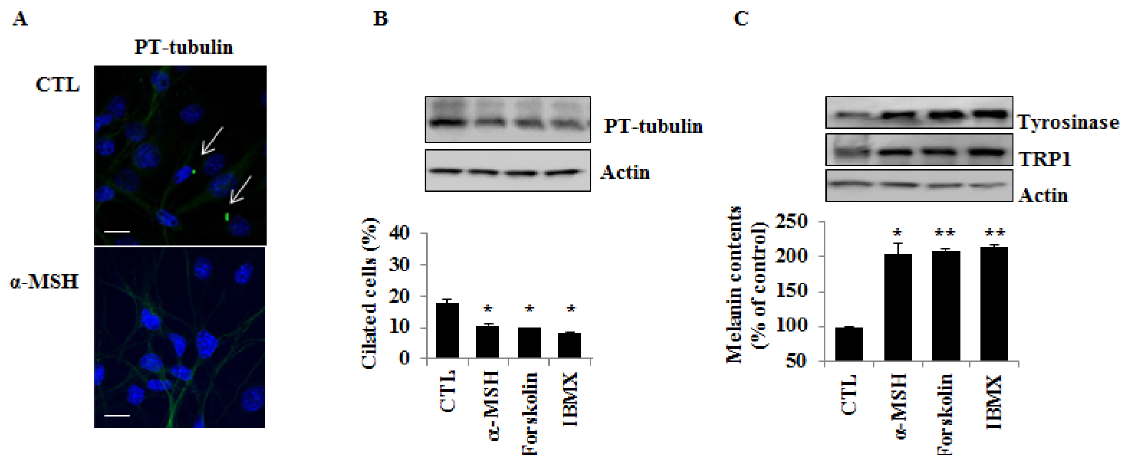

Figure 1. Melanogenic inducers inhibit primary cilium formation of Melan-A cells. Melan-A cells were treated with melanogenic inducers (α-MSH 1 μM, forskolin 10 μM, IBMX 100 μM) for 48 h. The induction of polyglutamylated-tubulin protein (PG-tubulin, green) was observed (A, scale bar = 10 μm). Ciliated cells and cellular melanin content were measured (B, C). The protein expression levels of PG-tubulin, tyrosinase, and TRP1 were analyzed. Data were obtained from at least 3 independent experiments and the values were presented as mean ± S.E. (* $p < 0.05$, ** $p < 0.01$).

Consequently, these data demonstrated that UV radiation increases skin pigmentation by elevating cAMP signaling, which leads to inhibition of the primary cilium formation.

3.2. Skin Whitening Components Induce Ciliogenesis in Normal Human Epidermal Melanocytes (NHEMs)

Primary cilium formation is induced by Smo-GLI2 signaling. Notably, the MITF and GLI2 genes are inversely expressed in various melanoma cell lines (Javelaud et al., 2011). MITF is a transcription factor of melanogenic proteins and cAMP was reported to inhibit the SHH signaling pathway and GLI2 expression (Mukhopadhyay et al., 2013). In previous report, the induction of primary cilium formation by Smo-GLI2 signaling suppresses melanin production (Choi et al., 2016). Therefore, we searched for activators of ciliogenesis in melanocytes as components to treat undesirable hyperpigmentation of skin. Methanol extract of *Glycyrrhiza glabra* leaves was reported to have antiviral activity against Newcastle disease virus (Ashraf et al., 2017). And glabridin, an isoflavan, isolated from the root of *Glycyrrhiza glabra*, has exhibited excellent inhibitory effects on tyrosinase (Chen et al., 2016). 3, 4, 5-trimethoxy cinnamate thymol ester (TCTE, Melasolv) is also known as a skin whitening agent (Kang et al., 2003). We treated NHEMs with an ethanol extract of Glycyrrhiza glabra (EGG) root and TCTE for 4 days. Primary cilia in NHEMs were observed by immunostaining for acetylated tubulin (the active form of tubulin) and ARL13B (a small G protein localized in the cilia). These skin whitening agents significantly induced primary cilium formation in NHEMs (Figure 2A and B). Cytochalasin D (Cyto D), an actin polymerization inhibitor that causes cell cycle arrest at the G1-S transition, is wildly used as an inducer of primary cilium formation (Kim et al., 2010). NHEMs increased ciliogenesis when cultured with Cyto D. And induction of primary cilium formation by these components was not inhibited by ciliobrevin A1 (Cilio A). Cilio A, the first specific small-molecule antagonist for the cytoplasmic dyneins, was reported to inhibit primary cilium formation (Firestone et al., 2012). When NHEMs were treated with Cilio A, ciliogenesis of the cells was inhibited (Figure 2A and 2B). Therefore, these results suggested that EGG and TCTE could suppress the inhibitory effects of Cilio A on primary cilium formation.

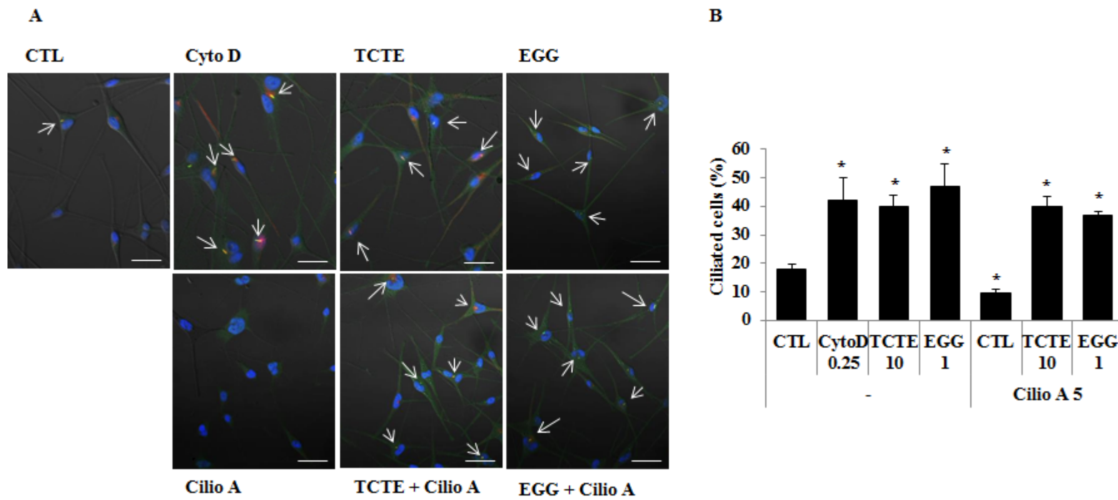

Figure 2. Skin whitening components induce ciliogenesis of normal human epidermal melanocytes. NHEMs were treated with cytochalasin D (Cyto D, 0.25 mM), TCTE (10 ppm), EGG (1 ppm) and ciliobrevin A1 (Cilio A, 5 μM) for 4 days. The induction of acetylated-tubulin (green) and ARL13B (red) was observed (A, scale bar = 20 μm). After immunostaining, ciliated cells were counted (B). Data represent the mean ± S.E. of 3 experiments (* $p < 0.05$).

3.3. Skin Whitening Components Suppress the Active Effects of Cilio A on Melanogenesis in NHEMs

Cilio A significantly induced melanin synthesis by increasing protein expression of tyrosinase in NHEMs (Figure 3A and B). However, this effect of Cilio A on melanogenesis was inhibited when NHEMs were incubated with EGG or TCTE for 4 days. EGG and TCTE inhibited melanin synthesis induced by Cilio A through impeding protein expression of tyrosinase. TCTE inhibited melanogenesis and tyrosinase with or without Cilio A at the concentration. Nevertheless, EGG did not inhibit tyrosinase at 1 ppm without Cilio A. It was attributed to low concentration because we observed inhibitory effect of EGG on protein expression of tyrosinase at high concentration (data not shown). Consequently, these data demonstrated that EGG and TCTE inhibited melanogenesis of NHEMs by increasing primary cilium formation.

4. Conclusion

Through the cilium, cells and tissues adapt to changes in the extracellular environment by regulating cell cycle, cellular function, cell shape, and movement. Primary cilia are involved in keratinocyte differentiation, hair follicle maturation and pigmentation in skin induced by UV radiation. In this report, we tested the effects of skin whitening components on ciliogenesis. EGG and TCTE inhibited melanogenesis effectively by inducing primary cilium formation. Therefore, melanogensis increased by Cilio A, an inhibitor of primary cilium formation was impeded by these whitening components. However, it was not investigated whether these components work as a direct inhibitor of Cilio A or not. Future studies on the molecular mechanism underlying ciliogenesis induced by these whitening components in melanocytes may need to investigate. Nevertheless, our results suggest a new mechanism for regulating undesirable skin pigmentation by inducing primary cilia for skin whitening agents EGG and TCTE.

Reference

1. A. Ashraf, M. M. Ashraf, A. Rafiqe, B. Aslam, S. Galani, S. Zafar, F. Asad, R. D. Asghar, S. Akram, H. Ahmed, S. M. A. Shah, and R. Asif, In vivo anti-

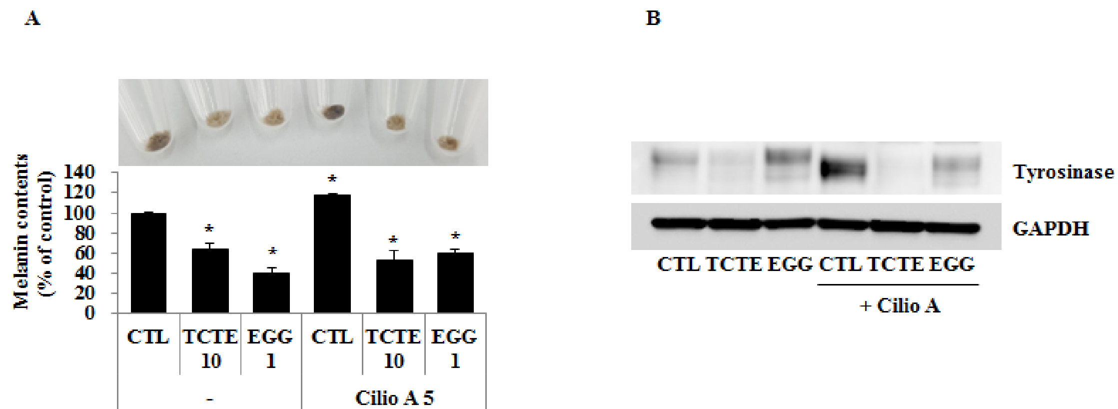

Figure 3. The inducers of ciliogenesis inhibit melanin synthesis in normal human epidermal melanocytes. NHEMs were treated with TCTE (10 ppm), EGG (1 ppm) and ciliobrevin A1 (Cilio A, 5 μM) for 4 days. Cellular melanin content (A) and protein expression of tyrosinase were analyzed (B). Data represent the mean ± S.E. of 3 experiments (* $p < 0.05$).

viral potential of *Glycyrrhiza glabra* extract against Newcastle disease virus, *Pak. J. Pharm. Sci.*, **30**(2(Suppl.)), 567 (2017).

2. C. Bertolotto, P. Abbe, T. J. Hemesath, K. Bille, D. E. Fisher, J. P. Ortonne, and R. Ballotti, Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes, *J. Cell Biol.*, **142**, 827 (1998).
3. J. Chen, X. Yu, and Y. Huang, Inhibitory mechanisms of glabridin on tyrosinase, *Spectrochim. Acta A Mol. Biomol. Spectrosc.*, **168**, 111 (2016).
4. H. Choi, S. Ahn, B. G. Lee, I. Chang, and J. S. Hwang, Inhibition of skin pigmentation by an extract of Lepidium apetalum and its possible implication in IL-6 mediated signaling, *Pigment. Cell Melanoma Res.*, **18**, 439 (2005).
5. H. Choi, M. Kim, S. I. Ahn, E. G. Cho, T. R. Lee TR, and J. H. Shin, Regulation of pigmentation by substrate elasticity in normal human melanocytes and melanotic MNT1 human melanoma cells, *Exp. Dermatol.*, **23**, 172 (2014).
6. H. Choi, J. H. Shin, E. S. Kim, S. J. Park, I. H. Bae, Y. K. Jo, I. Y. Jeong, H. J. Kim, Y. Lee, H. C. Park, H. B. Jeon, K. W. Kim, T. R. Lee, and D. H. Cho, Primary cilia negatively regulate melanogenesis in melanocytes and pigmentation in a human skin model, *PLoS One*, **11**(12), e0168025 (2016).
7. J. A. Follit, R. A. Tuft, K. E. Fogarty, and G. J. Pazour, The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly, *Mol. Biol. Cell*, **17**, 3781 (2006).
8. J. M. Gerdes and N. Katsanis, Ciliary function and Wnt signal modulation, *Curr. Top. Dev. Biol.*, **85**, 175 (2008).
9. A. Hamamoto, S. Yamato, Y. Katoh, K. Nakayama, K. Yoshimura, S. Takeda, Y. Kobayashi, and Y. Saito, Modulation of primary cilia length by melanin-concentrating hormone receptor 1, *Cell Signal*, **28**(6), 572 (2016).
10. H. Ishikawa and W. F. Marshall, Ciliogenesis: building the cell's antenna, *Nat. Rev. Mol. Cell Biol.*, **12**, 222 (2011).
11. D. Javelaud, V. I. Alexaki, M. J. Pierrat, K. S. Hoek, S. Dennler, L. Van Kempen, C. Bertolotto, R. Ballotti, S. Saule, V. Delmas, and A. Mauviel, GLI2 and M-MITF transcription factors control exclusive gene expression programs and inversely regulate invasion in human melanoma cells, *Pigment. Cell Melanoma Res.*, **24**, 932 (2011).
12. H. H. Kang, H. S. Rho, J. S. Hwang, and S. G. Oh, Depigmenting activity and low cytotoxicity of alkoxy benzoates or alkoxy cinnamte in cultured melano-

cytes, *Chem. Pharm. Bull. (Tokyo)*, **51**(9), 1085 (2003).

13. J. Kim, J. E. Lee, S. Heynen-Genel, E. Suyama, K. Ono, K. Lee, T. Ideker, P. Aza-Blanc, and J. G. Gleeson, Functional genomic screen for modulators of ciliogenesis and cilium length, *Nature,* **464**, 1048 (2010).
14. M. A. Lancaster, J. Schroth, and J. G. Gleeson, Subcellular spatial regulation of canonical Wnt signaling at the primary cilium, *Nat. Cell Biol.*, **13**, 700 (2011).
15. S. Mukhopadhyay, X. Wen, N. Ratti, A. Loktev, L. Rangell, S. J. Scales, and P. K. Jackson, The ciliary G-protein-coupled receptor Gpr161 negatively regulates the Sonic hedgehog pathway via cAMP signaling, *Cell*, **152**, 210 (2013).
16. P. Satir, L. B. Pedersen, and S. T. Christensen, The primary cilium at a glance, *J. Cell Sci.*, **15**, 499 (2010).