Interferon-gammasuppresses melanogenesis in B16 melanoma cells and primary human melanocytes

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This certifies that the dissertation ofJinyoung Son is approved.

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December, 14rd, 2012

- ABSTRACT -

Interferon-gamma suppressesmelanogenesisin B16 melanoma cells and primary human melanocytes

Inflammatory cytokines are closely related to melanogenesis in melanocytes. Here, I studied whether IFN-γ affectsmelanogenesis, and if so, what is the underlying mechanism involved in it. Using B16 melanoma cell lines and primary human melanocytes, I demonstrated that α-MSHinduced tyrosinase expression and activity and TRP-1, TRP-2 expressionwere suppressed by IFN-γ, which resulted from the inhibition of MITF expression, upstream molecule of tyrosinase. Moreover, the inhibitory effects on MITF expression by IFN-γoccuredthrough the disturbance of CREB binding ability on MITF promoter by reducing the binding of CBP and CREB.These results suggest that IFN-γ decreasesmelanogenesis through inhibiting the binding of CBP and CREB and block the MITF and tyrosinase expression.

Key words: Melanogenesis, α-MSH, IFN-γ, Tyrosinase, MITF, STAT1, CBP, CREB

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ABBREVIATION

IFN-γ: Interferon-gamma

α-MSH: Alpha-melanocyte-stimulating hormone

IL-6: Interleukin-6

TRP-1: Tyrosinase related protein-1

TRP-2: Tyrosinase related protein-2

MITF: Microphthalmia-associated transcription factor

CREB: cAMP response element binding protein

CBP: cAMP response element-binding protein-binding protein

STAT: Signal transducer and activator of transcription 1

Ⅰ. INTRODUCTION

Human skin color is primary determined by melanin produced in melanocytes. Different skin colors are caused by the number or the activity of melanocytes (Briganti et al., 2003). Hyper- and hypopigmentation of skin lead to melisma or solar lentigo and vitiligo, respectively(van den Boorn et al., 2009). Although most pigmentation disorders are not dangerous, patients often undergo serious psychological stress. For this reason, it is very important to understand the mechanism of melanin synthesis and the depigmentation pathway.

1. Roles of melanin

UV-radiation has strong energythat may cause diversedefects into our skins such as sunburn and local immune responses.More importantly, UV-radiation induces synthesis of reactive oxygen species (ROS) and also causes DNA damage in epidermis, which can be developed into skin cancer (Aubin, 2003; Halliday, 2005). Melanin, a pigment molecule synthesized in melanocytes, absorbs UV light to protect skin from the UV-radiation. Therefore, UV radiation does not effect on dermis(Tran et al., 2008).

2. Skin structure and melanin synthesis

Human skin consists of three layers; epidermis, dermis and hypodermis. The outmost layer, epidermis, contains keratinocytes that are capable of dividing. Melanocytes exist in the deepest layer of the epidermis, called stratum basale. It consists of a single layer of columnar

resting on basal lamina. Keratinocyte make up about 95 % of the total cell numbers in epidermis, while melanocyte only comprise of 1~5% cells(Costin and Hearing, 2007).

Melanocytes synthesize melanin using tyrosine as substrate. In human, two types of melanins, eumelanin and pheomelanin representing a dark brown-black insoluble and a light red-yellowsulfur-containing soluble polymer, respectively, are synthesized within melanosomes(Briganti et al., 2003). Both eumelanin and pheomelanin are actively produced by continued hydroxylation steps of tyrosine, and further matured in melanosome(Schiaffino, 2010).Several enzymes involved in the hydroxylation steps: tyrosinase acts in the two oxidative steps, from tyrosine to L-DOPA and from L-DOPA to DOPA quinone. Tyrosinase related protein 1 (TRP-1) and tyrosinase related protein 2 (TRP-2 or called DCT) act in the oxidative step, from DHICA to indole-5,6quinone carboxyl acid and from DOPA chrome to DHICA, respectively. The amount of melanin biosynthesis can be regulated by the expression of the three enzymes, tyrosinase, TRP-1 and TRP-2, which actively regulated by diverse stimuli(del Marmol and Beermann, 1996; Kobayashi et al., 1998).

3. Melanogenesis signaling pathway

Melanogenesis is induced by UV radiation. p53 plays a central role in the UV induced melanogenesis. In detail, DNA damage occurs in keratinocytes, the major constituent of epidermis, by UV radiation. The DNA damage induces activation of a tumor suppressor protein, p53, followed by inducing transcription of pro-opiomelanocortin (POMC) in keratinocytes. POMC is converted to melanocyte-stimulating hormone (α-MSH) by post-translational processes(Brenner and Hearing, 2008). α-MSH, released from keratinocyte,

effect on melanocytes located in stratum basale. Secreted α-MSH bind to melanocortin 1 receptor (MC1-R) in membrane of melanocyte, leading to elevates cAMP. Protein kinase A (PKA) is activated by cAMP, followed by phosphorylation of cAMP response element binding protein (CREB), which induces transcription of microphthalmia-associated transcription factor (MITF) gene through binding tocAMP response element (CRE) on the promoter of the MITF gene(Cannon et al., 1986; Busca and Ballotti, 2000). Besides CREB, MITFis also induced by other transcription factors such as paired box gene 3 (PAX3), SPY (sex-determining region Y) –box 10 (SOX10) and lymphoid enhancer binding factor 1 (LEF1) (Galibert et al., 1999). MITF is a basic-helix-loop-helix (bHLH) and leucine zipper transcription factor that binds to E-box (CACGTG) and M-box (AGTCATGTGCT), and regulates the mRNA transcription of tyrosinase, TRP-1 and TRP-2 (Levy et al., 2006).

4. Effects of interferon family on melanogenesis

Diverse molecules such as growth factors, hormones and cytokines are existed in epidermis. The elements are closely related with to cell growth and pigmentation. Inflammatory cytokines, IL-10, IL-6, TNF-α and interferon (IFN) family, has been suggested as regulators of melanogenesis(Yamaguchi and Hearing, 2009). In interferon family, IFN-β and IFN-γ, has been reported as modulators of melanogenesis, but specific mechanism is still unknown.

IFN family can be induced by viral glycoprotein, viral RNA, mitogen and other cytokines such as IL-1, IL-2, and TNF-α. IFN-α and β belong to type 1 interferon having

capacity to bind interferon α receptor (IFNAR1, IFNAR2), whereas IFN-γ constitutes the only type 2 interferon and binds to interferon γ receptor (IFNGR1, IFNGR2). IFN-γ, produced from NK cell and cytotoxic T cell, transduces their signal through well-defined pathway (Schroder et al., 2004): IFN-γ binds to IFNGR, followed by inducing auto-phosphorylation of Janus kinase (JAK). Phosphorylated JAK mediates a signal transducer and activator of transcription (STAT) activation, STAT1, by inducing its phosphorylation and dimerization. Activated STAT1 translocates into nucleus and binds to interferon-gamma activated sequence (GAS) on the promoter of target genes. STAT1 also associates with other transcriptional regulatory factors such as cyclic AMP response element-binding protein-binding protein (CBP), histone deacetylase 1 (HDAC-1), and N-myc-interactor(Lee et al., 1999; Chatterjee-Kishore et al., 2000; Nusinzon and Horvath, 2003; Schindler and Plumlee, 2008). STAT1, which binds toCBP, negatively regulates the transcriptional activity of CBP. In detail, IFN-γ suppresses MMP9 expression by association of STAT1 and CBP, inhibiting the binding activity of CBP to the target site (Ma et al., 2005).SR-A transcription suppress through competitive role of STAT1 on the binding of AP-1 with CBP (Horvai et al., 1997).

In this study, Ifound that IFN-γ inhibitmelanogenesis in B16 melanoma and primary human melanocyte. Treatment of IFN-γ co-treatment with α-MSH inhibited tyrosinase and MITF mRNA and protein expression.Furthermore, I found thatIFN-γ suppressed CREB bindingon the MITF promoterby inducing STAT1 and CBP binding.These results collectively suggest that IFN-γ, existed around melanocytes, impairsmelanogenesis.

Ⅱ. MATERIALS AND METHODS

A. Cell culture

The murine B16 melanoma cells were purchased from ATCC. B16 melanoma cells were cultured in DMEM (Hyclone) containing 10% fetal bovine serum (Gemini) and 1% antibiotics (GIBCO). Primary human melanocytes were derived from human adult foreskin. Culture was based on previously method(Martinez-Esparza et al., 1997). Primary human melanocytes were maintained in F12 media (GIBCO) containing 10% fetal bovine serum and 1% antibiotics and supplemented with 1.2ng/ml β-FGF, 80nM PMA, 24ug/ml IBMX, 0.1ug/ml cholera toxin. For experiment, media were changed to MCDB 153 containing 4% fetal bovine serum and 1% antibiotics and supplemented with 0.6ng/ml β-FGF, 0.5ug bovine insulin, 0.1ug/ml bovine transferrin, 0.1ug/ml α-tocopherol at 24h prior to drug treatment (Kim et al., 2010).

B. Reagent

Antibodies against Tyrosinase (Cat. #sc-7833), TRP-1 (Cat. #sc-25543) and DCT (Cat. #sc-10451) were purchased from Santacruze (CA, USA). CBP (Cat. #7389), STAT1 (Cat. #9172) and CREB (Cat. #9179) were purchased from Cell Signaling Technology (Danvers, MA, USA) and MITF (Cat. #ab12039) was purchased from abcam (Cambridge, UK). α-MSH, synthetic melanin, 3,4-digydroxy-phenylalanine (L-DOPA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mouse and Human IFN-γ, Mouse and Human IL-6 were purchased from R&D systems (Minneapolis, MN, USA).

C. Tyrosinase activity

Cells were washed twice with cold PBS and lysed in 0.1M phosphate buffer (pH 6.8) containing with 1% triton X-100, protease inhibitor cocktail (Roche Diagnostics, Sydney, NSW, Australia) for 1h at 4℃. The lysates were centrifuged at 12,000g for 15min at 4℃. The supernatant analyzed for protein concentration and each sample for 5ug protein were transferred to 96-well plates. Each sample was incubated with L-DOPA (2mM, in 0.1M phosphate buffer, pH 6.8) for 90min at 37℃. After incubation, tyrosinase activity was measured at 490nm.

D. Western immunoblot analysis

Culture plates were washed twice with cold PBS and lysed in RIPA buffer containing with protease inhibitor cocktail and phosphatase inhibitor cocktail for 1h at 4℃. Whole lysates were sonicated and centrifuged at 12,000g for 15min at 4℃. Supernatant correct new 1.5ml tube and protein concentration was determined using the Lawry assay and detected 650nm. Total protein (10~20ug) separated 8% SDS-polyacrylamide gel and transfer to NC membrane. Membrane was block Tris-buffered saline (TBS, pH 7.4) containing 0.05% tween-20, 5% skim milk powder for 1hr at room temperature. Primary antibodies (1:1,000~1:5,000) were used anti-tyrosinase, anti-TRP-1 and TRP-2, anti-MITF. Incubations were overnight at 4℃ and washing in TBS containing 0.05% tween-20. Secondary antibodies (1:10,000) were incubated 1hr at room temperature. Following washing membrane in TBST, protein were exposed enhanced chemiluminescence and detected band

using LAS imaging system.

E. RNA extraction and real-time PCR

Total RNA was isolated using TRI-zol (TaKaRa,Japan), following the instructions of manufacturer's. 1 ug of total RNA used for revers transcription reaction. Real-time RCR was performed using SYBR-Green (TaKaRa,Japan) following instructions of manufacturer's. mRNA level were normalized GAPDH.

F. Melanin content

Cells were washed with cold PBS twice and lysed with 0.1M phosphate buffer pH6.8 containing with 1% NP-40, protease inhibitor cocktail and incubation for 1h on ice. Lysates were centrifuged at 10,000g for 15min and supernatant measured protein concentration at 650nm. Pellet were solubilized 1N NaOH in 100ul for 3h at 60℃ and measuredabsorbance at 405nm. Melanin content was calculated from a standard curve using synthetic melanin.

G. Electrophoresis mobility shift assays (EMSA)

Cells were harvested in hypotonic buffer and correct the nucleus extracts. Nucleus extract was incubated for 1h in reaction buffer with or without [γ-^{32}P]-labeled probe such as CREB consensus oligonucleotides, CREB binding site, (Cat. #sc-2504, santacruz) 5'-AGA

GAT TGC CTG ACG TCA GAG AGC TAG- 3' and 3' −TCT CTA ACG GAC TGC AGT CTC TCG ATC- 5' or MITF oligonucleotides, MITF binding site of tyrosinase promoter, (purchased from Bioneer) 5'−TTA GTC ATG TGC TTT- 3' and 3'−AAA GCA CAT GAC TAA- 5'. For supershift assays, nuclear extract was incubated with antibodies for 1h. Incubated complexes were separated on 6% polyacrylamide gels and dried gels were exposed to X-ray film.

H. Duolink assay

Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 15min, permeabilized with 0.1% Triton X-100 in PBS for 5min and blocked with blocking solution (Olink Bioscience, Uppsala, Sweden) for 30min at 37°C. And two primary antibodies were incubated with antibody diluent (Olink Bioscience, Uppsala, Sweden) for 2h at room temperature. After three times wash in wash buffer A, cells were incubated with positive and negative PLA probes (Olink Bioscience, Uppsala, Sweden) for 1h at 37°C, washed three times with wash buffer, and incubated with ligation solution (Olink Bioscience, Uppsala, Sweden) for 30min at 37°C. Cells were washed two times in wash buffer A and then incubated in amplification solution (Olink Bioscience, Uppsala, Sweden) for 100 min at37°C. Cells were washed two times in wash buffer B and 1 times in 0.01x wash buffer B. Cells were mounted with mounting solution (Olink Bioscience, Uppsala, Sweden) containing with DAPI.

Ⅲ. RESULTS

A. IFN-γ suppresses tyrosinase expression in B16 melanoma cells

To test whether IFN-γ affectsmelanogenesis, IFN-γ was co-treated with α-MSH. α-MSH is well known melanogenesis inducer through cAMP pathway and CREB activation. B16 melanoma cells were stimulated withα-MSH and then treated with IFN-γfor 48h. The tyrosinase, TRP-1 and TRP-2 expressions were examined of the cells. As shown figure 1A,expressions of tyrosinase, TRP-1 and TRP-2 were induced by α-MSH treatment. IFN-γtreatment reduced the tyrosinase, TRP-1 and TRP-2 protein expression despite α-MSH treatment. IL-6 is well known cytokine for inhibits the melanogenesis through decrease of Pax3 protein and mRNA level (Swope et al., 1991). IL-6 reduced tyrosinase, TRP-1 and TRP-2 expression but does not above IFN-γ. I tested whether IFN-γ also affects tyrosinase mRNA. Tyrosinase mRNA was increased by α-MSH treatment after 6 h. IFN-γ treatment was inhibited the α-MSH induced tyrosinase mRNA expression approximately 50% inhibition compared with α-MSH only treated cell at 12 h after treatment (Fig. 1B). These results indicate that IFN-γ suppresses tyrosinase expression in B16 melanoma cells.

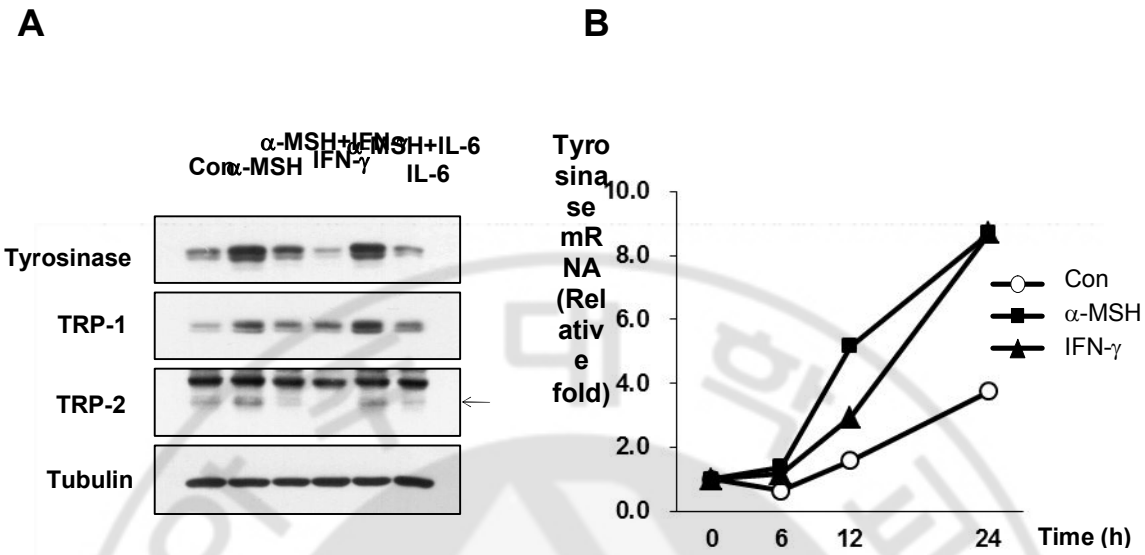


Figure 1.Effect of IFN-γ on melanogenic proteins and mRNA expression in B16melanoma cells.

(A) B16 melanoma cells were cultured at 24h and α-MSH (10nM/ml) treated with or without IFN-γ (10ng/ml) and IL-6 (10ng/ml) for 48h. Tyrosinase, TRP-1 and TRP-2 protein levels were determined using western blot. (B) For measuring mRNA levels, B16 melanoma cells were cultured at 24h and α-MSH treated with or without IFN-γ and IL-6. mRNAlevels were analyzed using real-time PCR and normalized relative to GAPDH.

B. IFN-γdoes not affect degradation of tyrosinase in B16 melanoma cells

Next, I test whether IFN-γ affects thedegradation of tyrosinase as well as the inhibition of tyrosinaseexpression. I inhibited protein degradation pathway.I treated the cells with proteasome inhibitor (MG132 and lactacystin), selective cysteine protease inhibitor (E-64) and lysosomal inhibitor (NH4Cl) 12h after α-MSH with or without IFN-γ stimulation. Cells were harvested after 12 h and analyzed using western blot. As shown figure 2, IFN-γ did not reverse tyrosinase protein through proteasome inhibitor, cysteine protease inhibitor and lysosomal inhibitor. These results demonstrate that IFN-γ does not affect degradation of tyrosinase in B16 melanoma cells.

C. IFN-γ inhibits tyrosinase activity and melanin production in B16 melanoma cells

To test whether IFN-γ regulates the tyrosinase activity, the tyrosinase activity was determined. As shown figure 3A, tyrosinase activity was increased by α-MSH treatment. Compared with control, IFN-γ was significantly decreased the tyrosinase activity stimulated by α-MSH. IFN-γ only also decreased the tyrosinase activity by approximately 50% of control. I also measured the melanin content, final event of melanogenesis. Melanin content was significantly decreased after IFN-γ treatment which is consistent with tyrosinase activity (Fig. 3B).These results demonstrate that IFN-γ reduces melanogenesis through inhibition of tyrosinase activity in the cells.

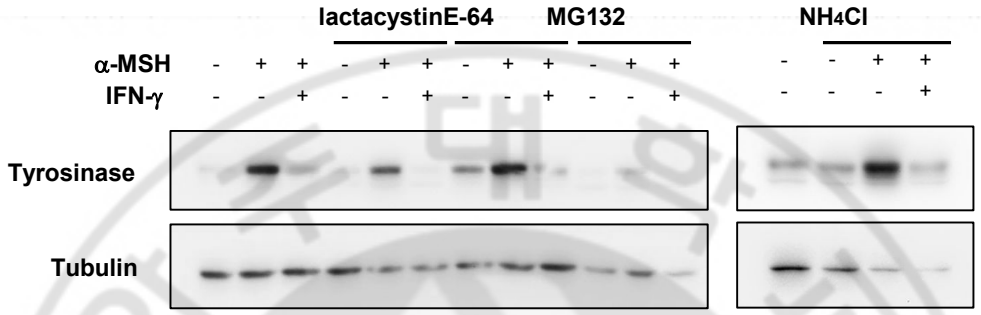


Figure 2. IFN-γ does not induce tyrosinase degradation in B16 melanoma.

Cell was seeded in 60mm dish and allowed to adhere for 24h. α-MSH (10nM) treated with or without IFN-γ (10ng/ml) for 12h and then treated with lactacystin (1μM/ml), E-64 (10μM/ml), MG132 (100nM/ml), NH_4Cl (10μM/ml). After 12h, Cells were harvested for western blot.

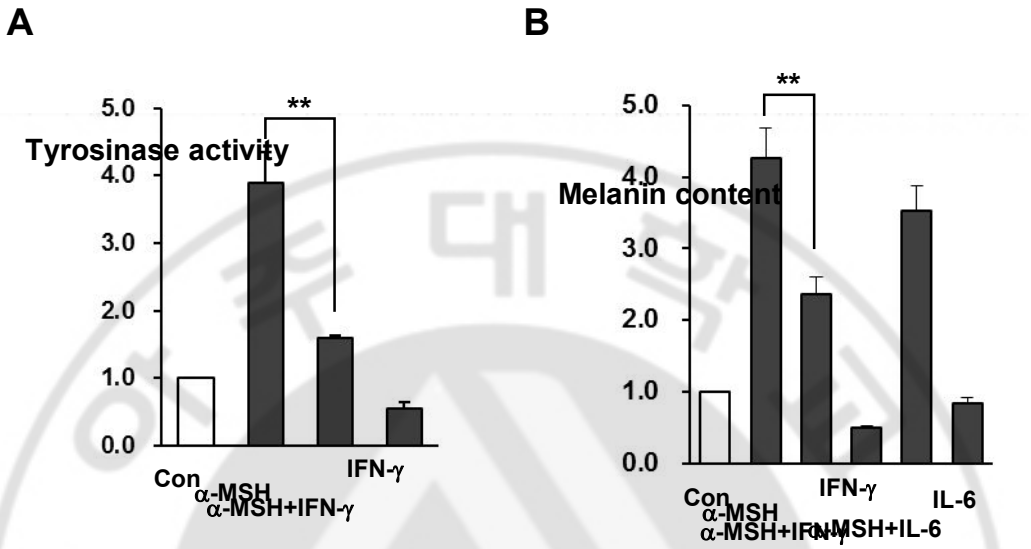


Figure 3.Effect of IFN-γ on tyrosinase activity and melanin content in B16 melanoma cells.

(A) B16 melanoma cells were cultured at 24h. Tyrosinase activity assay was determined using L-DOPA (2mM) and detected in 490nm. (B) Melanin content was determined using standard control of synthetic melanin and detected in 405nm. n=3. **P<0.01.

D. IFN-γ suppresses the tyrosinase expression and activity in primary human melanocytes

To confirm whether inhibitory effect of IFN-γ on melanogenesisaffects in primary human melanocyte, I treated cells α-MSH with or without IFN-γ and IL-6 every 2 days until 5day and determined melanogenic factors protein level and tyrosinase activity. Tyrosinase protein expression was increased by α-MSH compared with control. However, TRP-1, TRP-2 and control expression level was almost equal. α-MSH co-treatment with IFN-γ decreased compared with α-MSH only treatmentin primary human melanocyte (Fig. 4A). Between IL-6 treated sample and control sample was no differences. And tyrosinase activity was also inhibited by IFN-γ in primary human melanocyte (Fig. 4B). These results suggest that IFN-γ also reducesmelanogenesis in primary human melanocyte.

E. IFN-γ suppresses MITF binding ability in B16 melanoma cells

It is well known that MITF plays a role as a transcription factor of tyrosinase (Levy et al., 2006). MITF bind to E-box of tyrosinase promoter and induces tyrosinase transcription. To determine whether IFN-γ affects MITF binding abilityon the promoter of tyrosinase gene, I performed electrophoresis mobility shift assays (EMSA). B16 melanoma cells were treated α-MSH with or without IFN-γ and IL-6 for 3 h. Nucleus extract was incubated with or without [γ-^{32}P]-labeled probe such as E-box oligonucleotidesfor 1h in reaction buffer. MITF binding ability on the E-box, promoter of tyrosinase, was increased byα-MSH. IFN-

γ treatment decreased the α-MSH induced MITF binding ability (Fig. 5). These results indicate that IFN-γ attenuates MITF binding ability induced by α-MSH.

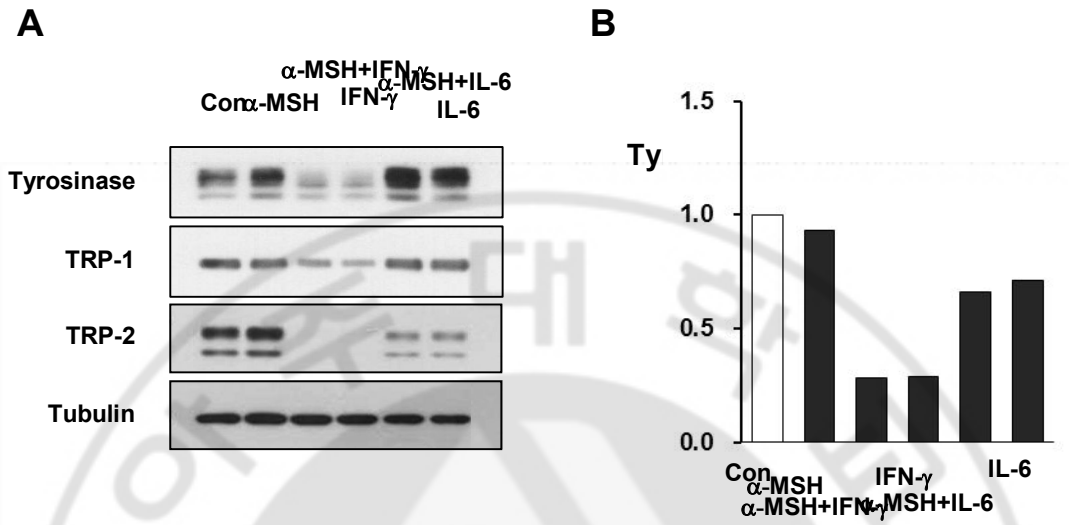


Figure 4. Effect of IFN-γ on melanogenic factors protein and tyrosinase activity in primary human melanocytes.

(A) For measuring protein levels, primary humanmelanocytes were cultured at 24h. α-MSH (10nM/ml) treated with or without IFN-γ (10ng/ml) and IL-6 (10ng/ml) every 2 days until 5 day. Tyrosinase, TRP-1 and TRP-2 protein expressions were determined using western blot. (B) α-MSH treated with or without IFN-γ and IL-6 every 2 days until 5 day, tyrosinase activity was determined using L-DOPA (2mM) and was detected in 490nm.

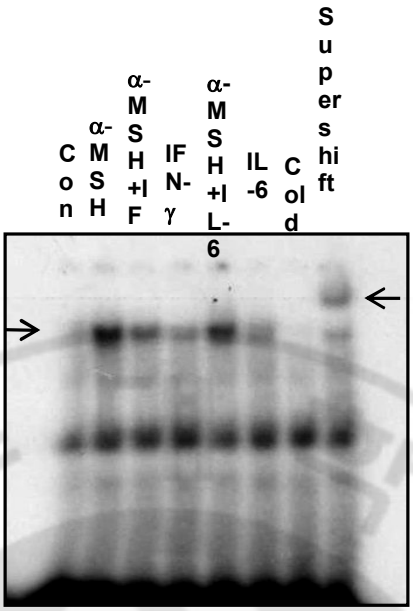


Figure 5. IFN-γ regulates MITF binding activity.

(A) Nuclear extract from α-MSH (10nM/ml), IFN-γ (10ng/ml), IL-6 (10ng/ml) treated cells for 3h and determined by EMSA to binding ability on MITF (E-box) promoter using oligonucleotide probes for MITF.

F. IFN-γ inhibits MITF expression in B16 melanoma cells

To testwhether IFN-γaffects MITF expression,the cells were stimulated with α-MSH and then treated with IFN-γ. The MITF protein expression level was increased by α-MSH treatment at 3 h. At 6 h, MITF does not increased anymore by α-MSH. IFN-γ treatment attenuated the α-MSH induced MITF expression (Fig. 6A). Namely, MITF expression by α-MSH peaked in 3h and that was markedly suppressed in the presence of IFN-γ.This was more clearly shown in real-time PCR (Fig. 6B).

G. IFN-γ does not regulate MITF mRNA stability in B16 melanoma cells

To determine whether IFN-γ affects the MITF mRNA stability, I checked the MITF mRNA decay rate (Fig. 7). I pre-treated α-MSH with or without IFN-γ for 3 h before treatment of actinomycin D, inhibitor of mRNA synthesis. The total RNA were isolated and detected by real-time PCR. IFN-γ does not affect the MITF mRNA stability.

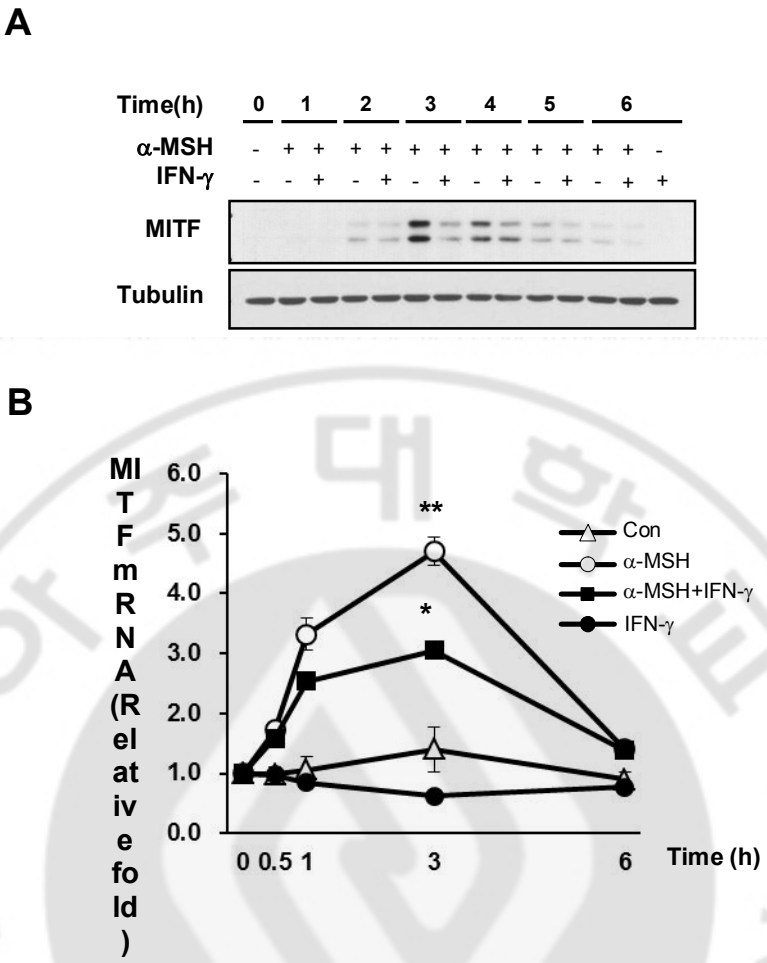


Figure 6. IFN-γ regulates MITF protein and mRNA expression.

(A) After treated α-MSH (10nM/ml) with or without IFN-γ (10ng/ml), IL-6 (10ng/ml) for 48h, cells were determined by western blot using MITF antibody in B16 melanoma cells. (B) After treated α-MSH with or without IFN-γ, total RNA isolated and determined MITF mRNA using real-time PCR analysis in B16 melanoma. n=9. *P<0.01

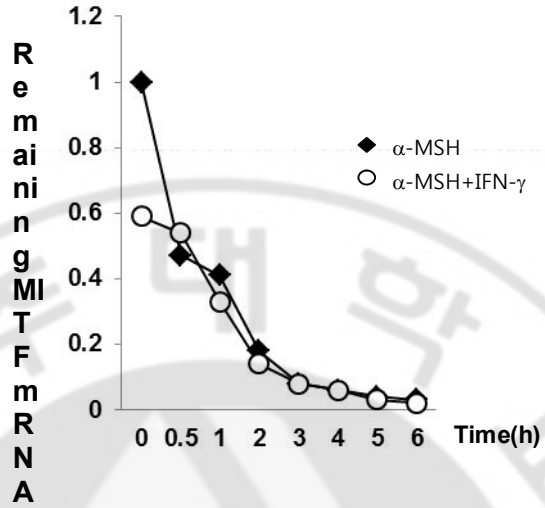


Figure 7. IFN-γdoes not suppress in MITF mRNA stability.

(A) For 3h before co-treatment with α-MSH (10nM/ml) and IFN-γ (10ng/ml), actinomycin D (2μg/ml) was treated in B16 melanoma. Total RNA isolated and determined MITF remaining mRNA using real-time PCR analysis in B16 melanoma. As shown data, IFN-γ does not changed MITF mRNA half-life.

H. CREB binding on promoter of MITF is decreased by IFN-γ treatment

Phosphorylated CREB through cAMP pathwaybinds to MITF promoter site and modulates the transcriptional level (Busca and Ballotti, 2000). To know whether IFN-γ regulates the CREB binding to the MITF promoter, CREB binding ability on promoter of MITF was investigated using EMSA.Cells are treated α-MSH with or without IFN-γ and IL-6 for 30 min. Nucleus extract was incubated for 1h in reaction buffer with or without [γ-^{32}P]-labeled probe such as CREB consensus oligonucleotides. As shown in figure 8, CREB binding abilityon the promoter of MITF was increased by α-MSH. However IFN-γtreatment reduced the binding activity induced by α-MSH. These results demonstrate that IFN-γaffectsCREB binding ability to the MITF.

I. JAK-STAT signaling may be involved in the IFN-γ induced inhibitor of melanogemesis

STAT1 is a representative transcription factor which is induced by IFN-γ (Schindler and Plumlee, 2008). STAT1 is induced by phosphorylation of JAK protein through IFN- γ. JAK inhibitor treatment reversed the IFN-γ effect on α-MSH induced tyrosinase expression compared with non-treated cells (Fig. 9A). STAT1 expression by IFN-γ treatment was induced. Also CREB phosphorylation more induced by IFN-γ treatment compared with α-MSH treatment. These results suggest that JAK-STAT1 signaling may play a role in the IFN-

γ effect on α-MSH induced melanogenesis. Recently, STAT1 not only well known bind to CBP but also negative regulate to several gene through binding of CBP (Ma et al., 2005). CBP is major transcription co-factor of CREB. So I determined whether STAT1 bind to CBP through competition to the CREB, I performed interaction test to STAT1 and CBP or CBP and CREB by proximity ligation assay (PLA) in B16 melanoma cells and primary human melanocytes. As shown figure 9B, STAT1 and CBP biding ability was increased by IFN-γtreatment in B16 melanoma cells. Also CREB and CBP interaction was decreased by IFN-γ treatment. Also figure 9 D showed same results as for IFN-γ negative regulate in primary human melanocytes. These results suggest that IFN-γ decreased melanogenesis by STAT1 and separate between CREB and CBP by doing that STAT1 interact with CBP.

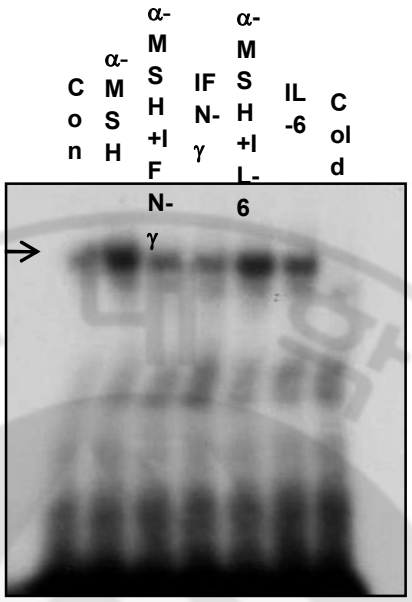
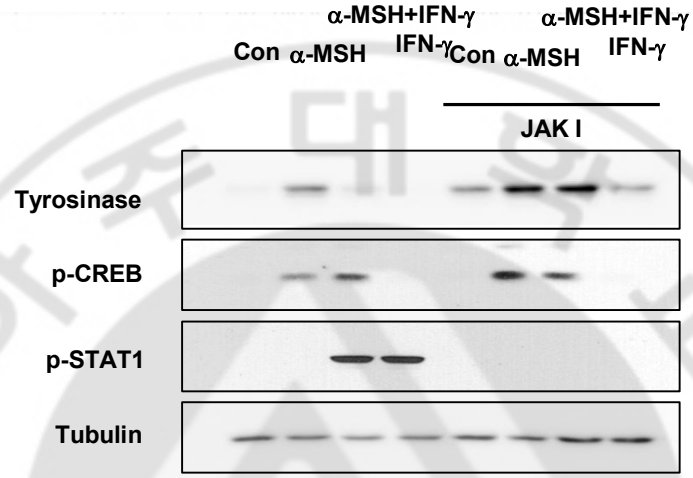


Figure 8. IFN-γ suppresses interaction between CREB and MITF promoter in B16 melanoma cells.

(A) Nuclear extracts from α-MSH (10nM/ml), IFN-γ (10ng/ml) and IL-6 (10ng/ml) treated cells for 30min were determined by EMSA to binding ability on CREB promoter using CREB consensus oligonucleotide probes.

A


α-MSH+IFN-γ
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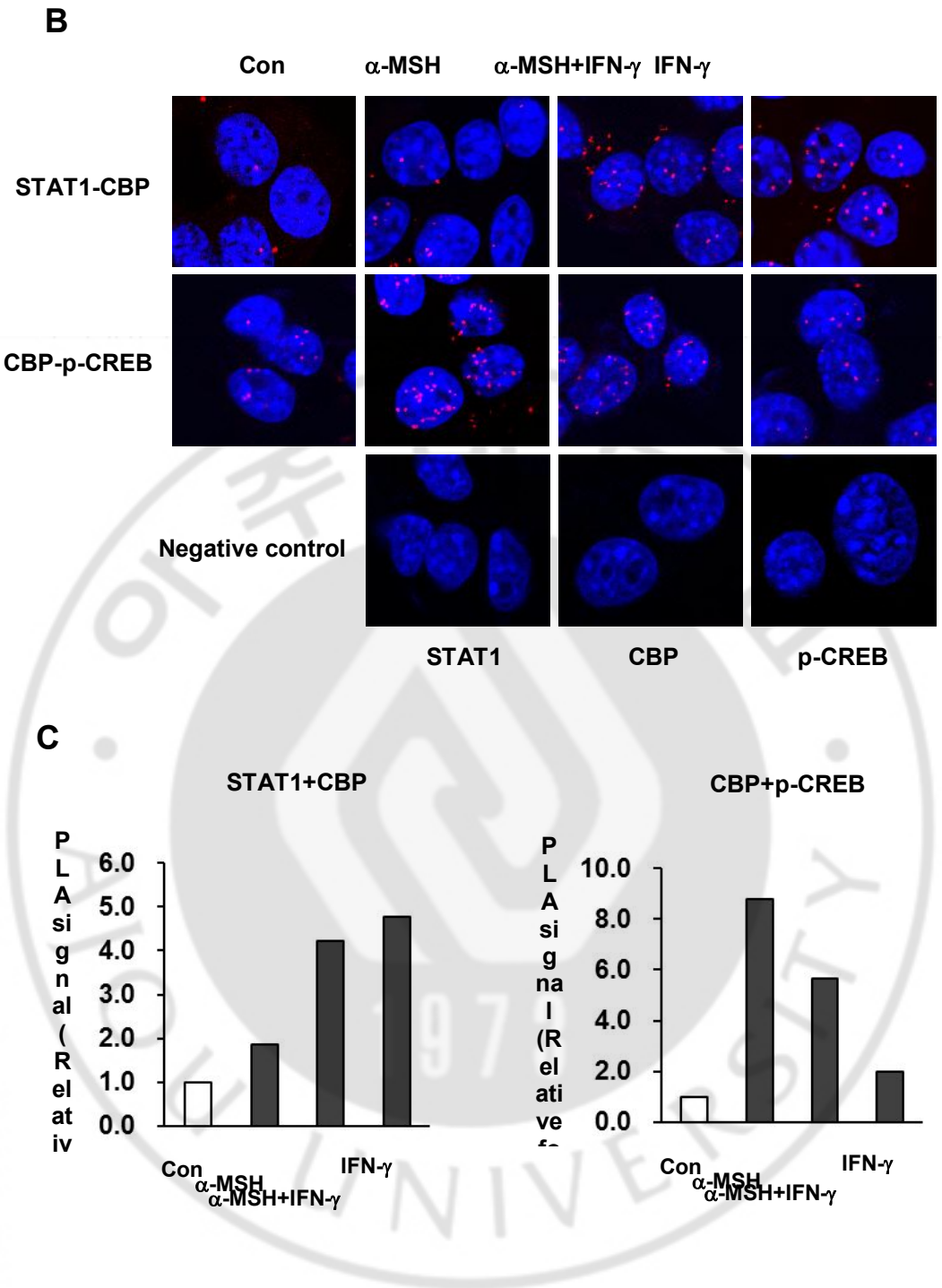
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α-MSH+IFN-γ
IFN-γ

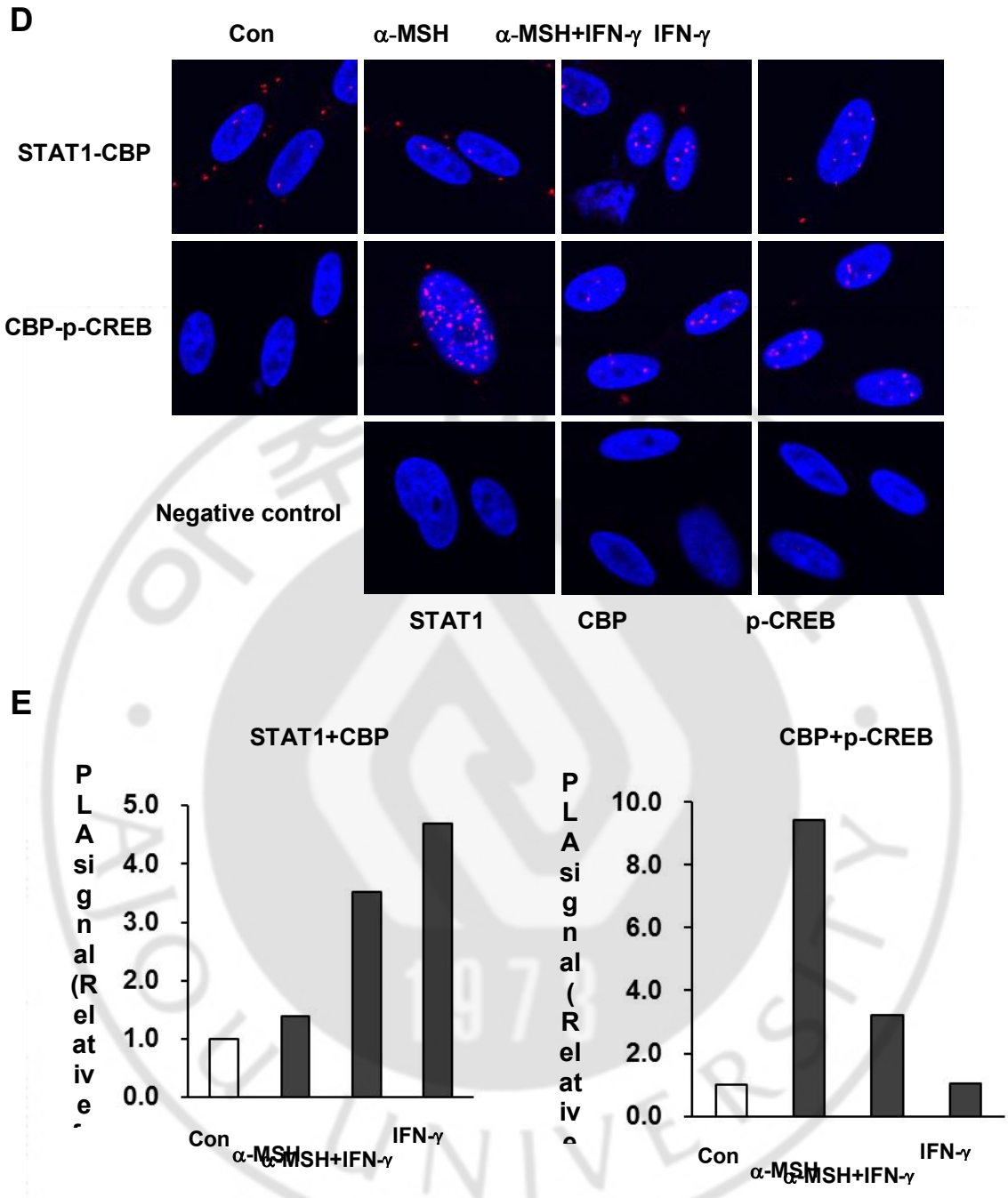
D
Con
α-MSH
α-MSH+IFN-γ
IFN-γ
STAT1-CBP
CBP-p-CREB
Negative control
STAT1
CBP
p-CREB
E
STAT1+CBP
PL Signal (Relative
5.0
4.0
3.0
2.0
1.0
0.0
Con
α-MSH
α-MSH+IFN-γ
IFN-γ
CBP+p-CREB
PL Signal (
10.0
8.0
6.0
4.0
2.0
0.0
Con
α-MSH
α-MSH+IFN-γ
IFN-γ

Figure 9. IFN-γ inhibits melanogenesis by association of STAT1 and CBP.

(A) For 1h before co-treatment with α-MSH (10nM/ml) and IFN-γ (10ng/ml), JAK inhibitor (10μM/ml) was treated in B16 melanoma cells. Total protein isolated and determined tyrosinase protein using western blot.(B)(D) For measuring PLA, B16 melanoma cells and primary human melanocytes were cultured at 24h in a 24well plate and α-MSH (10nM/ml) treated with or without IFN-γ (10ng/ml) and IL-6 (10ng/ml) for 30min. Double recognition PLA detected STAT1 and CBP antibodies, CBP and CREB antibodies in nucleus. STAT1 and CBP, CBP and p-CREB of interaction were determined using PLA signal. Red spot shows interaction between two protein using antibody and PLAprobes, respectively. DAPI shows nuclear stain (Blue). (C)(E) The number of PLA signals per cell of nucleus was quantified.

Ⅳ. DISCUSSION

1.Environment ofskin is closely related to mealanogenesis.

Melanocyte exists in stratum basal of epidermis having only 1 % of the whole cells of skin. The rest of skin cells are covered with keratinocytes and fibroblasts. Keratinocytes and fibroblasts are the cell types located in the outmost layer of epidermis. Therefore, they are constantly affected by diverse stimuli existed in outside of our body. It has been shown that the environment of skin was closely related to melanogenesis(Hearing, 2000; Yamaguchi and Hearing, 2009). One of the most well-known mechanisms is the melanogenesis by secreted molecules from keratinocytes. α-MSH released from keratinocyte binds to MC1R in melanocyte membrane and enhances mealanogenesis through cAMP and CREB activation. CREB is representative transcription factor of MITF and increases mealanogenesis through tyrosinase expression. ET-1, keratinocyte derived factor, binds to endothelin receptor B (ETBR) in melanocyte membrane. ET-1 induces proliferation and differentiation of melanoblast and melanocyte(Hirobe, 2005). Beside keratinocytes, other cell types in skin also secrete molecules having a potential of modulating melanogenesis. DKK1 and TGF-β, are released from fibroblast. DKK1 down-regulates GSK-3β phosphorylation and β-catenin activity by inhibiting Wnt signaling and MITF transcription, respectively.DKK1 also inhibits melanin uptake and increases thickness of keratinocyte. TGF-β down-regulates expression and activity of tyrosinase as well as TRP-1, but not TRP-2.(Martinez-Esparza et al., 1997; Yamaguchi et al., 2008).

Inflammatory cells located in skin also influence to mealanogenesis by releasing inflammatory mediators and cytokines. COX-2 can be produced in inflammatory cells by UV-irradiation as keratinocyte. COX-2 increases the expression and activity of tyrosinase, TRP-1, TRP-2 and MITF (Kim et al., 2012). IL-6 and IL-10 has been known as cytokines having inhibitory potential to mealanogenesis (Swope et al., 1991). IL-6 decreases MITF by inhibiting mRNA expression and by reducing protein stability. In detail, IL-6 inhibits Pax3 binding on MITF promoter(Galibert et al., 1999). IL-1, especially IL-1b, decreases mRNA expression of MITF, tyrosinase, gp100 and MLANA(Kholmanskikh et al., 2010). In this study, I tested other types of inflammatory cytokines,IFN-γ, and analyzed their roles in melanogenesis with IL-6.

2. IFN-γ focused on an autoimmune disease, vitiligo.

Vitiligo, a depigmentation disorder, occurred 1 to 2% of the general population. The lesions appear segmental type and non-segmental type(Huggins et al., 2005). Although the causative factors are largely unknown, it has been suggested that autoimmunity, genetic factors, oxidative stress, or viral infection is related with its occurrence. Recently, a line of evidence strongly suggests the importance of autoimmunity in vitiligo. Vitiligo shares common genetic etiologic links with other autoimmune disorders such as autoimmune thyroid disease and inflammatory bowel disease (Alkhateeb et al., 2003). In cancer animal models, vitiligo was presented along with the boundary of CD8 positive T cells activated

sites (Lengagne et al., 2004). Furthermore, T cells enhanced melanocyte antigen recognition and infiltration into the lesion of vitiligoin patient. Infiltrated T cells induced apoptosis of melanocyte as well as keratinocyte(van den Boorn et al., 2009). The activated T-cells secrete a number of inflammatory cytokines, including interferons (IFN). There are two types of IFNs, type 1 (IFN-α or IFN-β) and type 2 (IFN-γ). The correlation of IFNs with vitiligo, representative depigmentation disorder, has been reported. Injection of type 1 IFNs for the therapy of chronic hepatitis C and multiple sclerosis induced vitiligo at the local injected sites (Anbar et al., 2008; Kocer et al., 2009).However, the effect of type 1 IFNs was not influenced in mealanogenesis; no effect on the level of MITF mRNA(Kholmanskikh et al., 2010). UVB irradiation, the most common environmental factor, induced secretion of IFN-γin keratinocyte(Yoshizumi et al., 2008; Zaidi et al., 2011). IFN-γ was highly expressed in lesion of vitiligo patients (Harris et al., 2012).

In this study, I determined that IFN-γstrongly inhibits melanogenesis by reducing the effects of α-MSH on the expression of tyrosinase, TRP-1 and TRP-2 in melanocytes (Fig. 1). It suggests thatT cells may causevitiligo not only by death of melanocytes, but also by secretion of IFN-γ through inhibiting melanogenesis itself in melanocytes.

3. The mechanism of the inhibition of MITF mRNA by IFN-γ

I showed that IFN-γ reduced MITF mRNA inhibiting tyrosinase expression (Fig. 6B).To reveal the mechanism of IFN-γ mediated reduction of MITF mRNA, I analyzed stability of

MITF mRNA, but no effect on the MITF mRNA stability (Fig. 7). Subsequently, I checked MITF promoter activity using a luciferase reporter of human MITF promoter. However, the control level of the construct was too high, and there was no induction by α-MSH treatment.To analyze the effects of MITF transcription, I used another biochemical analysis, EMSA analysis using CREB binding consensus sequence, a well-known factor bound to MITF promoter site by α-MSH (Busca and Ballotti, 2000). Phosphorylated CREB through cAMP pathwaybinds to MITF promoter site and modulates the transcriptional level (Busca and Ballotti, 2000). I showed that IFN-γ specifically reduced the binding of CREB on the MITF promoter (Fig. 8). The binding of phospho-CREB, the active form of CREB, on a promoter often requires interaction with a cofactor, CBP (Chrivia et al., 1993). CBP stimulates gene expression through its histone acetyltransferase activity and recruiting other transcription factors (Vo and Goodman, 2001).CBP contained TAZ1/CH1 and TAZ2/CH3 domain which bind to transactivation domains (TADs). STAT1 is representative transcription factor, activated by IFN-γ. As STAT1 contains the TADs domain and activated STAT1 binds to CBP through the binding of TAD domain of activated STAT1 with TAZ2 domain of CBP(Wojciak et al., 2009), I analyzed the binding between STAT1, CBP and CREB using Duolink assay in melanocytes.Ifound that IFN-γinduces the interaction of STAT1 and CBP causing an inhibition of CBP and CREB binding (Fig. 9).

4. IFN-γ mediated inhibition of protein synthesis

IFN-γcan inhibit protein synthesis directly through IFN-γ-activated inhibitor of translation (GAIT) complex. The GAIT complex comprises glutamyl-prolyltRNAsynthetase (EPRS), NS1-associated protein 1 (NSAP1), ribosomal protein L13a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). IFN-γ induces phosphorylation of EPRS, which binds to NSAP1 to form a pre-GAIT complex. Several hours later, phosphorylated L-13a released from a large, 60S ribosomal subunit. Released L-13a binds to GAPDH and the L-13a-GAPDH complex combines to pre-GAIT complex to make functional GAIT complex.The GAIT complex attaches to 3 untranslated region of target mRNA and blockstranslation initiation bycircularization of target mRNA resulting from interaction of eIF4G and L-13a of GAIT complex (Mukhopadhyay et al., 2009).Interestingly, I found that IFN-γinhibits tyrosinase protein synthesis using methionine incorporation experiment. Radioactive methionine was added to B16 melanoma cells after treatment of α-MSH with or without IFN-γ. After 30 min, total cell lysates were immunoprecipitated with tyrosinase antibody, and the amounts of newly synthesized tyrosinase protein were analyzed through autoradiography. As a result, newly synthesized tyrosinase was significantly reduced by α-MSH and IFN-γ treatment groups compare to α-MSH group. Therefore, it suggests that inhibition of melanogenesis by IFN-γ may also be due to the effects of IFN-γ on the formation of GAIT complex in melanocytes. Further studies are required to reveal support to the hypothesis.

5. Other inhibiting mechanisms induced by IFN-γ

IFN-γ binds to IFNGR and activates JAK cross-phosphorylation of tyrosine residues. Activated JAK induces STAT1 phosphorylation and transfers STAT1 to nucleus in a form of homo- and heterodimers. Most of activated STAT1 promotes transcription of target genes for the proper inflammatory response. Activated STAT1 also binds to several transcriptional factors such as HDAC and N-myc-interactor(Lee et al., 1999; Nusinzon and Horvath, 2003).Concurrently, suppressors of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) are induced by activated STAT1 for the activation of negative feedback mechanism. In melanogenesis, SOCS and PIAS functions has been suggested on the expression and the activation of MITF(Vachtenheim and Borovansky, 2010). There are eight SOCS family members, SOCS1-7 and cytokine-induced SRC-homology 2 (SH2) protein, CIS. The proteins have a SH2 domain and SOCS-box. SOCS1inhibit JAK phosphorylation and activity through direct interactionwith. SOCS3 binds to cytokine receptor and JAK protein. CIS interacts with cytokine receptors and blocks recruitment of STAT1. Another inhibitory mechanism of SOCS families is through SOCS-boxbound to E3 ubiquitin ligase complex inhibiting STAT1 signaling by formation of ubiquitin complex(Alexander, 2002). Although IFN-α and IFN-γ induced SOCS1 and SOCS3 protein in human melanoma(Lesinski et al., 2010), the function of SOCS protein inmelanogenesisis largely unknown and remained to be clarified.

PIAS family isanother well-known negative regulator of STAT signaling. PIAS1 and PIAS3bind to STAT1 and STAT3, respectively, blocking STATs binding to the target promoter regions in a nucleus(Shuai and Liu, 2005). PIASsnegative regulates

melanogenesis:MITF is inactivated by interaction with PIASsunder resting states. STAT3 activation by IL-6 induces the release of MITF from MITF-PIASs complex for MITF activation. The released MITF binds to E-box on tyrosinase promoter and enhances tyrosinase transcription in primary human melanocyte(Sonnenblick et al., 2004). In this study, I focused on negative regulation of IFN-γ to the melanogenesis. IFN-γ suppresses MITF transcription. Suppression of MITF by IFN-γ was caused by STAT1 interaction with CBP in B16 melanoma, inhibiting CREB complex formation onto CRE element. Therefore, I determined a depigmentation mechanism that IFN-γ induced STAT1 is directly suppresses CREB activity through binding with CBP. This data help to understand the regulation of melanogenesis by activated immune system, especially for IFNs secreted by T-cells, and further provides therapeutic targets for modulating melanogenesis in patients.

Ⅴ. CONCLUSION

1. IFN-γ suppresses protein and mRNA expression of tyrosinase, TRP-1 and TRP-2.
2. IFN-γ suppresses tyrosinase activity and melanin content in B16 melanoma cells and primary human melanocytes.
3. IFN-γ does not affect degradation of tyrosinase protein in B16 melanoma cells.
4. IFN-γ attenuates MITF binding on tyrosinase promoter
5. IFN-γ inhibits MITF protein, mRNA expression.
6. IFN-γ does not affect mRNA stability of MITF in B16 melanoma cells.
7. CREB binding ability on MITF promoter is attenuated by IFN-γ treatment.
8. Negative regulation by IFN-γis induces STAT1 dependent manner and these results confirmed through JAK inhibitor test.
9. IFN-γis inducesthat STAT1 bind to CBP and these results confirmed through PLA (Duolink assay).

These results suggest that IFN-γ decrease melanogenesis through inhibiting the binding of CBP and CREB and blocking MITF and tyrosinase expression.

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- 국문요약 -

배양세포주를이용한인터페론감마의색소형성억제효과및 기전에관한연구

아주대학교의생명과학과

손진영

(지도교수 : 주일로)

멜라닌은 DNA 손상을유도하는 UV 자극으로부터피부를보호한다. 최근염증사이토카인이멜라닌생성억제와연관되어있다고보고된바있지만세부적인연구내용은아직밝혀지지않았다. 본연구자는멜라닌형성에염증사이토카인인인터페론감마가멜라닌색소생성에형성에미치는영향과그기전에관하여알아보았다. B16 악성흑색종세포와사람피부멜라닌형성세포를이용하여α-MSH 에의하여유도된멜라닌형성에주요한효소인tyrosinase, TRP-1, TRP-2 의발현이인터페론 감마에의하여억제되는현상을확인하

였으며tyrosinase의활성과 mRNA 발현이억제되는것을확인하였다. 이는tyrosinase 단백질의분해를통하여일어나는일은아님을밝혔다. 본연구자는인터페론감마가tyrosinase의상위전사인자인 MITF의단백질과 RNA의발현또한억제하는것을확인하였다. RNA의발현억제현상은 RNA의안정성억제와는무관하였다. MITF는전사인자인 CREB에의하여전사가조절됨이알려져있으며본연구자는인터페론감마가 CREB의활성을억제함을밝혔다. 또한인터페론 감마에의하여활성화되는 STAT1이 tyrosinase의발현억제에관여한다는사실을확인하였다. 활성화된 STAT1은 CREB의보조전사인자인 CBP와결합함으로써CBP와CREB과의결합을막아CREB의활성을떨어뜨리는것이밝혀졌다. 본연구자는결과를통하여인터페론감마가 CBP와결합함으로써MITF의전사인자인 CREB의활성을막아멜라닌형성을억제한다는사실을밝혀냈으며본연구는사이토카인에의한멜라닌조절메커니즘을밝히는연구에상당부분기여할수있을것으로기대된다.

핵심어 : Melanogenesis, α-MSH, IFN-γ, Tyrosinase, MITF, STAT1, CBP, CREB.