의학박사 학위논문

TGF-β3 의 멜라닌 생성 억제 효과와 각질형성세포와 섬유아세포의 기능에 미치는 영향에 대한 연구

TGF-β3 inhibits melanogenesis and improves cellular functions of keratinocyte and UV irradiated fibroblast

울산대학교 대학원

의 학 과

문 혜 림

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지도교수 이 미 우

이 논문을 의학박사 학위 논문으로 제출함

2017 년 12 월

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문 혜 림

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2017년 12월

Abstracts

TGF- β3 inhibits melanogenesis and improves cellular functions of keratinocyte and UV irradiated fibroblast

Background and Objectives: Transforming growth factor-β (TGF-β) is multifunctional growth factor with important roles in cell development, differentiation, and apoptosis. The role of TGF-β1 in melanogenesis has been already studied but functional property of TGF-β3 in melanogenesis and other effects on keratinocytes (KC) and fibroblasts (FB) are not fully understood. The present study aims to investigate the effect of TGF- β3 on melanogenesis in melanocytes (MC). In addition, we investigated the influence of TGF-β3 in differentiation of KC and cellular senescence of ultraviolet (UV)-damaged FB.

Materials and methods: Stimulated co-culture of normal human melanocytes (NHM) and KC or FB as well as B16F10 and Mel-Ab cell monoculture, anti-melanogenic property of TGF- β3 was investigated. In UV-treated KC and FB, expression change of proteins related to differentiation and senescence by treatment of TGF- β3 was determined.

Results: TGF- β3 inhibited melanin production and decreased tyrosinase activity in monoculture of mouse cells. As to co-culture of human melanocytes, in stem cell factor (SCF)/endothelin-1 (ET-1) stimulated NHM/KC or in UV stimulated NHM/FB, TGF-β3 effectively inhibited melanogenesis. We then assessed the impact of TGF-β3 on KC and FB. TGF- β3 increased the expression of involucrin, filaggrin, p21 and thymic stromal lymphopoietin (TSLP) in KC. In addition, the UVB-induced p53 expression was downregulated by TGF- β3 treatment in UVB-irradiated FB. As Smad pathways are known to be important players in TGF-β signaling, when we checked, TGF-β3 induced the expression of phosphorylated Smad 2 and 3 in both KC and FB.

Conclusion: Altogether, our findings suggest that TGF-β3 suppress melanogenesis while improving function of KC and photodamaged FB. Topical TGF-β3 may be useful for photoaging associated hyperpigmentation disorders.

Key words: Transforming growth factor; Melanogenesis; Photoaging; Paracrine growth

factor

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Introduction

The melanin pigment is produced in melanocytes (MC), transferred from MC to keratinocytes (KC) by melanosome and distributed in the epidermis[1]. Although melanin plays a crucial role in protecting from ultraviolet (UV) radiations, and reducing skin malignancies[2], excessive melanin production can induce pathologic hyperpigmentary conditions such as melasma and solar lentigo.

Several studies have shown that MC neighboring cells including KC, fibroblasts (FB), and immune cells can affect the MC's behavior via various paracrine factors[3,4]. These factors positively and negatively regulate the melanogenesis, dendricity and proliferation of MC. For example, KC-derived factors such as α-MSH, endothelin-1 (ET-1) and stem cell factor (SCF) are the representative paracrine factors, promoting the proliferation and differentiation of human MC[5-9]. Recently, FB have been shown to act on the functions of MC through various paracrine cytokines, proteins, and growth factors, including Dickkopf 1 (DKK1), secreted frizzle-related protein (sFRP), keratinocyte growth factor (KGF), neuregulin-1 (NGR-1), and basic fibroblast growth factor (bFGF)[10,11]. In addition, inflammatory mediators secreted by immune cells, such as histamines and IFN-γ, are thought to be associated with post-inflammatory hyper- or hypopigmentation[12,13].

Transforming growth factor-β (TGF-β) family is a group of cytokines which play an important role in various cellular functions including embryogenesis, immune response, wound healing, and carcinogenesis. TGF-β is mainly secreted by FB and to a lesser degree KC, and also has an important role in regulating MC[14,15]. Among the three isoforms of TGF-β, TGF-β1 is predominantly expressed in human skin. There have been several studies that TGF-β1 inhibits melanogenesis. TGF-β1 may reduce melanin synthesis by increasing tyrosinase degradation[16]. Another molecular mechanism associated with the TGF-β1 induced depigmentation via delayed extracellular signal-regulated kinase (ERK) activation has been also evaluated [17]. In spite of relatively small amount of TGF-β3 expression, TGF-β3 has been spotlighted as a novel therapeutic target of hypertrophic and keloid scars with its anti-fibrotic properties[18,19]. However, the effect of TGF- β3 on MC has not been fully understood.

In this study, in order to better clarify the role of TGF-β3 in the regulation of the melanin synthesis, we investigated the anti-melanogenic property of TGF-β3 on B16F10 cells and Mel-ab cells. Furthermore, to investigate paracrine effects of TGF-β3 between MC and

neighboring cells, FB and KC, we evaluated the roles of TGF-β3 in melanogenesis of normal human melanocytes (NHM) co-cultured with human dermal fibroblasts (HDF) or normal human keratinocytes (NHK). In addition, we assessed the influence of TGF-β3 in differentiation of KC and cellular senescence of UV-damaged FB, in order to explore the possibility of reversing photoaging phenotype of skin.

Materials and Methods

Materials

SCF was gained from R&D systems (Minnepolis, MN). ET-1 was gained from Sigma-Aldrich Co. (St. Louis, MO). Recombinant human bFGF, recombinant human KGF, recombinant human TGF- β3 were provided by Daewoong Pharmaceutical Company (Seoul, Korea). Antibodies specific for p21, involucrin were gained from genetex (Irvine, CA). Antibodies specific for filaggrin and TRP-1, TRP-2 were gained from abcam (Cambridge, MA). Antibodies specific for phospho-smad2, phospho-smad3 and smad 4 and β-catenin and phospho-β-catenin and phospho-ERK and phospho-AKT, phospho-glycogen synthase kinase 3β (GSK3β) were gained from Cell Signaling Technology (Beverly, MA). Collagen type 1 and melanogenesis associated transcription factor (MITF) were gained from Thermo Fisher Scientific Inc. (Waltham, MA). Antibodies specific for tyrosinase (C-19) was gained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA),

Cell culture

Human epidermal MC (neonatal - moderately pigmented) were cultured Medium 254 supplemented with Human Melanocyte Growth Supplement (HMGS; Cascade Biologics, Invitrogen) at 37 ℃ and 5% CO2. MC used passages between 3 and 7.

Human KC (neonatal) were cultured Epilife supplemented with Human keratinocyte Growth Supplement (HKGS; Cascade Biologics, Invitrogen) at 37 ℃ and 5% CO2. KC used passages between 2 and 5.

HDF from foreskin, were DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (invitrogen) at 37°C in 5% CO2.

Co-culture and treatments

The co-culture model of NHM and NHK was to proceed the method as follow.

NHM were into a 6-well plate at a density of 6×10^4 cells per well. Next day, NHK were added to each well at a density of 3×10^5 cells for the co-culture. Co-cultures of NHM and NHK were cultured in KC media, the seeding ratio of KC to MC being 5:1. After 24 h, stimulation factor (SCF and ET-1) and Growth factor (bFGF, KGF, TGF-β3) were treated

two times. Melanin contents was measured after 5 days.

Secondary, the co-culture model of FB and MC, FB suspended in 1ml collagen type1 matrix were seeded into a 6well plate. Next day, MC were then seeded onto collagen type 1 matrix. Co-culture of FB and NHM were cultured in FB media, the seeding ratio of 4:1. After starvation for 24 h, UV exposed once and next day TGF- β3 was treated. Melanin content was measured after 3 days.

Exposure of cells to UVB radiation

The cells were exposed to twice of TL20W/12RS UV lamps (Philips, Eindhoven, Netherlands). The cells were washed and added in phosphate-buffered saline (PBS) before exposure to UVB radiation. Non-exposed control samples were maintained in the dark under the same conditions. Following exposure to UVB radiation, the cells were grown in fresh medium.

MTT assay

Cell viability was measured using MTT assays. All cells were treated with 0.1 - 0.5 ng/ml of TGF-β3 for 5 days. MTT solution (5 mg/ml) was added with culture medium and incubation for 4 hr. MTT staining was extracted with DMSO. Absorbance was determined using microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Measurement of melanin contents

The cells were dissolved in 1 N NaOH at 100°C for 30 min and centrifuged at 13,000 rpm for 5 min. The optical densities (OD) of the supernatants were measured at an absorbance of 405 nm using a microplate reader. The protein amount of sample was used the Bradford assay (Bio-Rad. Hercules, CA, USA). Melanin was calculated by normalizing with the protein amounts. Cells were treated with various concentrations (0.1 -0.5 ng/ml) of TGF-β3 for 5 days.

Intracellular and cell-free tyrosinase activity assay

Tyrosinase activity was analyzed as described previously. TGF- β3 (0.1 - 0.5 ng/ml) was treated to cells for 5 days and lysed cells were prepared by phosphate buffer (pH 6.8) containing 1% Triton X-100. The protein levels of the lysate were measured. Following

adjustment of the protein concentrations with lysis buffer, lysate was treated with 5 mM L-DOPA. After incubation at 37°C, tyrosinase activity was measured by microplate reader at 475 nm. The direct effects of TGF-β3 on tyrosinase activity was assessed using cell-free mushroom tyrosinase and 5 mM L-DOPA.

Western blot analysis

The protein lysis buffer (Intron, Seongnam, Korea) was used and centrifuged at 13,000 rpm for 10 min. Separation of protein (10 - 20 μg) was handled with SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes. Primary antibody used blots were diluted following each protocol and incubated. Bound antibodies were detected using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Image analysis was perofrmed using Image J software (http://reb.info.nih.ogv/ij/).

ELISA assay

SCF and ET-1 secreted by UVB 20 mJ/cm^2 exposed human FB and NHM co-culture were detected using enzyme-linked immunosorbent assays (ELISA) using Human Magnetic Luminex Screening Assay kit (R&D Systems).

Statistics

The statistical significance of the differences was assessed by analysis of variance (ANOVA), followed by the Student's t-test. P values <0.05 were considered significant.

Results

TGF-β3 decreases the melanin contents in B16F10 cells and Mel-Ab cells

The effects of TGF-β3 on cell viability were determined by MTT assay (Fig. 1A). TGF-β3 had no effect on viability of B16F10 cells at the concentration up to 1000 ng/ml. α-MSH treatment significantly increased melanin contents in B16F10 cells, and TGF-β3 abolished it in a dose dependent manner (Fig. 1B). Moreover, TGF-β3 significantly inhibited tyrosinase activity *in vitro*, and decreased the mRNA expression of tyrosinase in a dose dependent manner (Fig. 1C-D).

In Mel-Ab cells, TGF-β3 had no cytotoxicity at the concentration of 0.1 - 1 ng/ml (Fig. 1E) and significantly decreased the melanin content in a dose dependent manner (Fig. 1F).

In NHM, TGF-β3 reduced melanin contents at the concentration of 0.2 – 0.4 ng/ml without cytotoxicity (Fig. 1H and G).

SCF and ET-1 decrease melanin contents in NHM monoculture, whereas increase melanin contents in NHM co-cultured with NHK

Even though α-MSH is a well-known pro-melanogenic protein, the melanogenic effect of α-MSH in NHM is quite smaller or no significant than in murine melanoma cells, B16F10 cells [20,21]. Previous studies have reported that human MC had relatively unresponsiveness to α-MSH stimulation and showed diverse responses according to different culture conditions. For example, a high concentrate of α-MSH down-regulated the MSH receptor on cultured human MC, rather than increasing melanogenesis.

In contrast with lower vertebrates and many mammals, melanogenesis in human might be affected by numerous cytokines and growth factors derived from environmental tissues via paracrine and autocrine regulation [3]. Previous studies reported that the increased expression of KC-derived ET-1 and SCF effectively activates epidermal MC in the lesional skin of UV induced melanosis and senile lentigo [7,9]. Therefore, we explored whether KC derived paracrine factors influenced the melanogenesis in NHM.

Exposure to SCF (Fig. 2A), and ET-1 (Fig. 2B), the well-known KC-derived paracrine factors, reduced the melanin contents in NHM monoculture. As shown in Fig. 2C, combination of SCF and ET-1 also decreased the melanin contents in NHM.

However, treatment with SCF and ET-1 for 5 days increased the melanin contents in NHK-

NHM co-culture conditions (Fig. 2D). Interestingly, lower doses of SCF (10 ng/ml) and ET-1 (0.1 nM) stimulated the melanin content more than higher doses (50 ng/ml and 1 nM). According to Fig. 2D, we determined adequate doses of SCF and ET-1 that markedly stimulated melanin production in NHM-NHK co-culture model, as the combination of 10 ng/ml SCF and 0.1 nM ET-1.

TGF-β3 decreases the SCF/ET-1 and UVB induced melanogenesis in NHM-NHK co-culture system

To assess the anti-melanogenic effects of various growth factors, NHM-NHK co-cultures were treated with bFGF, KGF, and TGF-β3 in the presence of SCF/ET-1 (10 ng/ml and 0.1 nM) (Fig. 3A). After stimulation with SCF/ET-1, melanin content in bFGF and KGF treatment groups was not found to be significantly decreased. Meanwhile, TGF-β3 significantly abolished SCF/ET-1 induced increase of melanin contents, which were even lower than the level of control group in the absence of SCF/ET-1 stimulation.

To explore whether other growth factors influenced the anti-melanogenic effects of TGF-β3, after SCF/ET-1 stimulation, melanin contents were demonstrated in NHM-NHK co-cultures treated with TGF-β3 combined bFGF or KGF, compared to TGF-β3 mono-treatment. As shown in Fig. 3B, combination treatment with bFGF or KGF resulted in no significant influence of anti-melanogenic effects of TGF-β3.

In NHM-NHK co-culture, SCF/ET-1 treatment significantly increased tyrosinase activity (Fig. 3C). TGF-β3 significantly reduced tyrosinase activity of NHM co-cultured with NHK in the presence of SCF and ET-1. In addition, melanin content was significantly increased in UV-irradiated NHK-NHM co-culture compared to non-irradiated control cells (Fig. 3D). Both pre- and post-treatment with TGF-β3 abolished UV-induced melanogenesis in NHK-NHM co-culture system. On the contrary, in the cell-free system, TGF-β3 mono-treatment increased tyrosinase activity (Fig. 3E).

Anti-melanogenic effects of TGF-β3 were associated with ERK and PI3K/Akt/GSK3β signaling pathway

To address whether TGF-β3 affected melanogenesis related proteins in NHM, we first determined expression levels of MITF, a crucial transcription factor regulating melanogenesis, and tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 by western

blot analysis (Fig. 4A). TGF-β3 treatment was shown to decrease expression of MITF, tyrosinase and TRP-1/2 proteins, indicating that TGF-β3 inhibited melanogenesis via the down-regulation of MITF signaling pathway.

To investigate the action mechanism of antimelanogenic properties of TGF-β3 on NHM, expression levels of β-catenin, phosphor-ERK, phosphor-GSK3β and phosphor-Akt were detected in NHM. As shown in Fig. 4B, TGF-β3 treatment enhanced phosphorylated ERK level in NHM as early as 30 min after treatment and increasing up to 6 hr. Moreover, when we determined the effect of TGF-β3 on PI3K/Akt/GSK3β signaling pathway, phospho-GSK3β and Akt levels were found to be decreased in NHM treated with TGF-β3. Our finding revealed that anti-melanogenic effect of TGF-β3 in NHM is associated with activation of ERK and PI3K/Akt/GSK3β signaling pathway.

TGF-β3 regulates the proliferation, differentiation and inflammation functions of NHK

We next investigated whether TGF-β3 was involved in cellular function of NHK. TGF-β3 had no effect on cell viability of NHK at the lower concentration of 0.1 - 0.2 ng/ml, while 0.3 ng/ml of TGF-β3 slightly affected cell viability of NHK (Fig. 5A).

To assess expression of KC differentiation marker after TGF-β3 treatment, NHK were treated with 0.2 ng/ml of TGF-β3. As shown in Fig. 5B, expression of KC differentiation markers, including involucrin and filaggrin, was significantly upregulated after TGF-β3 treatment (1.6-fold and 7.8-fold, relatively). Moreover, TGF-β3 also induced expression of p21, which plays a crucial role in cell cycle regulation to inhibit proliferation and promote differentiation in NHK. Moreover, TGF-β3 resulted in significantly increases in thymic stromal lymphopoietin (TSLP) (3.9-fold), which is expected to play an important role in regulation of inflammation and antimicrobial properties.

We next investigated the signaling pathways by which TGF-β regulated cellular functions of NHK. Our study determined phosphorylation of Smad 2 and 3 because Smad 2/3 signaling is known as the representative pathway induced by TGF-β activation. Our study revealed TGF-β3 could increase the expression of phosphorylated form of Smad 2 and 3 (Fig. 5C).

TGF-β3 reduces melanin production and reverses cellular senescence in UVB-irradiated HDF-NHM co-culture

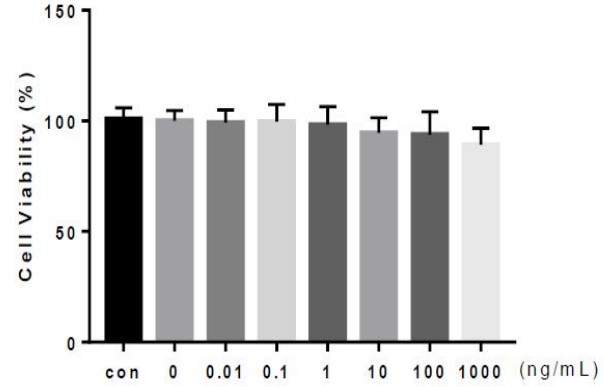
To evaluate the effects of TGF-β3 on photo-damaged skin, co-cultured HDF and NHM were

exposed to a 20 mJ/cm^2 dose of UV irradiation (the maximal dose without cytotoxicity). Melanin content irradiated with UVB was significantly increased in HDF-NHM co-culture compared to non-irradiated control cells (Fig. 6A). Conversely, TGF-β3 treatment could reverse UV-induced melanogenesis in HDF-NHM co-culture system.

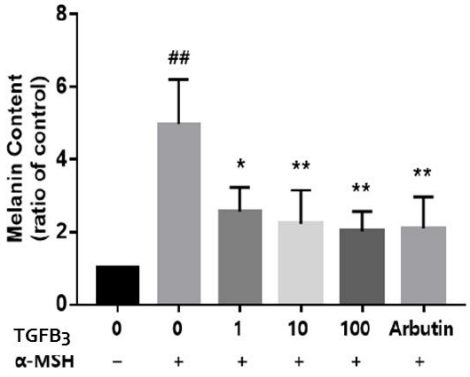
To evaluate whether TGF-β3 influenced cellular senescence of UV-irradiated HDF, we detected the expression of p53, which is a key regulator of cell cycle and expressed in senescence cells. Our results demonstrated that UVB irradiation resulted in a significant increase of p53 in HDF co-cultured with NHM. However, decreased p53 expression was observed in irradiated HDF-NHM co-culture system in response to TGF-β3 treatment (Fig. 6B).

As shown in Fig. 5C, in UV- non-irradiated NDFs, TGF-β3 induced phosphorylation of Smad 2 and 3, which was the most remarkable at 30 and 60 minutes (Fig.6C).

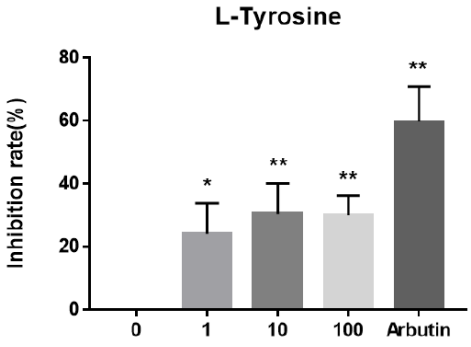
(A)

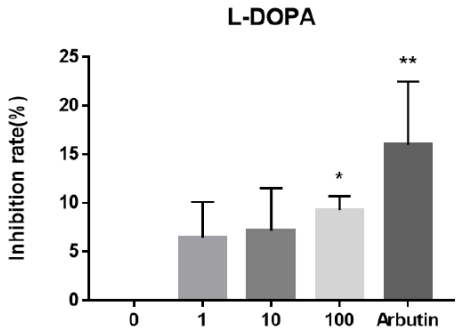

Cell Viability (%)
150
100
50
0
con
0
0.01
0.1
1
10
100
1000
(ng/mL)

(B)

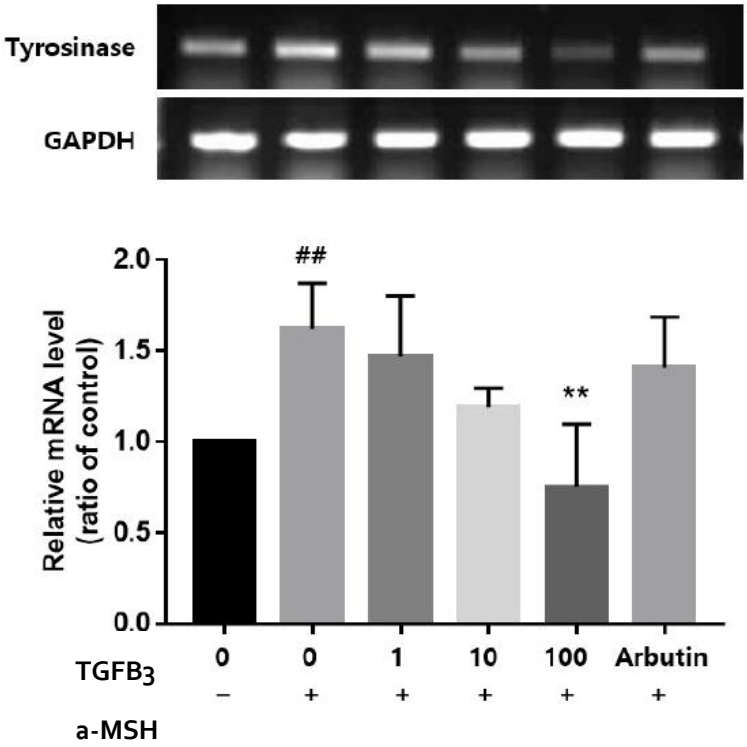

Melanin Content
(ratio of control)
8
6
4
2
0
##
*
**
**
**
TGFB3
α-MSH
0
0
1
10
100
Arbutin
–
+
+
+
+
+

(C)

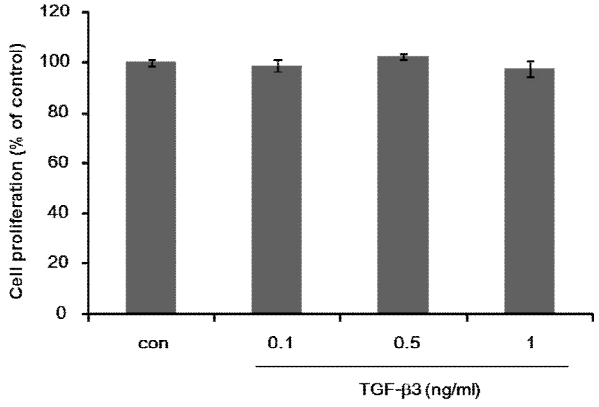

L-Tyrosine
Inhibition rate(%)
80
60
40
20
0
*
**
**
**
0
1
10
100
Arbutin


L-DOPA
Inhibition rate(%)
25
20
15
10
5
0
**
*
0
1
10
100
Arbutin

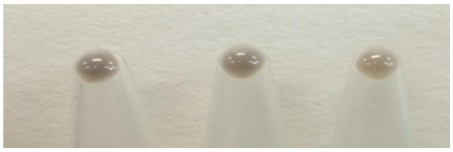
(D)

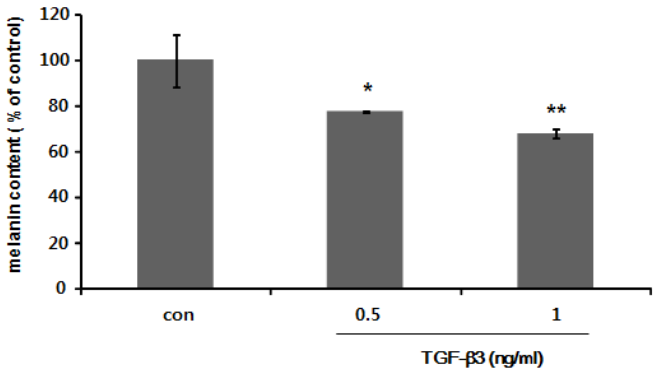

Tyrosinase
GAPDH
Relative mRNA level
(ratio of control)
2.0
1.5
1.0
0.5
0.0
##
**
0
0
1
10
100
Arbutin
TGFB3
–
+
+
+
+
+
a-MSH

(E)

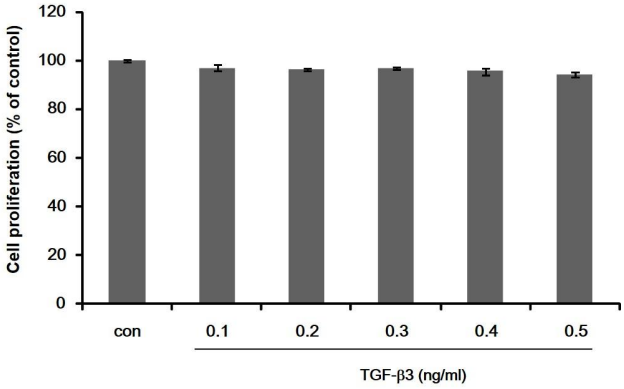

Cell proliferation (% of control)
120
100
80
60
40
20
0
con
0.1
0.5
1
TGF-β3 (ng/ml)

(F)





(G)



(H)

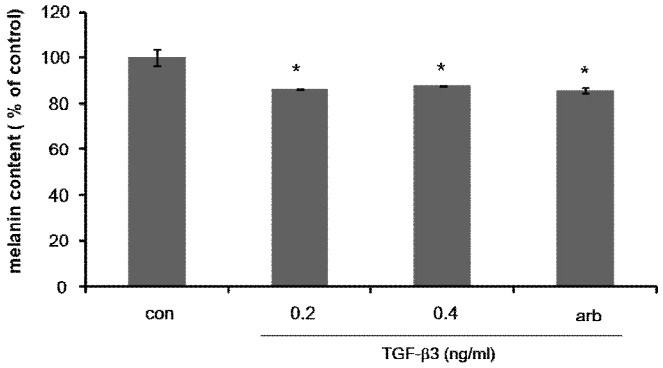
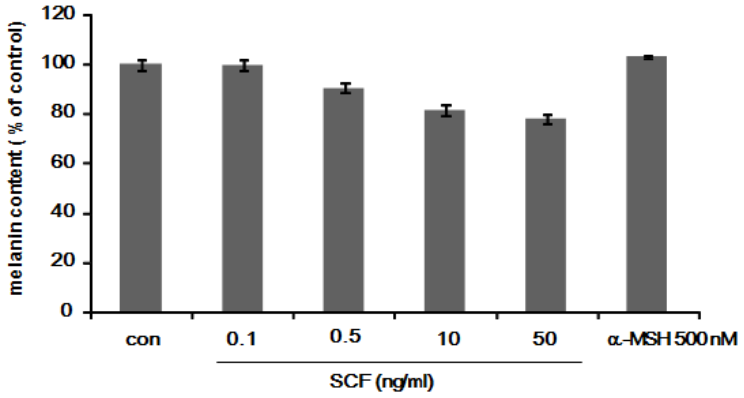


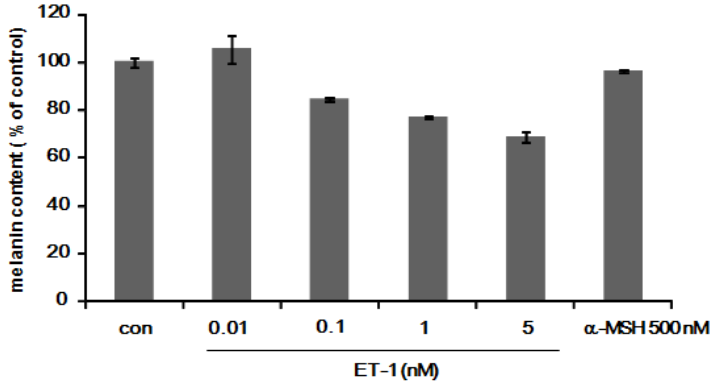
Fig 1. The anti-melanogenic effects of transformation growth factor- β3 (TGF-β3) in B16F10 cells, Mel-Ab cells and normal human melanocytes (NHM). (A) TGF-β3 showed no

cytotoxicity at 0.01 – 1,000 ng/ml in B16F10 cells. (B) After α-MSH stimulation, melanin contents were increased in B16F10 cells, and TGF-β3 abolished it in a dose dependent manner. (C) TGF-β3 significantly inhibited tyrosinase activity *in vitro*, in B16F10 cells. (D) TGF-β3 decreased the mRNA expression of tyrosinase in a dose dependent manner in B16F10 cells. (E) TGF-β3 did not affect cell viability in Mel-Ab cells at 0.1 - 1 ng/ml. (F) TGF-β3 decreased melanin contents of Mel-Ab cells in a dose dependent manner. (G) TGF-β3 showed no cytotoxicity at 0.1 – 0.5 ng/ml in NHM. (H) TGF-β3 decreased melanin contents of NHM in a dose dependent manner.

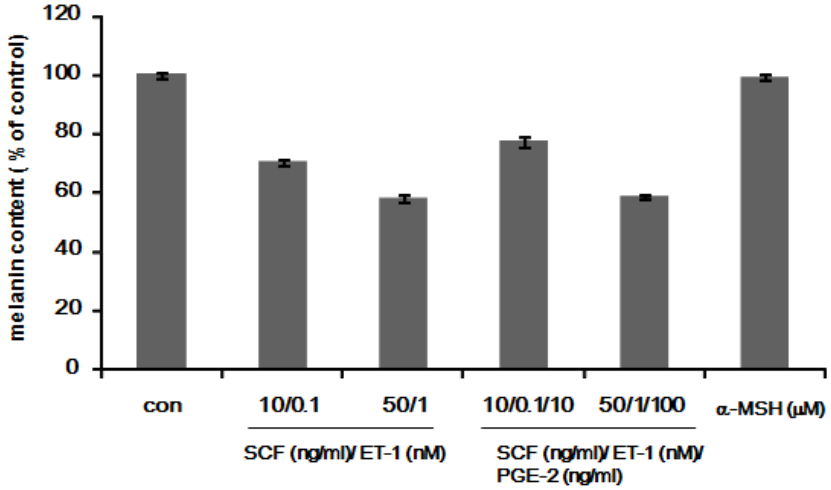
(A)


melanin content (% of control)
120
100
80
60
40
20
0
con
0.1
0.5
10
50
α-MSH 500 nM
SCF (ng/ml)

(B)


melanin content (% of control)
120
100
80
60
40
20
0
con
0.01
0.1
1
5
α-MSH 500 nM
ET-1 (nM)

(C)


melanin content (% of control)
120
100
80
60
40
20
0
con
10/0.1
50/1
10/0.1/10
50/1/100
α-MSH (μM)
SCF (ng/ml)/ ET-1 (nM)
SCF (ng/ml)/ ET-1 (nM)/
PGE-2 (ng/ml)

(D)

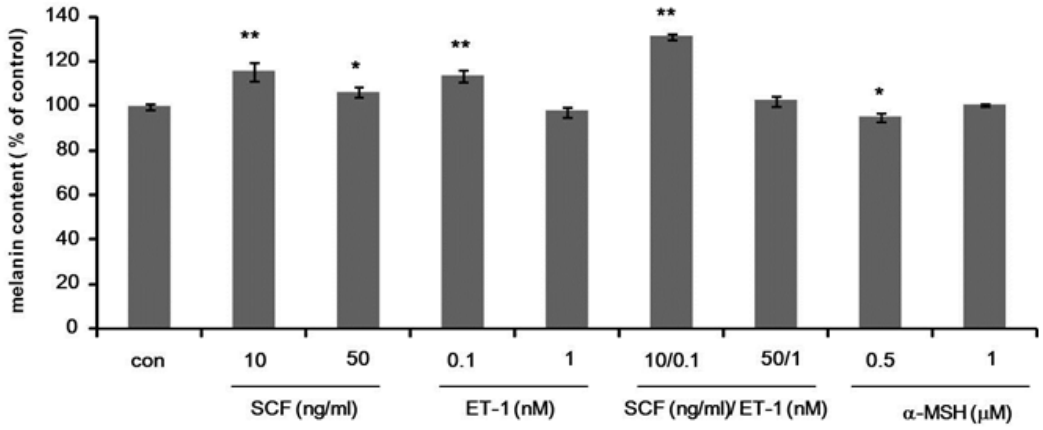
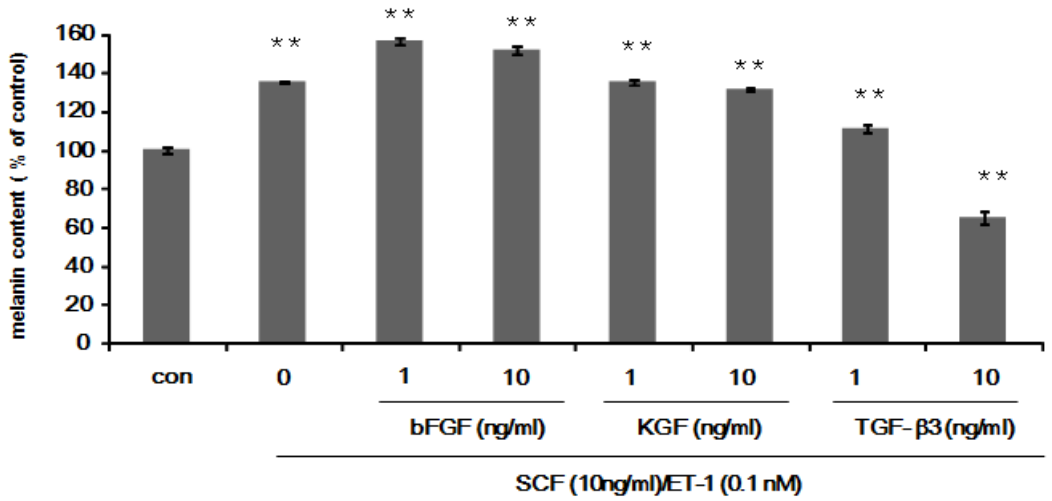
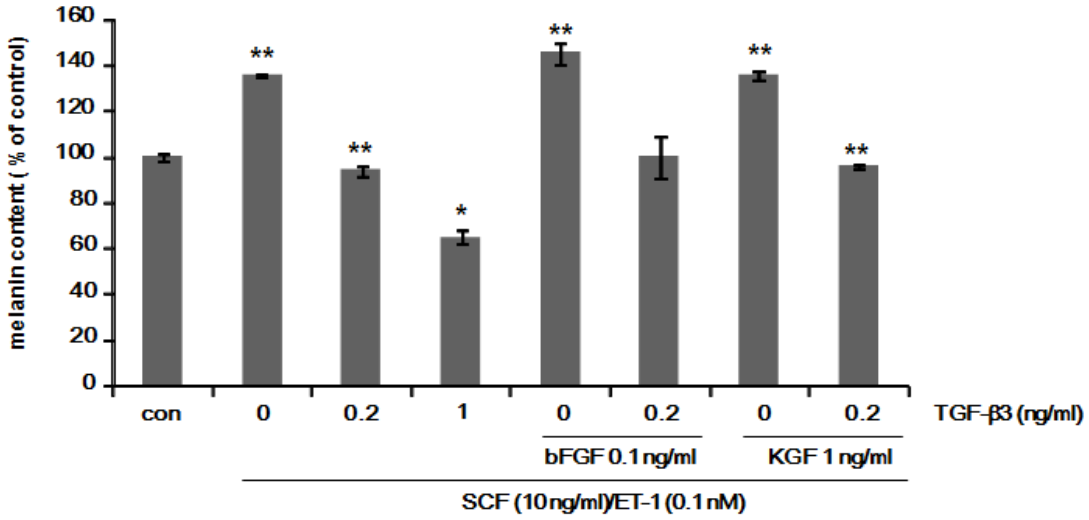


Fig 2. The effects of stem cell factor (SCF) and endothelin-1 (ET-1) on melanogenesis in NHM. Dose dependent effects of SCF (A) and ET-1 (B) on the melanin content in NHM alone. Melanin content were reduced in both groups. (C) Combination treatment with SCF and ET-1 revealed the decrease of melanin content in NHM monoculture. (D) In NHM co-cultured with NHK, melanin contents were significantly increased following 5 days incubation of SCF and ET-1.

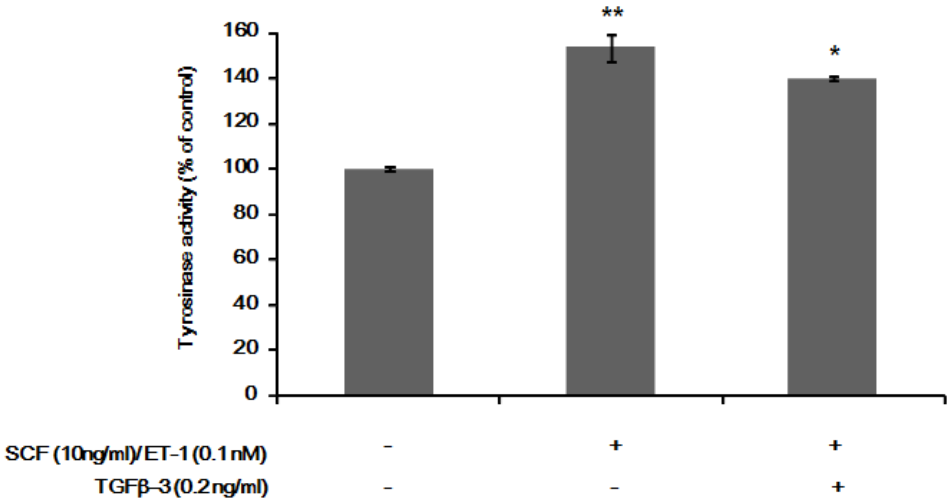
(A)


melanin content (% of control)
160
140
120
100
80
60
40
20
0
**
con
0
1
10
1
10
1
10
bFGF (ng/ml)
KGF (ng/ml)
TGF-β3 (ng/ml)
SCF (10ng/ml)/ET-1 (0.1 nM)

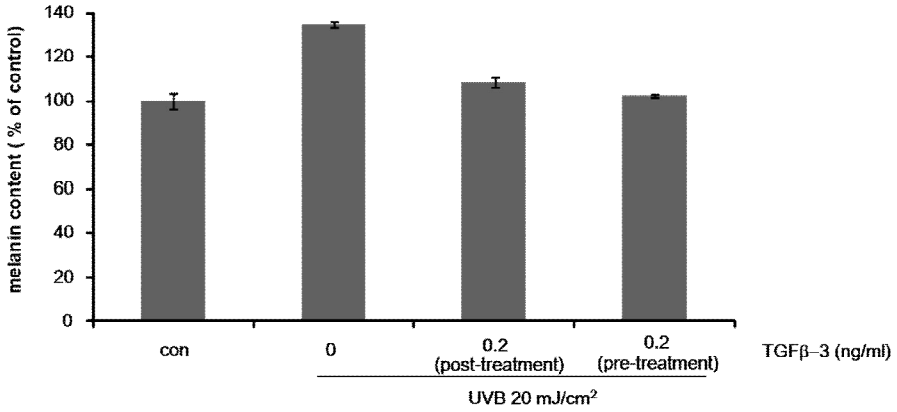
(B)


melanin content (% of control)
160
140
120
100
80
60
40
20
0
**
*
con
0
0.2
1
0
0.2
0
0.2
TGF-β3 (ng/ml)
bFGF 0.1 ng/ml
KGF 1 ng/ml
SCF (10 ng/ml)/ET-1 (0.1 nM)

(C)


Tyrosinase activity (% of control)
160
140
120
100
80
60
40
20
0
**
*
SCF (10ng/ml)/ET-1 (0.1nM)
TGFβ-3 (0.2ng/ml)
-
+
+
-
-
+

(D)


melanin content (% of control)
140
120
100
80
60
40
20
0
con
0
0.2
(post-treatment)
0.2
(pre-treatment)
TGFβ-3 (ng/ml)
UVB 20 mJ/cm²

(E)

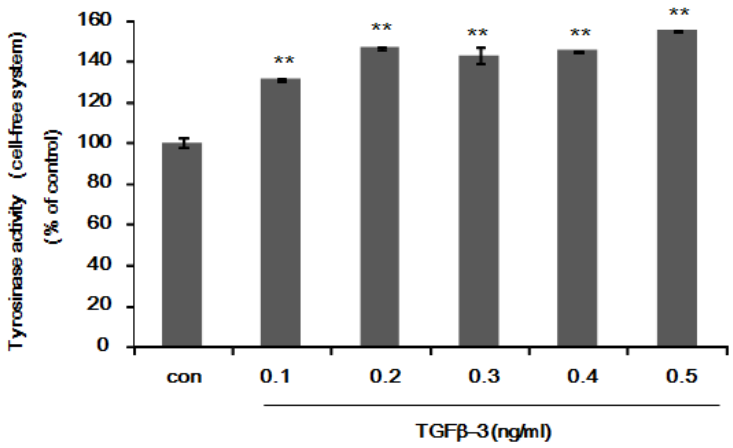
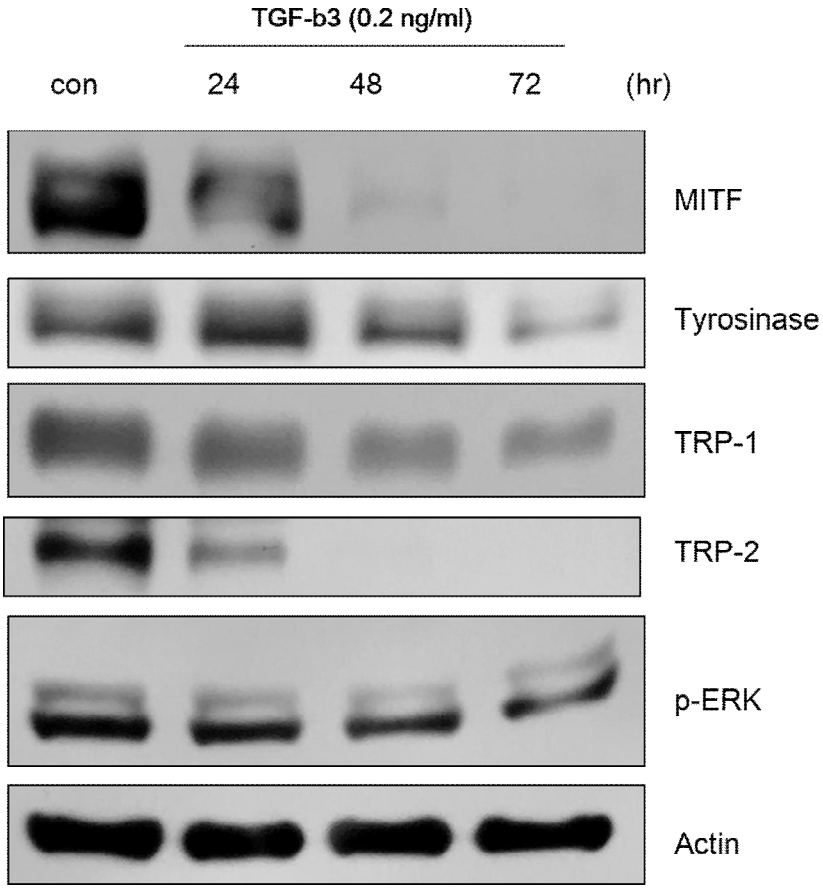

Tyrosinase activity (cell-free system)
(% of control)
160
140
120
100
80
60
40
20
0
**
**
**
**
**
con
0.1
0.2
0.3
0.4
0.5
TGFβ-3 (ng/ml)

Fig 3. The effects of various paracrine growth factors on melanogenesis in NHM co-cultured with NHK. (A) NHM-NHK co-cultures were treated with various growth factors, including basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), and TGF-β3 in the presence of 10 ng/ml SCF and 0.1 nM ET-1. Whereas bFGF and KGF significantly increased melanin content, TGF-β3 abolished SCF/ET-1 induced melanin production. (B) NHM-NHK co-cultures were treated with TGF-β3 combined bFGF or KGF for determining influences of other growth factors on anti-melanogenic properties of TGF-β3. Combination treatment with bFGF or KGF resulted in no significant influence of anti-melanogenic effects of TGF-β3. (C) In NHM-NHK co-culture, SCF/ET-1 treatment significantly increased tyrosinase activity. TGF-β3 could reverse the increases of tyrosinase activity in SCF/ET-1 treated NHM-NHK co-cultures. (D) Melanin content was significantly increased in UV-irradiated NHK-NHM co-culture compared to non-irradiated control cells, whereas both pre- and post-treatment with TGF-β3 reversed UV-induced melanogenesis in NHK-NHM co-culture system. (E) In the cell-free system, TGF-β3 monotreatment increased tyrosinase activity.

(A)


TGF-b3 (0.2 ng/ml)
con
24
48
72
(hr)
MITF
Tyrosinase
TRP-1
TRP-2
p-ERK
Actin

(B)

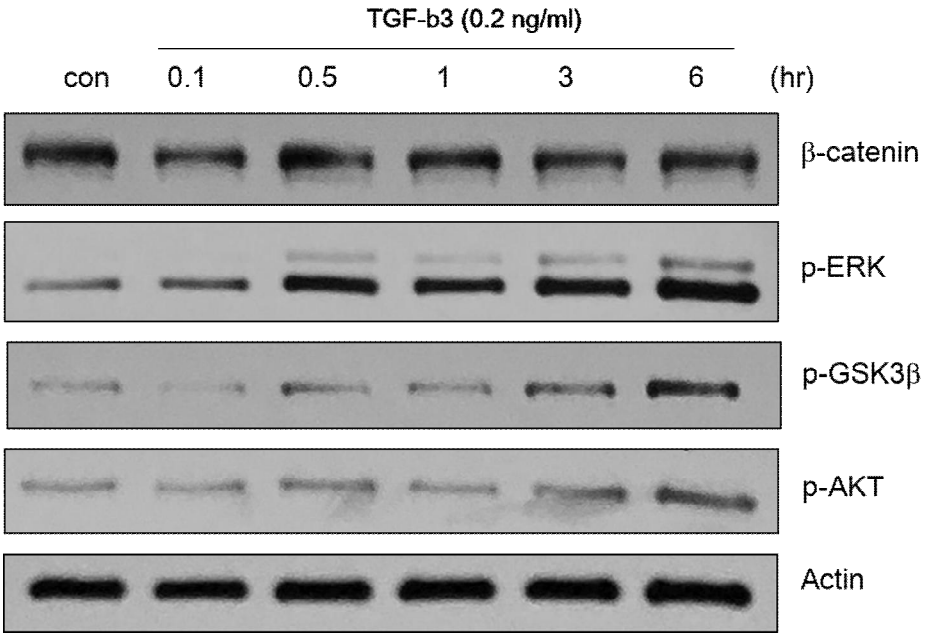
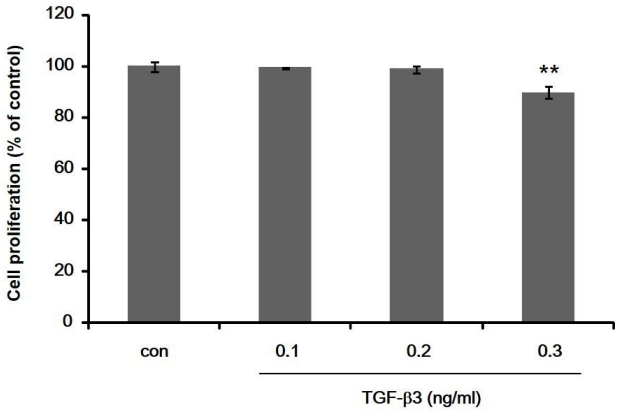
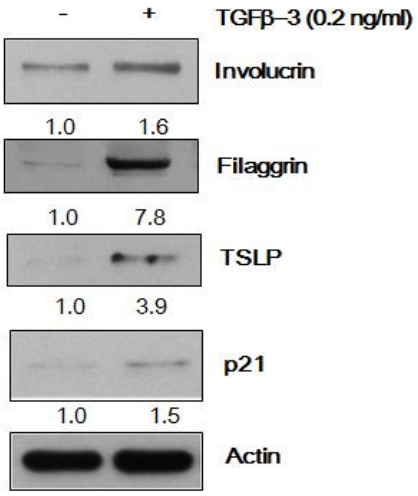

TGF-b3 (0.2 ng/ml)
con
0.1
0.5
1
3
6
(hr)
β-catenin
p-ERK
p-GSK3β
p-AKT
Actin

Fig 4. The effects of TGF-β3 on expression of melanogenesis-related proteins and activation of ERK, GSK3β and Akt in NHM. (A) TGF-β3 down-regulated expression of MITF, tyrosinase and tyrosinase-related proteins (TRP). (B) To determine the signaling pathway involved in the antimelanogenic effect of TGF-β3, the expression levels of β-catenin, phosphor-ERK, phosphor- GSK3β and phosphor-Akt were detected using Western blot analysis. Phosphorylated ERK, GSK3β and Akt were upregulated after TGF-β3 treatment, whereas the expression levels of β-catenin was not changed.

(A)


120
100
80
60
40
20
0
Cell proliferation (% of control)
**
con
0.1
0.2
0.3
TGF-β3 (ng/ml)

(B)


-
+
TGFβ–3 (0.2 ng/ml)
Involucrin
1.0
1.6
Filaggrin
1.0
7.8
TSLP
1.0
3.9
p21
1.0
1.5
Actin

(C)

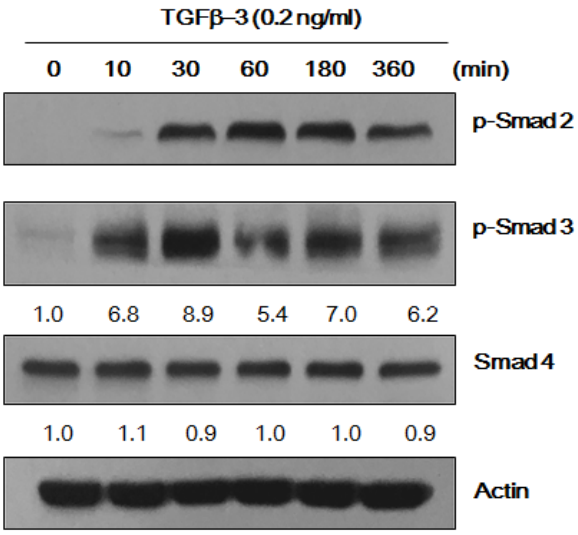
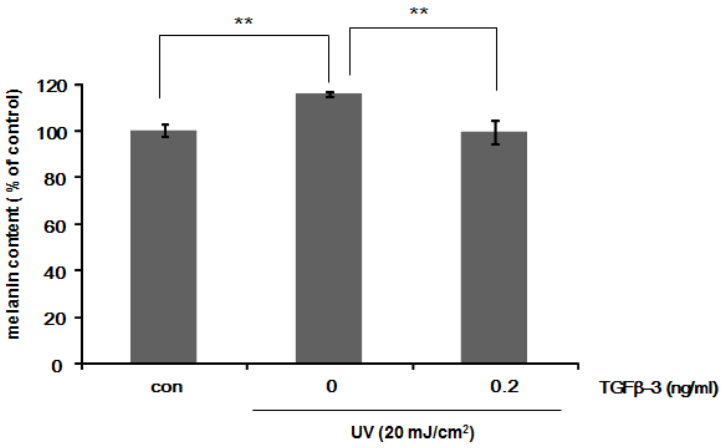
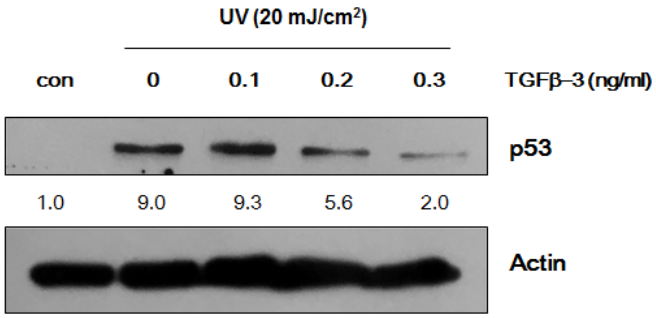


Fig 5. The effects of TGF-β3 on proliferation, differentiation and inflammatory functions of NHK. (A) Cell viability of NHK was not affected at the lower concentration of 0.1 - 0.2 ng/ml TGF-β3. (B) Expression of involucrin, filaggrin, TSLP, and p21 in NHK. TGF-β3 significantly induced expression of involucrin (1.6-fold), filaggrin (7.8-fold), TSLP (3.9-fold), and p21 (1.5-fold). (C) Signaling pathway of TGF-β3 in NHK. Phosphorylation of Smad 2 and 3 were up-regulated in NHK treated with TGF-β3.

(A)


**
**
melanin content (% of control)
120
100
80
60
40
20
0
con
0
0.2
TGFβ-3 (ng/ml)
UV (20 mJ/cm²)

(B)


UV (20 mJ/cm²)
con
0
0.1
0.2
0.3
TGFβ-3 (ng/ml)
p53
1.0
9.0
9.3
5.6
2.0
Actin

(C)

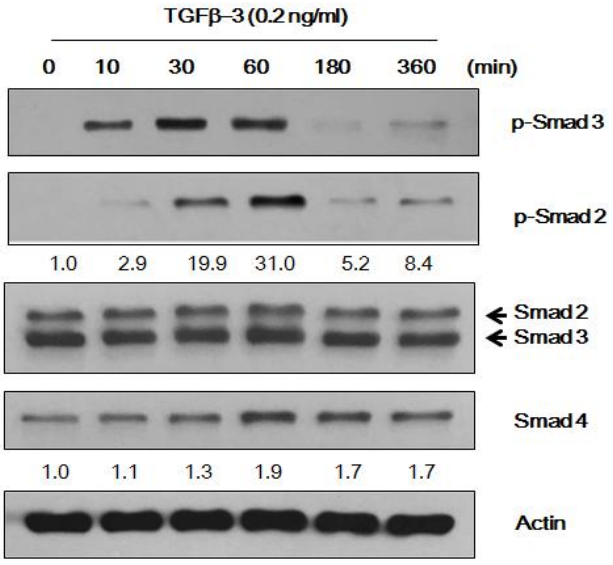

TGFβ-3 (0.2 ng/ml)
0
10
30
60
180
360
(min)
p-Smad3
p-Smad2
1.0
2.9
19.9
31.0
5.2
8.4
Smad2
Smad3
Smad4
1.0
1.1
1.3
1.9
1.7
1.7
Actin

Fig 6. The effects of TGF-β3 on melanogenesis and cellular senescence in UVB-irradiated human dermal fibroblasts (HDF)-NHM co-culture system. (A) The effects of TGF-β3 on UV-induced melanin production in NHM co-cultured with HDF. Melanin content was significantly increased in UV-irradiated HDF-NHM co-culture compared to non-irradiated control cells, whereas TGF-β3 reversed UV-induced melanogenesis in HDF-NHM co-culture system. (B) The effects of TGF-β3 on UV-induced cellular senescence of HDF co-cultured with NHM. TGF-β3 abolished UV-induced expression of p53 in a dose dependent manner. (C) Signaling pathway of TGF-β3 in HDF. TGF-β3 induced phosphorylation of Smad 2 and 3 in HDF.

Discussion

Skin damage induced by UV exposure is a major cause of photoaging and photocarcinogenesis. Cutaneous responses to the UV radiation are quite different between differing ethnic skin types[22]. Although the MC numbers in epidermis between dark and white skin are relatively even, darker complex has larger MC with abundant melanin and diffusely distributed melanosomes in KC[23]. Because of the photoprotective effect of an abundant melanin, Asian populations including Korean tend to more resistant to wrinkles and have lower incidence rate of skin malignancies than white populations[24,25]. Meanwhile, photodamaged skin in Asian populations is relatively associated with pigmentary changes, including melasma, solar lentigo, and motteled pigmentation[22]. Therefore, therapeutic strategies aimed at treatment of pigmentary disorders in photoaged skin need to be a comprehensive approach for MC and its surrounding cells.

Melanogenesis is influenced by numerous paracrine factors derived from neighboring cells, such as epidermal KC and dermal FB[3,10]. The TGF-β, a multifunctional growth factor with important roles in cell development, differentiation, and apoptosis in various cells[14,16,17,26]. In addition to normal cell biology, TGF-β is thought to be associated with cancer development. A recent study showed that the TGF-β could inhibit the growth of melanoma by directly repressing expression of PAX3, which is highly expressed in melanoma cells[27].

In MC, most of previous studies about the influence of TGF-β in MC has been mainly focused on TGF-β1. TGF-β1 may inhibit melanogenesis although the details of the study results are somewhat different according to the previous reports. TGF-β1 has been reported to decrease melanin synthesis via down regulation of MITF expression via delayed ERK activation in immortalized mouse MC cell line, Mel-Ab cells[17]. In addition, TGF-β1 was postulated to be associated with whitening effect of adipose-derived stem cells (ADSC)[28]. In their article, a conditioned medium of ADSCs (ADSC-CM) treatment reduced the melanin synthesis and tyrosinase activity in melanoma B16 cells, and neutralizing antibodies of TGF-β1 abolished the ADSC-CM induced whitening effect. Together, the anti-melanogenic effect of TGF-β1 in human skin has been suggested but not fully clarified.

Among all three isoforms of TGF-β familly, TGF-β1 has a key function in embryogenesis, wound healing, and immunologic regulation. TGF-β1 activation through Smad 2/3 pathway has been well researched in wound healing process. Although TGF-β signaling is essential

for wound healing, prolonged activation of TGF-β1 is related to unwanted scarring and fibrosis. Increased expression of TGF-β1 is associated with the most skin diseases, in which dermal fibrosis is a majority of pathophysiology[26]. In addition, TGF-β1 is predominantly expressed in immune cells, and negatively regulates inflammation[29]. In skin, overexpression of TGF-β1 by keratinocytes initiates skin inflammation and keratinocyte hyperproliferation[30]. In contrast, TGF-β3 has been extensively studied about its anti-fibrotic property and association with fetal scarless wound healing[18]. Moreover, recent studies have reported the regulatory functions of TGF-β3 and its contribution in the adaptive immune system[31]. Therefore, we focused on anti-melanogenic effects and therapeutic potential of TGF-β3, which has been listed in International Cosmetic Ingredient Dictionary (ICID).

Our study found that TGF-β3 had the significant anti-melanogenic effects in multiple types of MC including NHM, B16F10 cells and Mel-Ab cells. Furthermore, TGF-β3 reversed the UVB or SCF/ET-1 induced melanogenesis in the NHM co-cultured with NHK or HDF. Co-culture system is more physiologic to MC experiments as MC behaves closely in association with KC or FB, neiboring cells. These expanded results more confirmed anti-melanogenic properties of TGF-β3.

In order to more mimic real skin milieu of MC, in co-culture system using NHM and NHK, we introduced to use mixture of SCF and ET-1 rather than α-MSH considering the importance of a complex paracrine networks of skin cells beyond the single paracrine mediator and NHM's relative refractoriness to α-MSH[20,21]. Mixture of SCF and ET-1 treatment resulted in quite consistently and significantly increased melanin content. SCF and ET-1 are mostly derived from epidermal KC and may have melanogenic roles in UVB-induced hyperpigmentary lesions, such as melasma and senile lentigo [7,9]. Several potential anti-pigmentary agents inhibited melanogenesis only in the presence of ET-1 and SCF stimulation, whereas did not show significant reduction of melanin in the absence of ET-1 and SCF[32,33]. Furthermore, ET-1 is a downstream of TNF-α/NF-kB pathway and might be associated with post-inflammatory hyperpigmentation[34]. In addition, substance P, a representative neuropeptide in the wound healing process, promotes melanogenesis through ET-1 and ET receptor type B in NHM[35]. These findings altogether indicate that SCF and ET-1 is essential for melanin production in diverse environment stimuli such as sun exposure, inflammation, and neural stress. Interestingly, on the other hand, in NHM monoculture, treatment of ET-1 and SCF resulted in reduction of melanin content possibly by more

increasing cellular proteins, working as growth factors of MC.

Previous studies have reported that UV-irradiated FB induced melanogenic gene expression[36,37]. Salducci *et al*[36] showed that UV-irradiated old FB conditioned media induced main histopathological features of solar lentigo in reconstructed epidermis. Similarly, Duval *et al*[37] proposed that pigmentation of pigmented reconstructed skin model using UV irradiated FB significantly increased compared to that of same model with non-irradiated young FB. Although various FB derived paracrine factors, including DKK1, sFRP, KGF, NGR-1, and bFGF, could be possible candidates for a key player in melanogenesis[11], their exact involvement in photodamaged FB induced hyperpigmentation has not yet been disclosed. That's why we used NHM co-cultured with UV-irradiated FB rather than mixtures of different paracrine factors.

When we confirmed that TGF-β3 has anti-melanogenic effects in multiple types of monoculture of melanin-producing cells and co-cultures adding paracrine stimulation or UVB irradiation, we intended to know whether TGF-β3 may be useful for topical anti-melanogenic agent in real clinical setting such as photodamage-associated hyperpigmentary skin disorders. Then, we evaluated the role of TGF-β3 in regulation of cellular functions in NHK and HDF.

Our study revealed TGF-β3 increased the expression level of involucrin, filaggrin, p21, and TSLP. These results indicate that TGF-β3 might be involved in promoting differentiation and improving skin barrier function in KC. Human TSLP is translated in two forms, the long form TSLP and the short form TSLP, which exhibits potential antimicrobial peptide activity[38]. When evaluating the role of TGF-β3 in HDF, TGF-β3 reversed UVB-induced p53 expression in HDF, indicating protective effects of TGF-β3 against UV-induced cellular senescence of FB. In terms of cellular signaling of KC and FB experiments by treatment of TGF-β, it is well known that activated TGF-β family binds to TGF-β Receptor I and II and forms TGF-βR complex, which subsequently leads to phosphorylation of Smad 2 and 3[39]. Our results also showed that TGF-β3 initiated phosphorylation of Smad 2/3 in NHK and HDF.

Conclusion

Taken together, our results on TGF-β3 have shown (1) that TGF-β3 exhibits potent anti-melanogenic activity in co-cultured NHM with KC or FB as well as in B16F10 cells and Mel-Ab cells; (2) that TGF-β3 reverses melanin production stimulated by UVB irradiation or SCF/ET-1 (UVB-induced melanogenic paracrine factors) in co-culture; (3) that TGF-β3 regulates cellular functions of KC, promoting differentiation and defense; and finally (4) that TGF-β3 has protective effects against UVB-induced cellular senescence of FB.

Because clinically, UV exposure results in hyperpigmentation, skin barrier impair and increased skin laxity, TGF-β3 could provide a comprehensive defensive approach against skin photoaging.

Abbreviations

adipose-derived stem cells (ADSC)

basic fibroblast growth factor (bFGF)

conditioned medium of ADSCs (ADSC-CM)

dickkopf 1 (DKK1)

endothelin-1 (ET-1)

extracellular signal-regulated kinase (ERK)

fibroblasts (FB)

frizzle-related protein (sFRP)

glycogen synthase kinase 3β (GSK3β)

human dermal fibroblasts (HDF)

International Cosmetic Ingredient Dictionary (ICID)

keratinocyte growth factor (KGF)

keratinocytes (KC)

melanocytes (MC)

melanogenesis associated transcription factor (MITF)

neuregulin-1 (NGR-1)

normal human keratinocytes (NHK)

normal human melanocytes (NHM)

stem cell factor (SCF)

thymic stromal lymphopoietin (TSLP)

transforming growth factor-β (TGF-β)

ultraviolet (UV)

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Korean Abstracts

TGF-β3 의 멜라닌 생성 억제 효과와 각질형성세포와 섬유아세포의 기능에 미치는 영향에 대한 연구

연구목적: Transforming growth factor-β (TGF-β)는 세포의 발달, 분화, 자연사 등의 기능에 있어 중요한 역할을 수행하는 성장인자이다. TGF-β1 이 멜라닌세포에 작용하여 멜라닌생성과정에 미치는 영향에 대해서는 이전부터 연구되어 왔으나, TGF-β3 가 멜라닌생성과정에 미치는 영향이나, 멜라닌 세포 주변의 각질형성세포, 섬유아세포에서 어떤 역할을 하는지는 아직 연구되지 않고 있다. 본 연구는 멜라닌세포에서 TGF-β3 가 멜라닌생성과정에 미치는 영향에 대해 조사하였다. 더불어, TGF-β3 가 각질형성세포의 분화과정이나, 섬유아세포의 광노화에 미치는 영향에 대해 알아보고자 하였다.

연구방법: TGF-β3 의 항멜라닌생성 작용을 알아보기 위해, B16F10 세포, Mel-Ab 세포와 더불어, 정상 인체 멜라닌세포를 각질형성세포 또는 섬유아세포와 함께 배양하여 실험하였다. 또한 각질형성세포와 자외선 조사 섬유아세포에서 분화와 세포노화와 관련된 단백질 발현의 변화를 TGF-β3 처리 전 후로 비교분석 하였다.

연구결과: TGF-β3 는 사람멜라닌세포, B16F10 세포, Mel-Ab 세포에서 멜라닌 생성을 감소시키고, tyrosinase 활성을 감소시켰다. SCF 와 ET-1 으로 멜라닌 생성을 자극한 정상 인체 멜라닌세포를 각질형성세포와 함께 배양하였을 때, TGF-β3 는 멜라닌 생성을 유의하게 감소시켰다. 또한 TGF-β3 가 각질형성세포와 섬유아세포에 미치는 영향에 대해 알아보았을 때, TGF-β3 는 각질형성세포의 involucrin, filaggrin, p21 and TSLP 의 발현을 증가시켰으며, 자외선 조사 섬유아세포에서 p53 발현을 효과적으로 감소시켰다. TGF-β3 가 각질형성세포와 섬유아세포에서 작용하는 신호전달경로를 살펴보았을 때, 두 세포 모두에서 TGF-β3 는 Smad 2 와 3 의 인산화를 유도하는 것이 발견되었다.

결론: TGF-β3 는 멜라닌 형성을 효과적으로 감소시키며, 각질형성세포와 광노화된 섬유아세포에 기능을 향상시키는 작용을 하였다. 따라서 TGF-β3 는 향후 광노화와 연관된 과색소 질환의 효과적인 치료 방법으로 사용하는데 유용할 것이다.

중심단어: TGF-β3, 멜라닌억제, 광노화, 과색소질환