의학석사 학위논문

The effects of calcineurin and MEF2A signaling on melanogenesis in human epidermal melanocytes

피부 표피 멜라닌세포에서 Calcineurin 및 MEF2A 신호전달이 멜라닌 합성에 미치는 영향

2020년 2월

서울대학교 대학원

의과학과 의과학 전공

김고은

피부 표피 멜라닌세포에서 Calcineurin 및 MEF2A 신호전달이 멜라닌 합성에 미치는 영향

지도교수 정진호

이 논문을 의학석사 학위논문으로 제출함

2020년 2월

서울대학교 대학원

의과학과 의과학전공

김고은

김고은의 석사 학위논문을 인준함

2020년 2월

위 원 장 권오상 (인)

부 위 원 장 정진호 (인)

위 원 이동훈 (인)

ABSTRACT

The effects of calcineurin and MEF2A signaling on melanogenesis in human epidermal melanocytes

Goeun Kim

Department of Biomedical Sciences

The Graduate School

Seoul National University

Calcineurin (CaN) is a calcium/calmodulin regulated serine/threonine protein phosphatase that has been widely studied in calcium signaling pathways. For the treatment of Vitiligo that is a depigmenting autoimmune disease in skin, calcineurin inhibitors such as cyclosporin A and FK506 are used to prevent the transcription of proinflammatory cytokines. However, there is some controversy about the effect of these inhibitors on melanogenesis and little has been reported the effect of calcineurin itself on melanogenesis in human skin. The downstream targets of calcineurin in calcium pathways have been variously ascribed to members of the NFAT and MEF2 families of transcription factors. Although the role of NFAT in human skin has been reported, the effect of MEF2 remains unknown. Among the MEF2

transcription factor members, MEF2A has been reported to be expressed higher than other members in human epidermal melanocytes.

To investigate the role of calcineurin and MEF2 in melanogenesis, knockdown and overexpression experiments were performed. A decrease in expression of calcineurin by siRNA of calcineurin B subunit gene, PPP3R1, caused a decrease in melanin synthesis and resulted in the downregulation of mRNA and protein expression of tyrosinase which is a crucial enzyme for melanogenesis in human epidermal melanocytes. Likewise, knockdown of MEF2A decreased the melanin synthesis and resulted in the downregulation of mRNA and protein expression of tyrosinase. To complement knockdown experiments of calcineurin and MEF2A, lentiviral overexpression experiments were performed. The overexpression of both calcineurin A subunit and B subunit in human melanocytes didn't change the melanin synthesis as well as tyrosinase, while the overexpression of MEF2A resulted in increase of melanin synthesis. In addition, the mRNA and protein expression of tyrosinase was increased by overexpression of MEF2A. Furthermore, MEF2 luciferase reporter assay was performed to examine whether calcineurin regulates MEF2A transcriptional activity. Calcineurin overexpression enhanced MEF2A transcriptional activity in B16 melanoma cells.

Taken together, these results suggest that calcineurin regulates melanin synthesis and tyrosinase expression through activation of MEF2A pathway. We suggest that MEF2A could be a new candidate gene that regulates skin pigmentation.

Key words

Melanogenesis, melanin, tyrosinase, calcineurin, myocyte enhancer factor-2 (MEF2), calcium pathway, skin pigmentation

Student ID : 2017-20815

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Pigmentation

Melanocytes are melanin producing dendritic cells involved in pigmentation and determine skin, hair and eye color [1]. One melanocyte can contact and distribute melanin to approximately 36 neighboring keratinocytes, which is called the epidermal-melanin unit. Melanin is synthesized in melanosomes, transferred to the dendrite tips, and translocated into keratinocytes forming caps over the keratinocyte nuclei [2]. Skin pigmentation is the most important photoprotective factor, since melanin has antioxidant and radical scavenging properties. Pigmentation differences can arise from variation in the number, size, composition and distribution of melanosomes, whereas melanocyte numbers typically remain relatively constant [3].

Melanogenesis

Melanocytes contain melanosomes, which are subcellular lysosome-like organelles in which melanin pigments are synthesized and stored [4]. There are two types of melanin in mammals, the brownish black eumelanin and the reddish yellow pheomelanin whose synthesis is

dependent on the availability of sulfhydryl compounds in melanosomes [4, 5]. Melanin biosynthesis can be initiated from either the hydroxylation of L-phenylalanine to L-tyrosine (nonobligatory step, operative in vivo) or directly from L-tyrosine (obligatory step both in vitro and in vivo) [6]. Melanogenesis is a complex biochemical event, involving a series of enzymatic and chemical catalyzed reactions. Three enzymes, tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and TRP-2 (also known as dopachrome tautomerase, DCT), are important mediators of melanogenesis. Above all, TYR is exclusively necessary for melanogenesis since it controls at the first step [4, 7].

Regulation of melanogenesis

Upon UV irradiation, human epidermal keratinocytes produce various factors that regulate melanogenesis, including proopiomelanocortin (POMC)-derived peptides such as α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) [8]. Both peptides induce cyclic AMP (cAMP) formation and increase the proliferation and melanogenesis of human melanocytes [9]. Human epidermal keratinocytes also produce another important POMC-derived peptide, β-endorphin [10], which has potent melanogenic, mitogenic, and

dendritogenic effects in human epidermal melanocytes [11]. Moreover, transforming growth factor (TGF)-β is an important keratinocyte-derived cytokine that regulates melanocyte differentiation and inhibit melanin synthesis [12]. On top of that, there are many other important keratinocyte-derived factors such as basic fibroblast growth factor (bFGF), stem cell factor (SCF), endothelins (EDNs), prostaglandins (PGs) and so on [13-16]. Furthermore, neighboring cells such as fibroblasts and immune cells as well as extracellular matrix (ECM) proteins positively or negatively influence melanogenesis by secreting paracrine factors in response to UV irradiation or inflammatory conditions [17].

Besides external factors, internal factors in melanocytes such as cAMP, PKA, β-catenin are also crucial to regulate melanogenesis [18]. Ca^{2+} levels in cytosol of melanocytes also serve as secondary messengers that affect melanogenesis. Changes in cytosolic Ca^{2+} are known to regulate cAMP levels and induce a dose-dependent increase in filopodia formation, melanin transfer, as well as β-catenin levels [19, 20].

Calcineurin

Calcineurin (CaN) is a calcium/calmodulin regulated serine/threonine protein phosphatase that has been widely studied in calcium signaling pathway and a heterodimer of a catalytic A subunit (calcineurin A, CnA) and regulatory B subunit (calcineurin B, CnB) [21]. Full activation of CaN requires Ca^{2+} binding to the CnB and calmodulin binding to CnA is Ca^{2+} dependent [22]. CaN-dependent regulation of gene transcription was originally attributed solely to the activation of the transcription factor nuclear factor of activated T-cells (NFAT) [23]. Subsequently, recent studies suggest that CaN also regulates gene expression by another important downstream transcription factor, myocyte enhancer factor 2 (MEF2) [24].

In human skin, the effects of CaN immunosuppressive drugs like cyclosporin A, tacrolimus (FK506) and pimecrolimus are clinically reported. The topical application of CaN inhibitors has been used as the anti-inflammatory therapy for moderate-to-severe atopic dermatitis (AD) and tacrolimus is reported to be particularly effective at psoriasis [25, 26].

Myocyte enhancer factor 2 (MEF2)

MEF2 belongs to a family of MADS (MCM1, agamous, deficiens, and serum response factor) box transcription factors and plays central roles in cell differentiation, proliferation, morphogenesis, survival and apoptosis of a wide range of cell types [24, 27]. The four vertebrate MEF2 genes (*MEF2a, b, c* and *d*) display distinct, but overlapping, temporal and spatial expression patterns in embryonic and adult tissues with highest expression in striated muscles and brain [28]. MEF2 has been also reported to be expressed in lymphocytes, neural crest, smooth muscle, endothelium and bone [27]. MEF2 proteins bind to DNA as homo- and heterodimers through the consensus MEF2 binding sequence YTA$(A/T)_4$TAR to regulate gene expression [24].

There are conflicting results about the effect of calcineurin inhibitors on human melanocytes. Cyclosporin A does-dependent decreases melanogenesis through decrease both expression and activity of tyrosinase in vitro [29], while FK5056 and pimecrolimus increases pigmentation via facilitating melanin transfer [30, 31]. Thus, the purpose of this study was to investigate the role of calcineurin itself on melanogenesis in human epidermal melanocytes. Furthermore, because

of the lack of research on the presence and role of MEF2A which is a downstream of calcineurin in skin, we examined whether calcineurin also controls the MEF2A transcriptional activity in human epidermal melanocytes and MEF2A affects melanogenesis as well. This study suggests a new role of MEF2A in human skin and suggests a mechanism which the calcium dependent calcineurin can control the melanin synthesis through MEF2A transcription factor in human skin.

MATERIALS AND METHODS

Cell culture

Human Epidermal Melanocytes isolated from moderately pigmented (MP) neonatal foreskin (HEMn-MP cells, HEMn) were purchased from Thermo Fisher Scientific (Waltham, MA). Two different HEMn cells obtained from different donors were used (lot#1913945, lot#1949265) and cultured in Medium 254 (Thermo Fisher Scientific, Waltham, MA) with human melanocyte growth supplement (HMGS) which is an ionically balanced supplement containing bovine pituitary extract (BPE), fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and phorbol 12-myristate 13-acetate (PMA). Cultured HEMn cells were used for the experiments at passages 6-7.

Cloning of lentiviral vectors and lentivirus production

pET15b CnA CnB plasmid was a gift from Anjana Rao (Addgene plasmid # 11787) and pCMV3-MEF2A-Flag including full length clone DNA of human myocyte enhancer factor 2A with C terminal Flag tag was purchased from Sino Biological (cat#HG14869-CF, Wayne, PA). pET15b CnA CnB plasmid and pCMV3-MEF2A plasmid DNA were transformed into DH5α competent cells. After transformation by way

of heat shock protocol, 150μl of LB with non-antibiotics was added to transformed cells and incubated for 1hour at 37℃ for cell recovery and for expression of antibiotic resistance. The transformed cells in LB was spread to antibiotic-selective agar plate and incubated overnight at 37℃. Next day, a single colony was inoculated in LB medium with antibiotics for overnight at 37℃ and then plasmid DNA was obtained by DokDo-Prep Plasmid Mini-Prep Kit (Elpisbio, Daejeon, South Korea). 20ng of plasmid DNA was cloned by specific primers including restriction enzyme sequences (Table 2) using Pfu Plus DNA polymerase (Elpisbio) according to the manufacturer's instruction. After the pCDH-CMV-MCS-EF1-Puro lentivector and synthesized PCR products were digested by appropriate restriction enzymes at 37℃ overnight, digested recipient lentivector was treated with CIP (calf alkaline phosphatase) to prevent self-ligation. Next, control lentivector DNA and target inserted lentivector DNA (pCDH-CnA,CnB or pCDH-MEF2A) were isolated by gel purification and ligated using T4 DNA ligase (Enzynomics, Daejeon, South Korea) overnight at 16℃.

HEK293TN cells were transfected with 9μg of pCDH-CnA,CnB or pCDH-MEF2A, 6.75μg of packaging vector (GAG) and 2.25μg of

envelope vector (VSVG) using Lipofectamine® 3000 reagent (Invitrogen, Thermo) in Opti-MEM reduced serum media (Gibco, Thermo) for 6 hours. After that, media were replaced with DMEM (Gibco, Rockville, MD) containing glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL), and 10% FBS (Gibco). Viral packaging media were first collected after 24 hours and second collected after 52 hours. After filtering the mixed viral packaging media, media were subdivided and stored at −80℃ deep freezer.

Transfection with small interfering RNA (siRNA)

For knockdown of PPP3R1 and MEF2A, HEMn cells were seeded into $2x10^5$ cells per 35mm cell culture dish two days before transfection. HEMn cells were transfected simultaneously with the scrambled negative control siRNA (AccuTarget™ negative control siRNA, Bioneer, Daejeon, South Korea) or a PPP3R1/MEF2A-specific siRNA (Bioneer) at 150mM using Lipofectamine® 2000 Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) in Opti-MEM reduced serum media(Gibco, Thermo). At 6 hours after transfection, media were replaced with Medium 254 and incubated until cell harvest for RNA and protein extraction.

Transduction with lentivirus

HEMn cells were seeded on 35mm dishes two days before and calcineurin or MEF2A inserted lentivirus was treated with polybrene (6μg/ml). The non-inserted lentivirus was used for the control. 8h after the virus treatment, HEMn cells were washed twice with PBS and cultured in Medium 254 with HMGS. Media were replaced with Medium 254 at every two days and incubated until cell harvest for RNA and protein extraction.

Luciferase reporter assay

B16 melanoma cells were seeded into $1x10^5$ cells per 24 well plate dish the day before transfection. MEF2 transcriptional activity was examined by using the plasmid 3XMEF2-Luc which contains MEF2-binding boxes cloned upstream of the firefly luciferase reporter gene. 3XMEF-luciferase vector was a gift from Ron Prywes (Addgene plasmid # 32967). B16 cells were transfected with 300ng of 3XMEF-luc per well together with total 450ng of pCDH-control or pCDH-MEF2A, pCDH-calcineurin A&B using Lipofectamine® 3000 reagent (Invitrogen, Thermo) in Opti-MEM reduced serum media (Gibco, Thermo) for overnight. The pRL-TK (Renilla luciferase) plasmid was used as an internal control for transfection efficiency. After overnight

transfection, the medium was changed into DMEM with 10% FBS and antibiotics. After 24h, the cells were lysed and MEF2-Luc and Renilla luciferase activities were measured using the dual-luciferase kit Assay Reporter System (Promega, Madison, WI, United States) and a luminometer (Perkinelmer, Waltham, MA).

RNA extraction and RT-qPCR

Total RNA was extracted from cultured HEMn cells using RNAiso Plus from Takara Bio (Shiga, Japan). 0.6 μg of total RNA was reverse transcribed into cDNA using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Quantitative real-time PCR was performed on a 7500 Real-time PCR system (Applied Biosystems, Life Technologies, Foster City, CA) using TB green™ Premix Ex Taq™ (Takara Bio, Seoul, South Korea) according to the manufacturer's instruction. The PCR conditions were 50 ℃ for 2 minutes, 95 ℃ for 2 minutes, followed by 40 cycles at 95 ℃ for 15 seconds and 60 ℃ for 1 minute. All of primer sequences are showed at Table 1.

Western blot analysis

HEMn cells were lysed on ice with 1X RIPA lysis buffer (Millipore, Darmstadt, Germany) containing protease/phosphatase inhibitor cocktail (Sigma-Aldrich, ST. Louis, MO). The total cell extract protein concentration was quantified using a bicinchoninic acid solution (BCA) containing cooper(II) sulfate solution (Sigma-Aldrich, ST. Louis, MO). 30μg of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Roche Applied Science, Rockford, IL). The membranes were blocked with 5% skim milk solution and incubated overnight with primary antibodies at 4℃. Membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Immune complexes were detected using ECL reagents (Pico; Thermo, Femto; Biomax, Seoul, South Korea).

Melanin content analysis

HEMn cells were seeded on 35mm dishes two days before transfection and melanin contents were measured at day 8 after the siRNA or lentiviral transfection. Cells were washed twice and harvested in phosphate buffered saline solution (1X PBS). After cell

pellets were obtained by centrifuge and lysed by 1X RIPA buffer (Millipore, Darmstadt, Germany), melanin pellets were obtained by centrifuge once again. For analysis of melanin contents, cell pellets were solubilized in 500μl of 1 N NaOH in 70 ℃ for 1 hours to dissolve melanin, and 150μl of aliquots were transferred to 96-well plates in three repetitions. The absorbance was measured at 405 nm by using a microplate reader. Relative melanin production was calculated by normalizing with protein concentrations (absorbance/μg protein).

WST1 proliferation assay

The same number of HEMn cells were seeded two days before the transfection with calcineurin B siRNA or MEF2A siRNA. A WST1 assay was performed on these cells according to the manufacturer's instructions (DoGEN, Seoul, South Korea). 8 days after the siRNA transfection, the WST1 reagent was added and incubated for a further 2 h before reading the plate. Each assay was conducted in sets of three and the experiment was performed twice. The proliferative index is the fold induction of proliferation of calcineurin B siRNA or MEF2A siRNA transfected cells compared to negative siRNA transfected cells.

Statistical analysis

Statistical analysis was performed using the paired *t*-test by Prism GraphPad Software (San Diego, CA). *P*-value of less than 0.05 was considered statistically significant. Results are presented as mean ± standard error of the mean (SEM).

Table 1. Primer sequences of human genes used for RT-qPCR.

Target of primer	Primer sequences
PPP3R1	Forward : 5′-GAG GGC GTC TCT CAG TTC AG-3′ Reverse : 5′-GCT GGA CGT CTT GAG CAG AT-3′
MEF2A	Forward : 5′-AGC TCC TCA GAG ACC ACC AA-3′ Reverse : 5′-GGA GGG GGA GAC TTT GTA GG-3′
36B4	Forward : 5′-TCG ACA ATG GCA GCA TCT AC-3′ Reverse : 5′-TGA TGC AAC AGT TGG GTA GC-3′
TYR	Forward : 5′-TGC CAA CGA TCC TAT CTT CC-3′ Reverse : 5′-CCA TGT AGG ATT CCC GGT TA-3′
TRP-1	Forward : 5′-GCT CCA GAC AAC CTG GGA TA-3′ Reverse : 5′-TCA GTG AGG AGA GGC TGG TT-3′
MITF-M	Forward : 5′-TCT ACC GTC TCT CAC TGG ATT GG-3′ Reverse : 5′-GCT TTA CCT GCT GCC GTT GG-3′

Table 2. Primer sequences necessary to DNA cloning for lentiviral overexpression.

Vector source	Cloned sequences
pET15b CnA CnB	5′-NheI-His-**CnA**-BamHI-3′
Primer sequences	Forward : 5′-CTA GCT AGC TAG GCC GCC GCG ATC GCC ATG GGC AGC AGC CAT CAT C-3′ Reverse : 5′-CGC GGA TCC GCG TCA CTG AAT ATT GCT GCT ATT A-3′
pET15b CnA CnB	5′-NheI-**CnB**-BamHI-3′
Primer sequences	Forward : 5′-CTA GCT AGC TAG GCC GCC GCG ATC GCC ATG GGA AAT GAG GCA AGT TAT C-3′ Reverse : 5′- CGC GGA TCC GCG TCA CAC ATC TAC CAC CAT CTT TTT G-3′
pCMV3 MEF2A Flag	5′-EcoRI-**MEF2A**-Flag-NotI-ZhoI-3′
Primer sequences	Forward : 5′-CCG GAA TTC GCC GCC GCG ATC GCC ATG GGG CGG AAG AAA ATA C-3′ Reverse : 5′-CTG CTC GAG CGG CCG CTT ACT TAT CGT CGT CAT CCT TGT AAT C-3′

Table 3. Primary antibodies used for western blot analysis.

Target of antibody	Host	Catalog #	Manufacturer
Calcineurin A	Rabbit polyclonal IgG	PA5-17446	Thermo
Calcineurin B	Rabbit polyclonal IgG	154650	Abcam
MEF2A	Mouse monoclonal IgG	sc-17785	Santa Cruz
TYR	Goat polyclonal IgG	sc-7833	Santa Cruz
TRP-1	Rabbit polyclonal IgG	sc-25543	Santa Cruz
MITF	Mouse monoclonal IgG	sc-17588	Santa Cruz
NFAT4 (NFATc3)	Rabbit polyclonal IgG	4498s	Cell signaling
α-tubulin	Mouse monoclonal IgG	sc-23948	Santa Cruz

RESULTS

Knockdown of calcineurin B subunit decreases phosphatase activity of calcineurin and melanin synthesis in human epidermal melanocytes.

Calcineurin is a heterodimer of a catalytic subunit, calcineurin A that is encoded by separate genes (PPP3CA, PPP3CB, and PPP3CC) and a regulatory subunit, calcineurin B that is also encoded by separate genes (PPP3R1, PPP3R2) [21]. Among the genes that encode calcineurin B, PPP3R1 is analyzed as the only expressed gene in whole skin including melanocytes by transcriptome analysis [32]. Thus, we expected that the expression of total calcineurin would be reduced by knockdown of PPP3R1 to inhibit calcineurin B subunit (CnB) in human epidermal melanocytes. Human epidermal melanocytes derived from neonatal foreskin of moderately pigmented donors (HEMn-MP cells, HEMn) were transfected with the negative control siRNA or the PPP3R1(Calcineurin B, CnB) siRNA 150nM for 6 hours. The results showed that the mRNA expression of CnB was significantly reduced at 24 hours (Fig. 1a) and protein expression of CnB was also decreased consistently from day 2 to day 8 after the siRNA transfection (Fig. 1b). To investigate whether the phosphatase activity of calcineurin is

reduced by knockdown, we examined the phosphorylation state of nuclear factor of activated T cell cytoplasmic (NFATc) which is best known as a downstream factor of calcineurin and activated when dephosphorylated by calcineurin [33]. As a result, western blot analysis showed that knockdown of CnB led to an increase in NFATc3 phosphorylation state (Fig. 1b). Thereafter, to confirm the effects of calcineurin on melanogenesis, we measured a melanin content after CnB knockdown. As CnB expression is reduced, a melanin content assay revealed that knockdown of CnB inhibited melanin synthesis in HEMn cells at day 6 and day 8 after the siRNA transfection by an average of 19% and 30% respectively (Fig. 1c). Compared with the control group, relative proliferation level was also increased in the knockdown group of CnB at day 8 (Fig. 1d).

Figur e 1

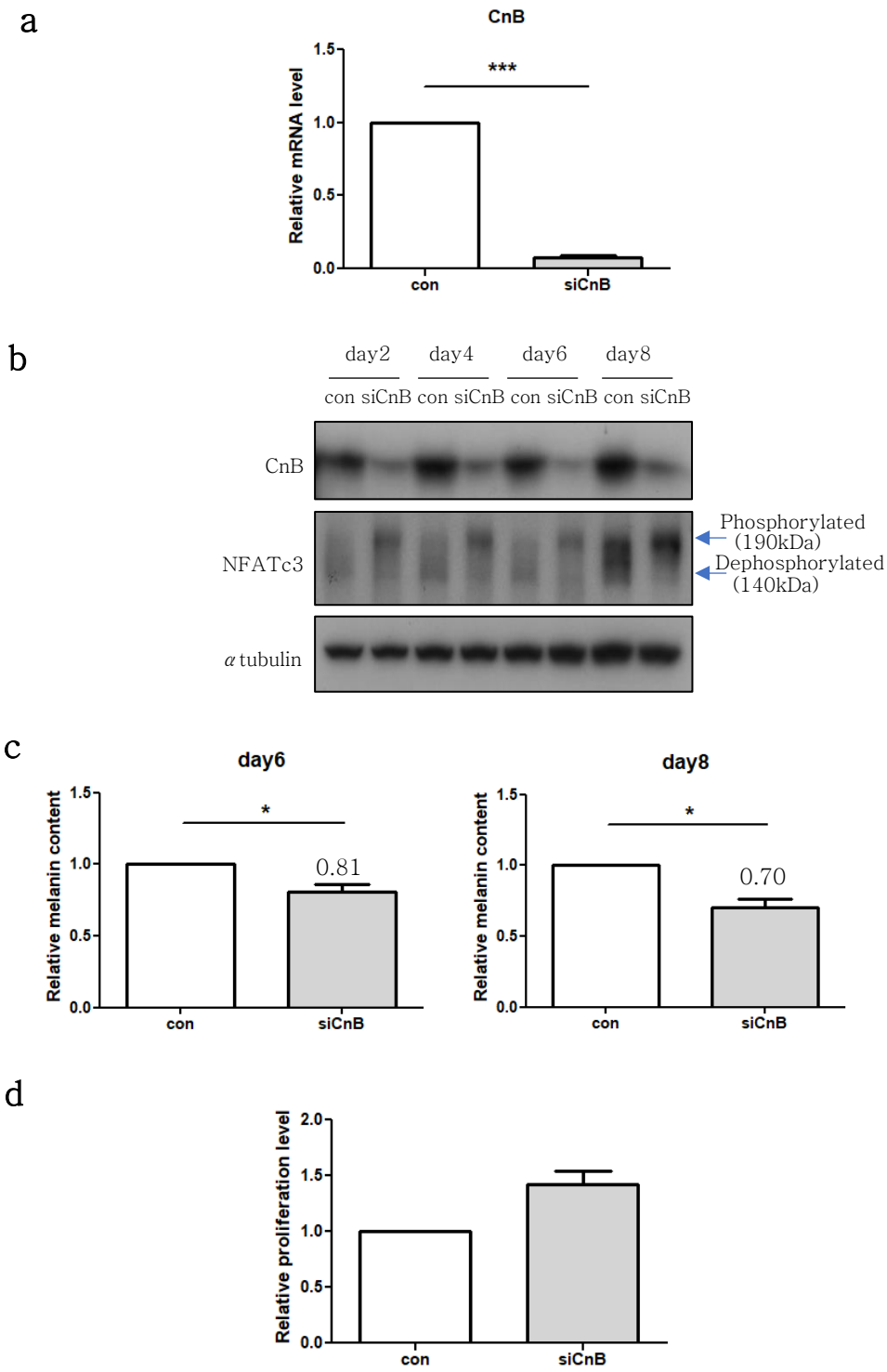

a
CnB
***
Relative mRNA level
1.5
1.0
0.5
0.0
con
siCnB
b
day2
day4
day6
day8
con siCnB con siCnB con siCnB con siCnB
CnB
NFATc3
α tubulin
Phosphorylated
(190kDa)
Dephosphorylated
(140kDa)
c
day6
*
0.81
Relative melanin content
1.5
1.0
0.5
0.0
con
siCnB
day8
*
0.70
Relative melanin content
1.5
1.0
0.5
0.0
con
siCnB
d
Relative proliferation level
2.0
1.5
1.0
0.5
0.0
con
siCnB

Figure 1. Knockdown of calcineurin B subunit decreases phosphatase activity of calcineurin and melanin synthesis in human epidermal melanocytes.

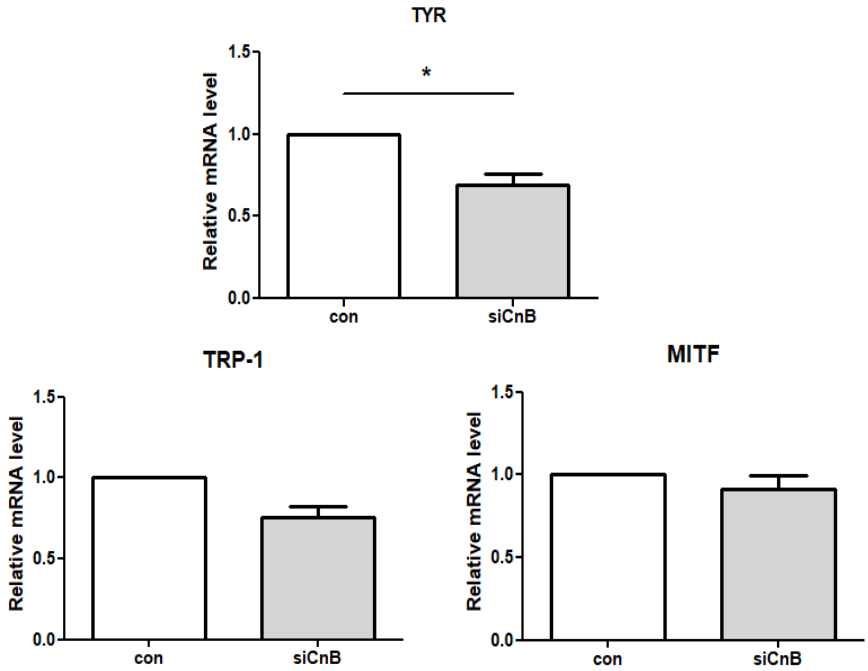
HEMn cells were transfected with the negative control siRNA or the calcineurin B subunit (CnB) siRNA (siCnB) 150nM for 6 hours. (a) CnB mRNA were obtained at 24h after the transfection. CnB mRNA expression levels were analyzed by RT-qPCR and normalized by 36B4 control. (***$p<0.0001$ versus negative control siRNA, n=3, paired t-test) (b) NFATc3 protein expression was detected to indicate change of phosphatase activity of calcineurin. (c) Day6, 8 after the siCnB transfection, the melanin contents were measured and normalized to total protein concentration. (*$p<0.05$, n=4, paired t-test) (d) Day8 after the siCnB transfection, relative proliferation level was measured by WST1 proliferation assay. Graphs show mean ±SEM. ($p=0.0671$, n=3, paired t-test)

Knockdown of calcineurin B downregulates both mRNA and protein expression of tyrosinase in human epidermal melanocytes.

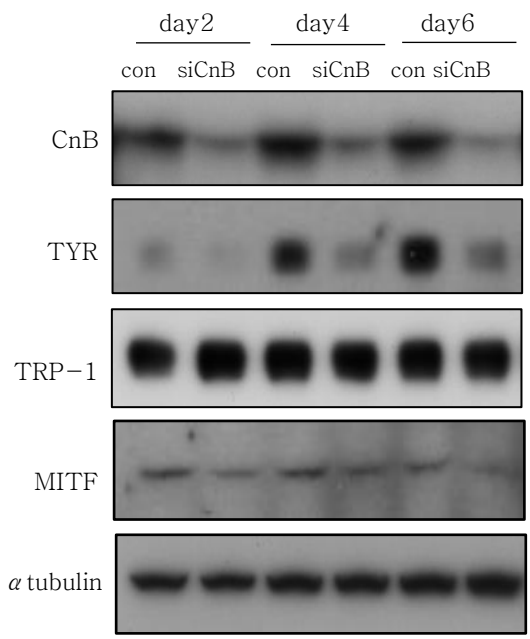
To investigate whether the decrease of melanin synthesis was caused from the downregulation of essential melanogenic enzymes such as tyrosinase (TYR), tyrosinase related protein-1 (TRP-1) and microphthalmia-associated transcription factor (MITF), we performed the RT-PCR and western blot assay. HEMn cells were harvested at every two days until day 6 after CnB siRNA transfection (siCnB) and both mRNA and protein expression level were analyzed by comparing with the control siRNA transfected cells. In accordance with the reduction of CnB expression, mRNA expression of TYR was downregulated significantly at 48 hours (Fig. 2a) and protein expression was decreased significantly from day 2 to day 6 (Fig. 2b). However, both mRNA and protein expression of TRP-1 and MITF was not decreased constantly and significantly in four independent experiments. Statistical analysis was performed to compare the statistical difference and results showed that the protein expression of TYR was significantly decreased at day 4 (Fig. 2c). These data suggest that the reduction of melanin synthesis after knockdown of calcineurin was due to downregulation of tyrosinase expression.

Figure 2

a


TYR
Relative mRNA level
1.5
1.0
0.5
0.0
*
con
siCnB
TRP-1
Relative mRNA level
1.5
1.0
0.5
0.0
con
siCnB
MITF
Relative mRNA level
1.5
1.0
0.5
0.0
con
siCnB

b


day2
day4
day6
con siCnB con siCnB con siCnB
CnB
TYR
TRP-1
MITF
α tubulin

C

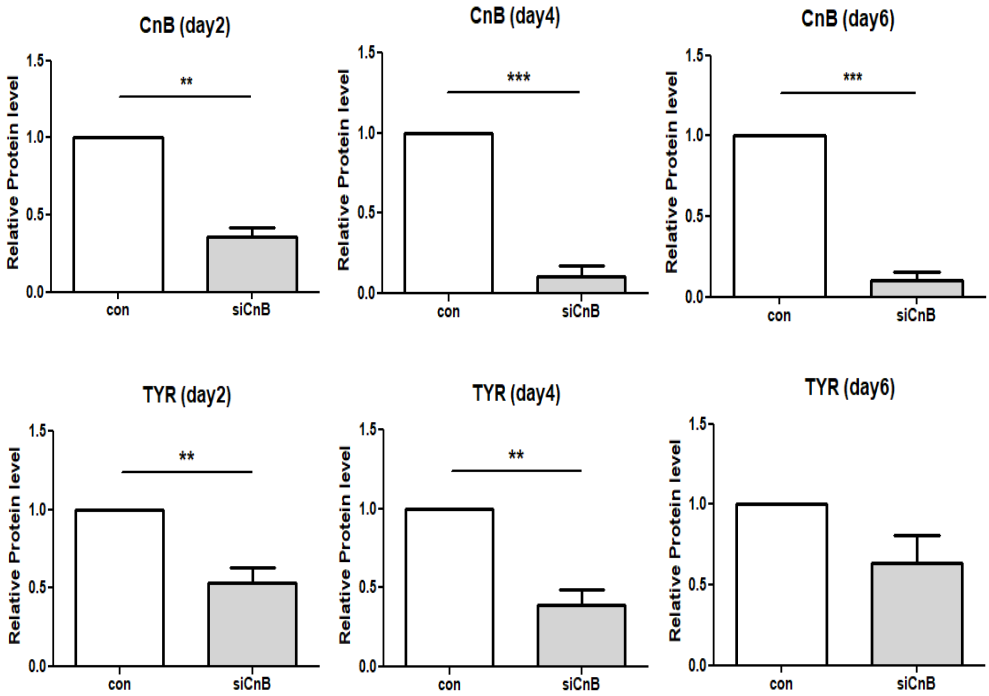

CnB (day2)
CnB (day4)
CnB (day6)
TYR (day2)
TYR (day4)
TYR (day6)
Relative Protein level
1.5
1.0
0.5
0.0
**
***
con
siCnB

Figure 2. Knockdown of calcineurin B downregulates both mRNA and protein expression of tyrosinase in human epidermal melanocytes.

HEMn cells were transfected with the calcineurin B subunit (CnB) siRNA (siCnB) 150nM for 6h and then harvested at 48h, the point after the decrease of CnB mRNA expression. HEMn cells were also harvested at day2, 4, 6 after the transfection for the analysis of protein expression levels. **(a)** tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and MITF mRNA expression levels were obtained using RT-qPCR and normalized by 36B4 control. (*$p<0.05$ versus negative control siRNA, n=3, paired t-test) **(b)** Western blot assay was performed to examine the protein levels of CnB, TYR, TRP-1 and microphthalmia-associated transcription factor (MITF). Alpha-tubulin (α-tubulin) was used as a loading control. **(c)** Relative band density was normalized by a-tubulin and analyzed using Image J software. Graphs show mean ±SEM. (**$p<0.01$, ***$p<0.001$ versus negative control siRNA, n=4, paired t-test)

Overexpression of calcineurin has no effect on melanin synthesis in human epidermal melanocytes.

To complement the effect of calcineurin knockdown experiments, we performed the calcineurin overexpression experiments. We cloned the calcineurin A subunit (CnA) cDNA and calcineurin B subunit (CnB) cDNA into lentiviral vector respectively (Fig. 3a) and treated both CnA lentivirus and CnB lentivirus to HEMn cells with polybrene(6ug/ml) for 8 hours (CnA+B) (Fig. 3b). To examine whether the both subunits of calcineurin were overexpressed correctly, HEMn cells were harvested at day 2 and day 4 after the treatment of viral media. Western blot analysis showed that both subunits were increased by lentiviral overexpression (Fig. 3c). Since calcineurin protein is activated by binding of Ca^{2+} and calmodulin, different media were treated depending on whether Ca^{2+} (0.2mM) was in media or not. The results showed that Ca^{2+} increase slightly the protein expression of calcineurin in control, while Ca^{2+} did not affect that of calcineurin in overexpressed statement. The melanin contents were measured at day 8 after the treatment of viral media and were not changed by calcineurin overexpression (Fig. 3d). These data suggest that just increased expression of calcineurin has no effects on melanin synthesis.

Figure 3

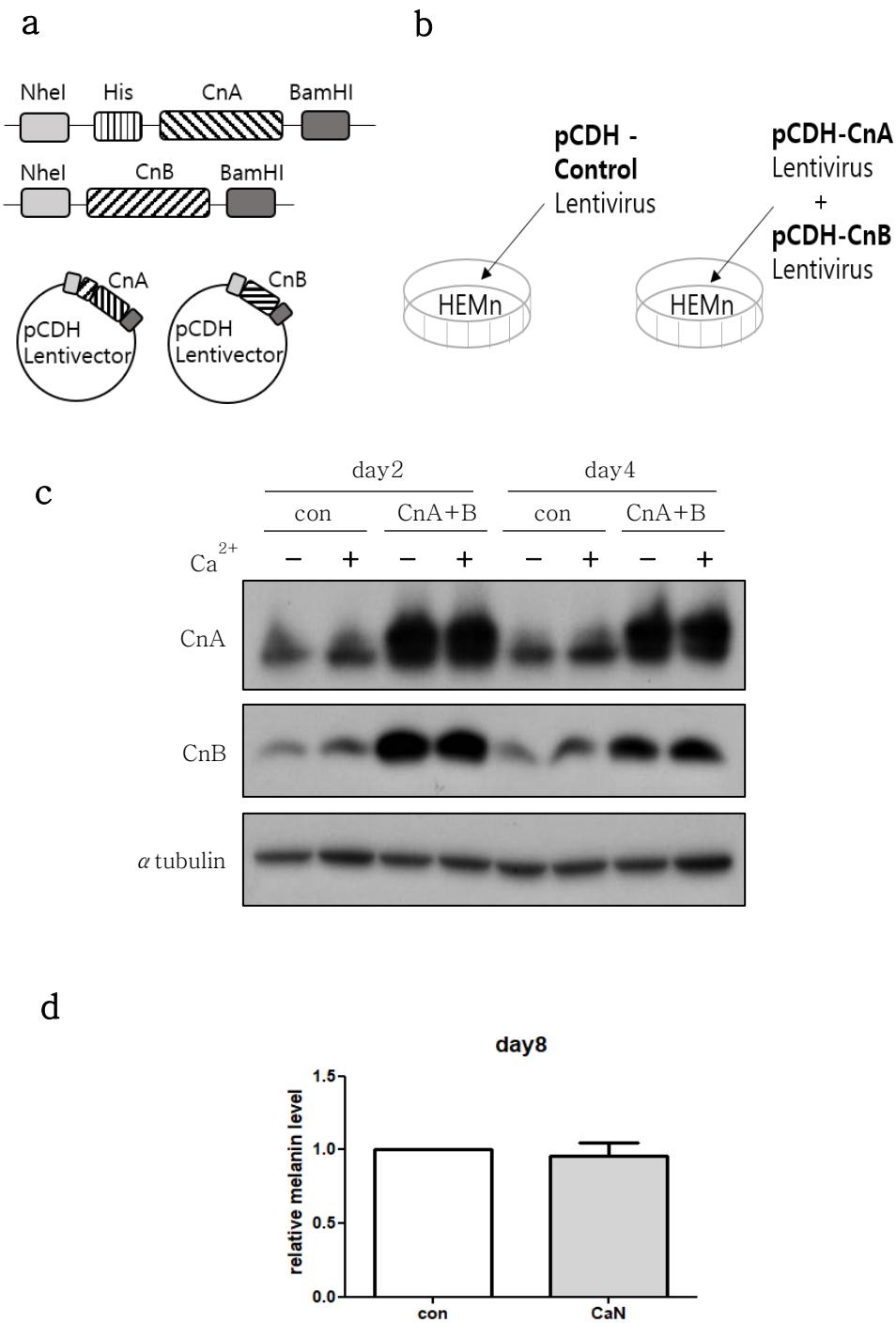

a
NheI
His
CnA
BamHI
NheI
CnB
BamHI
CnA
pCDH
Lentivector
CnB
pCDH
Lentivector
b
pCDH -
Control
Lentivirus
pCDH-CnA
Lentivirus
+
pCDH-CnB
Lentivirus
HEMn
HEMn
c
day2
day4
con
CnA+B
con
CnA+B
Ca2+
− + − + − + − +
CnA
CnB
α tubulin
d
day8
relative melanin level
1.5
1.0
0.5
0.0
con
CaN

Figure 3. Overexpression of calcineurin has no effect on melanin synthesis in human epidermal melanocytes.

(a) Calcineurin A subunit (CnA) cDNA and Calcineurin B subunit (CnB) cDNA was amplified respectively with restriction enzymes (Nhe Ⅰ/BamH Ⅰ) sites from pET15b CnA CnB vector by PCR and recombined to pCDH-CMV-MCS-EF1-Puro lentiviral vector separately. (pCDH-CnA, pCDH-CnB) (b) HEMn cells were treated with the pCDH-Control lentivirus packaging media (CON) or half of pCDH-CnA lentivirus packaging media and pCDH-CnB lentivirus packaging media (CnA+B) for 8 hours. (c) CnA and CnB protein were detected according to overexpression and Ca2+ treatment by western blot analysis. Alpha-tubulin (α tubulin) was used as a loading control. (d) Day8 after the pCDH-CnA and pCDH-CnB lentiviral overexpression (CaN), the melanin contents were measured and normalized to total protein concentration. Graphs show mean ±SEM. (versus with the pCDH-Control lentivirus packaging media, n=4, paired t-test)

MEF2A is highly expressed in human epidermal melanocytes and calcineurin enhances MEF2A transcriptional activity in melanocytes.

Because the myocyte enhancer factor 2 family A (MEF2A) has not been reported to be expressed in human skin, we compared the relative MEF2A mRNA expression levels in human epidermal keratinocytes (HEKs), human dermal fibroblasts (HDFs) and human epidermal melanocytes (HEMs). The results showed that MEF2A was highly expressed in melanocytes, which was about four times higher than that in keratinocytes (Fig. 4a).

Even though MEF2A transcription factor has been shown to be regulated by calcineurin in various tissues like muscles and neurons, regulation of its transcriptional activity has not been reported in human skin. To confirm that MEF2A is one of downstream targets of calcineurin in melanocytes, MEF2 luciferase reporter activity was examined in the presence or absence of both calcineurin A and B plasmids. B16 and G361 melanoma cells were transfected with pGL3-3XMEF2-luciferase vector and at the same time each pCDH-MEF2A, pCDH-CnA and pCDH-CnB vectors were transfected to induce MEF2A and calcineurin overexpression. The pCDH-control vector was used at final DNA amount of overexpression vector. As a result, calcineurin overexpression increased baseline MEF2 transcriptional

activity by 1.7−fold in B16 melanoma cells. Furthermore, MEF2A and calcineurin overexpression at the same time induced about 2.4−fold increase of MEF2 transcriptional activity compared with the control group in B16 melanoma cells (Fig. 4b). In G361 cells, overexpression of calcineurin and MEF2A together increased MEF2 transcriptional activity, whereas overexpression of calcineurin alone did not make a difference (Fig. 4b). Taken together, like other muscle and neuron cells, calcineurin regulates MEF2A transcriptional activity in human epidermal melanocytes.

Figure 4

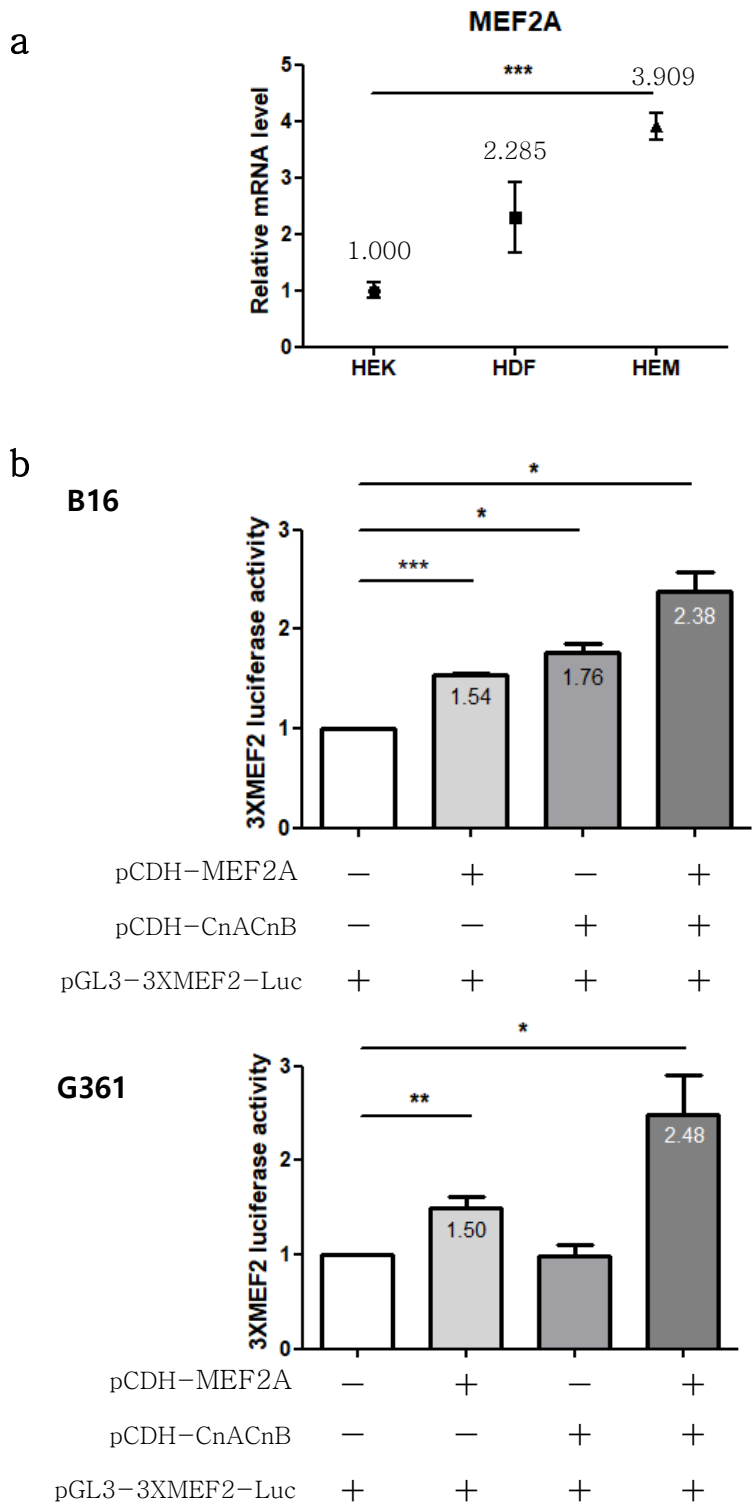

a
MEF2A
Relative mRNA level
***
3.909
2.285
1.000
HEK
HDF
HEM
b
B16
3XMEF2 luciferase activity
*
*
***
2.38
1.76
1.54
pCDH-MEF2A
pCDH-CnACnB
pGL3-3XMEF2-Luc
G361
3XMEF2 luciferase activity
*
**
2.48
1.50
pCDH-MEF2A
pCDH-CnACnB
pGL3-3XMEF2-Luc

Figure 4. MEF2A is most expressed in human epidermal melanocytes and calcineurin enhances MEF2A transcriptional activity in melanocytes.

(a) Human epidermal keratinocytes (HEKs), human dermal fibroblasts (HDFs) and human epidermal melanocytes (HEMs) was harvested and MEF2A mRNA expression levels were obtained using RT-qPCR. The Ct value of 36B4 control was set to the same value at all skin cells and used as normalization. The average Ct value in HEK was set to 1 and the relative mRNA expression of MEF2A in other cells was compared. (***p<0.001 versus expression in HEK, n=4 at each HEK, HDF, HEM cells, paired t-test) (b) All of B16 and G361 melanoma cells was transfected with pGL3-3XMEF-Luc and pRL-TK. pCDH-CON was used as empty plasmid control for the MEF2A (pCDH-MEFA) and calcineurin A&B expression vectors (pCDH-CnACnB), respectively. The MEF2A binding dependent firefly luciferase activities were normalized by renilla luciferase activities. (*p<0.05, ***p<0.001 versus empty plasmid control, n=3 in B16 and n=6 in G361, paired t-test)

Knockdown of MEF2A decreases melanin synthesis in human epidermal melanocytes.

Although it is known that calcineurin is expressed in melanocytes and the effect of topical calcineurin inhibitors on human skin, the role of MEF2A which is known as downstream of calcineurin has not been determined. To confirm MEF2A's role in melanin synthesis, knockdown of MEF2A in human epidermal melanocytes derived from neonatal foreskin of moderately pigmented donors (HEMn-MP cells, HEMn) was performed. HEMn cells were transfected with the negative control siRNA or the MEF2A siRNA 150nM for 6 hours. The mRNA expression of MEF2A was significantly reduced from 24 hours to 48 hours after the transfection (Fig. 5a). The cell pellet color became lighter at day 8 after transfection of siMEF2A and melanin content assay revealed that knockdown of MEF2A inhibited melanin synthesis in HEMn cells at day 6 and day 8 after the transfection by an average of 17% and 26% respectively (Fig. 5b). Compared with the control group, relative proliferation level was also increased in the knockdown group of MEF2A at day 8 (Fig. 5c).

Figure 5

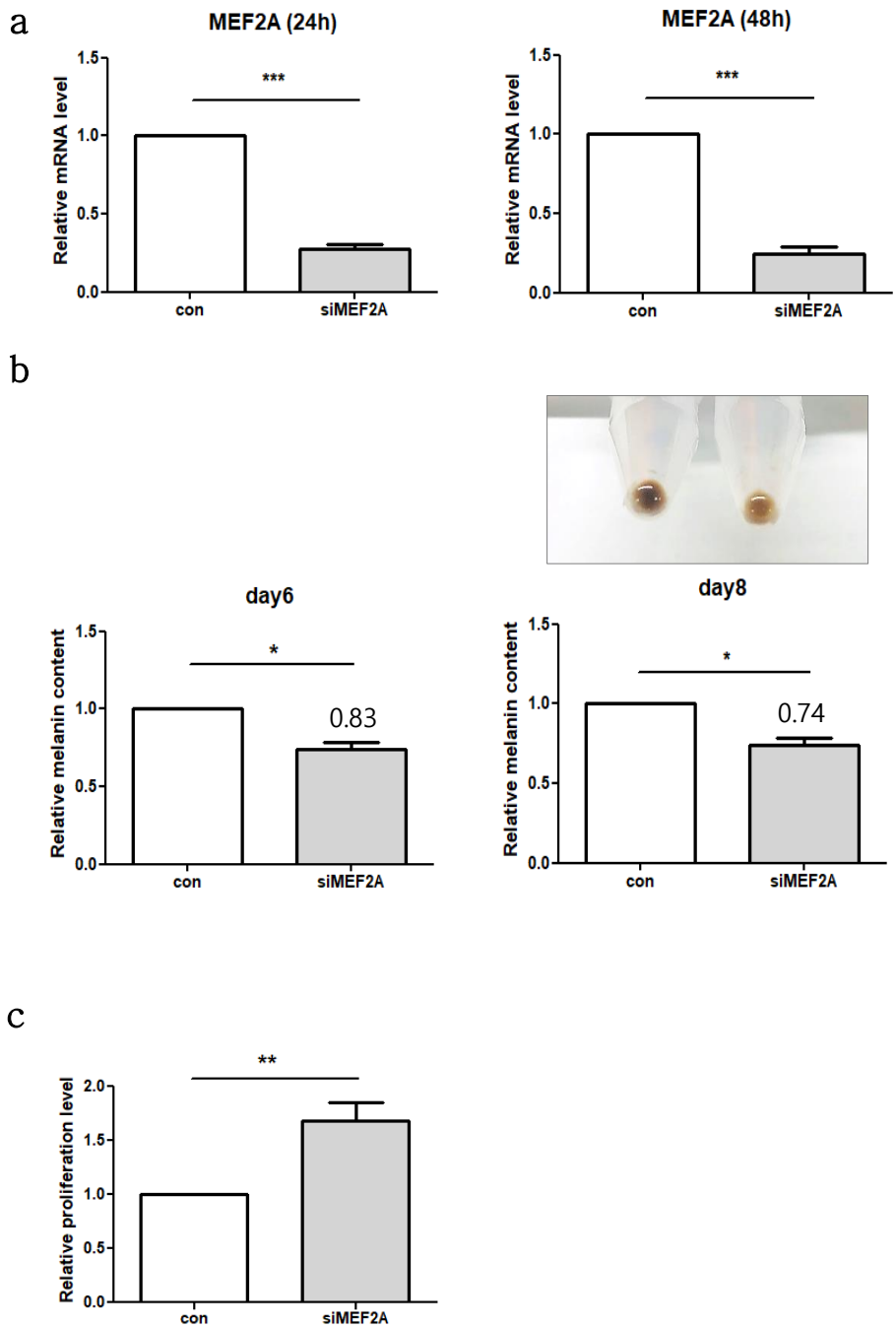

a
MEF2A (24h)
Relative mRNA level
1.5
1.0
0.5
0.0
***
con
siMEF2A
MEF2A (48h)
Relative mRNA level
1.5
1.0
0.5
0.0
***
con
siMEF2A
b
day6
Relative melanin content
1.5
1.0
0.5
0.0
*
0.83
con
siMEF2A
day8
Relative melanin content
1.5
1.0
0.5
0.0
*
0.74
con
siMEF2A
c
Relative proliferation level
2.0
1.5
1.0
0.5
0.0
**
con
siMEF2A

Figure 5. Knockdown of MEF2A decreases melanin synthesis.

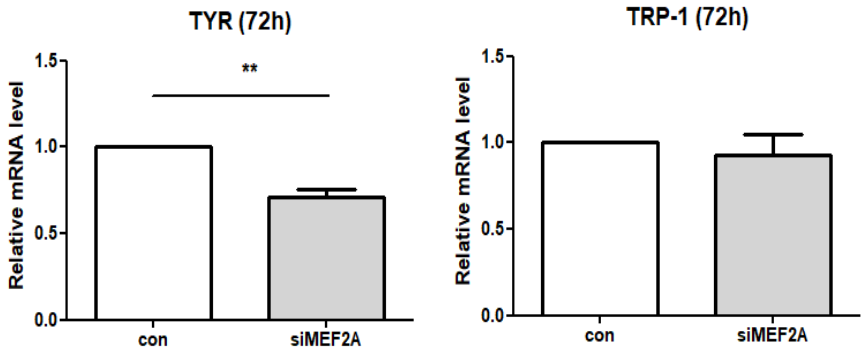
HEMn cells were transfected with the negative control siRNA (con) or the MEF2A siRNA (siMEF2A) 150nM for 6 hours. **(a)** MEF2A mRNA expression levels were obtained using RT-qPCR and normalized by 36B4 control. (***p<0.001 versus negative control siRNA, n=5, paired t-test) **(b)** Day 8 after the siMEF2A transfection, total harvested cell was collected by centrifuge in eppendorf tubes. And day6, 8 after the transfection, the melanin contents were measured and normalized to total protein concentration. (*p<0.05 versus negative control siRNA, n=4, paired t-test) **(c)** Day8 after the siMEF2A transfection, relative proliferation level was measured by WST1 proliferation assay. Graphs show mean ±SEM. (**p<0.01 versus negative control siRNA, n=3, paired t-test)

Knockdown of MEF2A downregulates both mRNA and protein expression of tyrosinase in human epidermal melanocytes.

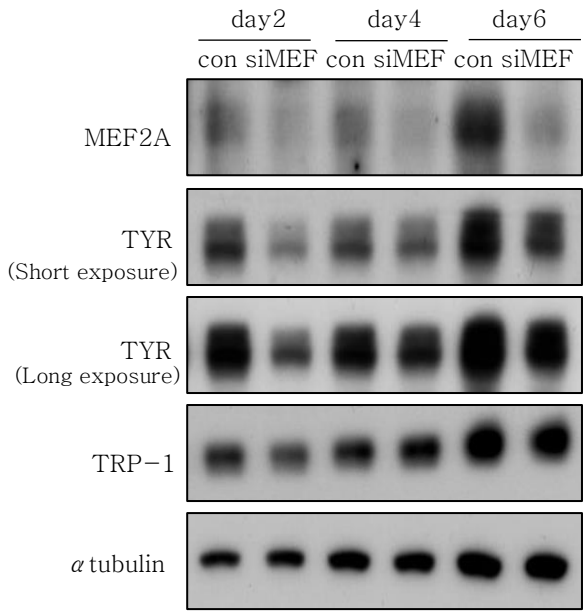
To investigate whether the decrease of melanin synthesis was caused from the changes of critical melanogenic enzymes such as tyrosinase (TYR) and tyrosinase related protein-1 (TRP-1), we performed the RT-PCR and western blot assay. HEMn cells were harvested at 72 hours to observe mRNA levels after the MEF2A siRNA transfection. Since mRNA expression of MEF2A was significantly reduced at 48 hours, the mRNA expression of tyrosinase (TYR) was expected to decrease at least after that time. The mRNA expression of tyrosinase was decreased significantly, while the mRNA expression of TRP-1 was not changed (Fig. 6a). According to reduction of MEF2A expression, protein expression of TYR was also decreased steadily from day 2 to day 6 and protein expression of TRP-1 was slightly decreased at day 2 but not consistent (Fig. 6b,c). Alpha-tubulin (α tubulin) was used as a loading control and quantification. These data suggest that the reduction of melanin synthesis after knockdown of MEF2A is due to downregulation of tyrosinase expression.

Figure 6

a


TYR (72h)
**
Relative mRNA level
1.5
1.0
0.5
0.0
con
siMEF2A
TRP-1 (72h)
Relative mRNA level
1.5
1.0
0.5
0.0
con
siMEF2A

b


day2
day4
day6
con siMEF con siMEF con siMEF
MEF2A
TYR
(Short exposure)
TYR
(Long exposure)
TRP−1
α tubulin

c

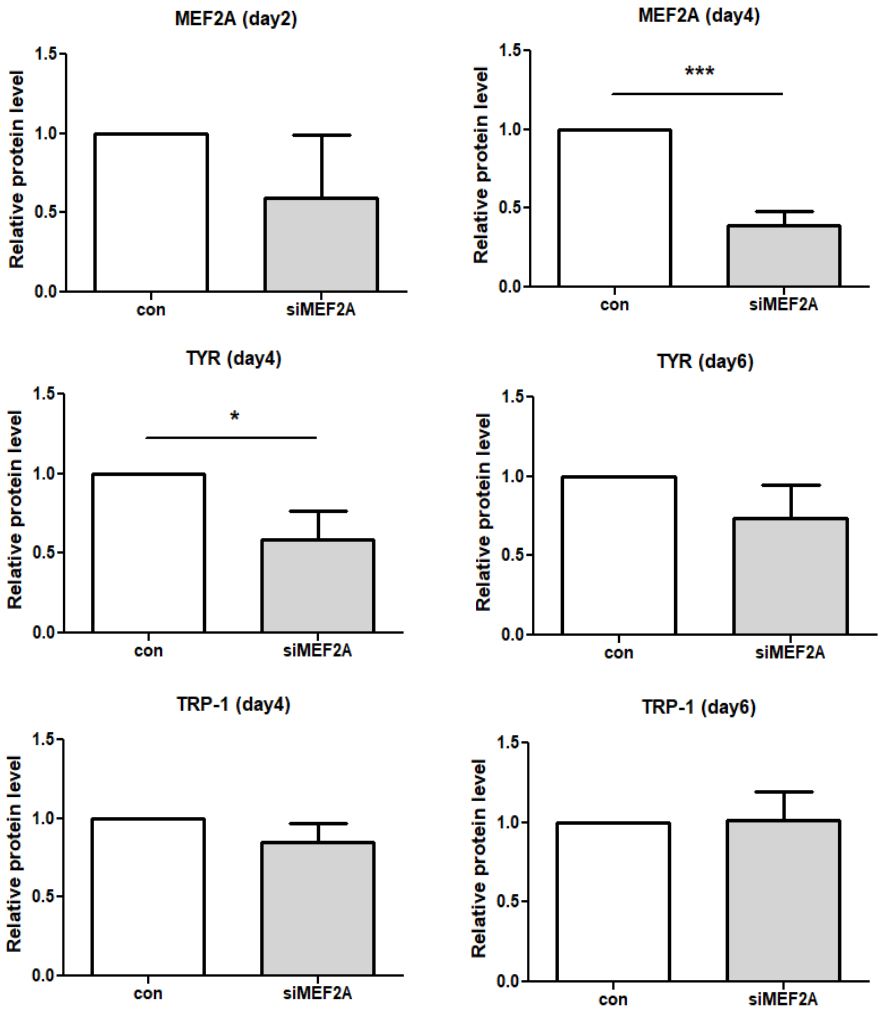

MEF2A (day2)
Relative protein level
1.5
1.0
0.5
0.0
con
siMEF2A
MEF2A (day4)
***
TYR (day4)
*
TYR (day6)
TRP-1 (day4)
TRP-1 (day6)

Figure 6. Knockdown of MEF2A downregulates both mRNA and protein expression of tyrosinase in human epidermal melanocytes.

HEMn cells were transfected with 150nM of MEF2A siRNA (siMEF) for 6 hours and harvested at 72 hours after the transfection for the analysis of mRNA expression and harvested at day2, 4, 6 for the analysis of protein expression levels. (a) Tyrosinase (TYR) and tyrosinase-related protein-1(TRP-1) mRNA expression levels were obtained using RT-qPCR and normalized by 36B4 control. Graphs show mean ±SEM. (**p<0.01 versus negative control siRNA, n=5, paired t-test) (b) Western blot assay was performed to examine MEF2A, TYR and TRP-1 protein levels. Alpha-tubulin(α tubulin) was used as a loading control. (c) Relative band density was normalized by α tubulin and analyzed using Image J software. Graphs show mean ±SEM. (*p<0.05, **p<0.01, ***p<0.001 versus negative control siRNA, n=4, paired t-test)

Overexpression of MEF2A increases melanin synthesis in human epidermal melanocytes.

As we confirmed that knockdown of MEF2A decreases melanin synthesis through downregulation of tyrosinase which is a key enzyme regulating melanogenesis, we performed the MEF2A overexpression experiments to complement the effect of MEF2A knockdown experiments. We cloned the MEF2A cDNA into lentivector (Fig. 7a) and treated MEF2A lentivirus to HEMn cells with polybrene(6ug/ml) for 8 hours (Fig. 7b). HEMn cells were harvested at 24, 48, 72 hours after media change and we confirmed that mRNA expression of MEF2A was considerably increased (Fig. 7c). On the contrary to knockdown experiments, a melanin content assay revealed that overexpression of MEF2A increased melanin synthesis in HEMn cells at day 8 after the transfection (Fig. 7d).

Figure 7

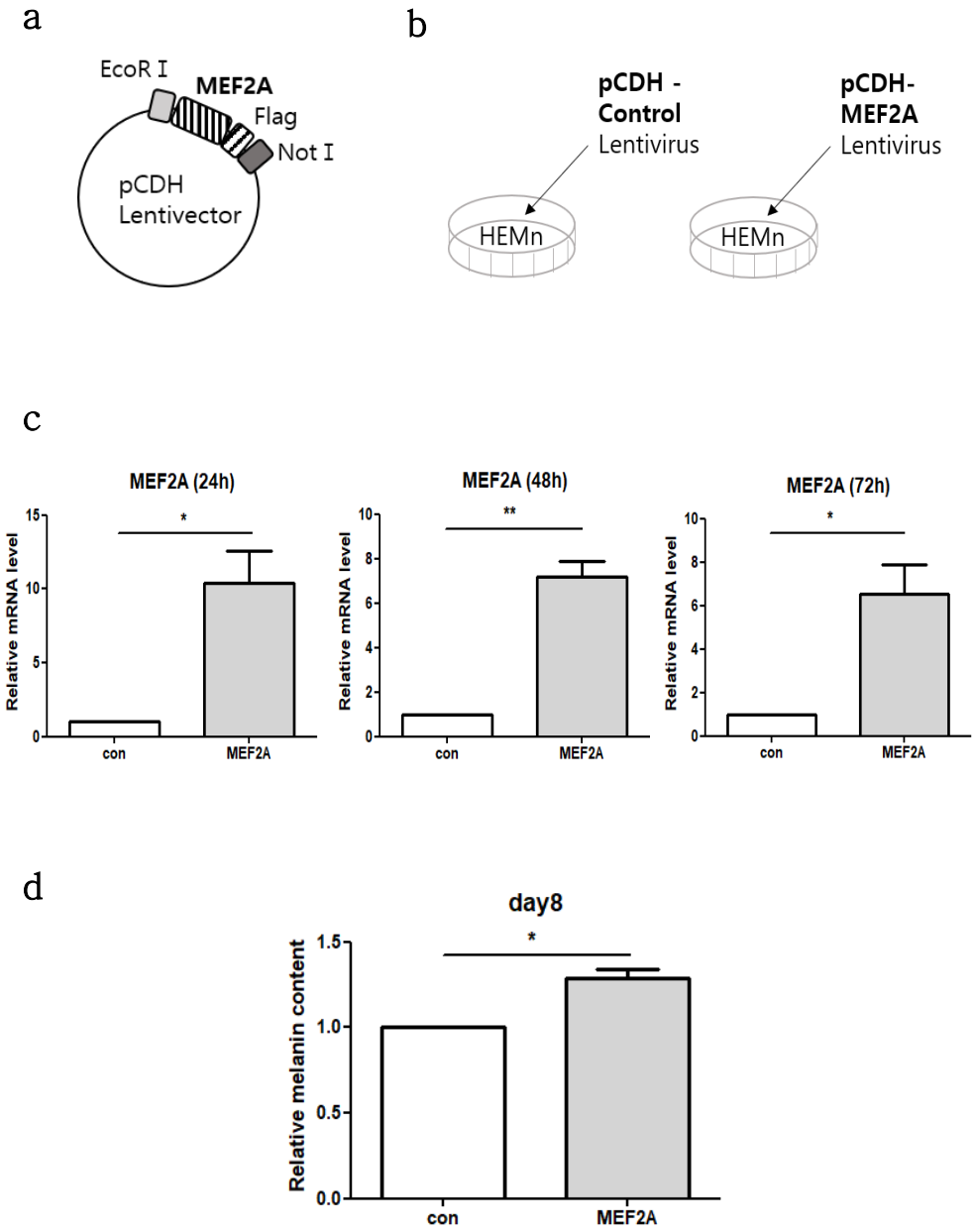

a
EcoR I
MEF2A
Flag
Not I
pCDH
Lentivector
b
pCDH -
Control
Lentivirus
pCDH-
MEF2A
Lentivirus
HEMn
HEMn
c
MEF2A (24h)
MEF2A (48h)
MEF2A (72h)
Relative mRNA level
con
MEF2A
d
day8
Relative melanin content
con
MEF2A

Figure 7. Overexpression of MEF2A increases melanin synthesis.

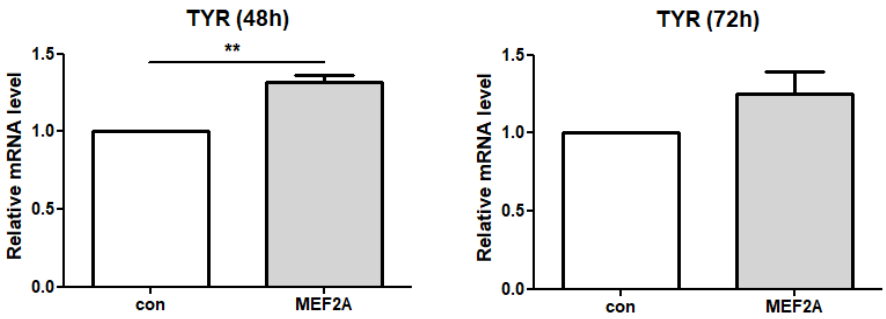
(a) MEF2A cDNA ORF was amplified with restriction enzymes (EcoRI/NotI) sites by PCR and recombined to pCDH-CMV-MCS-EF1-Puro lentiviral vector. (pCDH-MEF2A) (b) HEMn cells were treated with the pCDH-Control lentivirus (con) or pCDH-MEF2A lentivirus (MEF2A) for 8 hours. (c) MEF2A mRNA expression levels were obtained using RT-qPCR and normalized by 36B4 control. (d) Day8 after the pCDH-MEF2A lentiviral overexpression, the melanin contents were measured and normalized to total protein concentration. Graphs show mean ±SEM. (*$p<0.05$, **$p<0.01$ versus pCDH-control viral overexpression, n=4, paired t-test)

Overexpression of MEF2A upregulates both mRNA and protein expression of tyrosinase in human epidermal melanocytes.

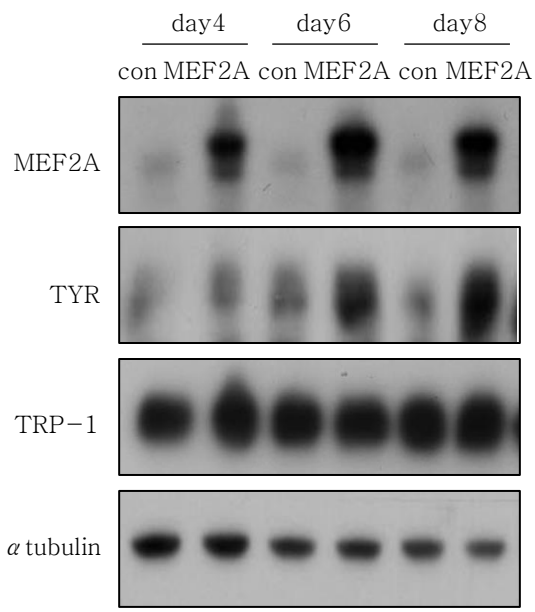
To investigate whether the increase of melanin synthesis was caused from the changes of tyrosinase (TYR) like the previous experiment, we performed the RT-PCR and western blot assay. HEMn cells were harvested at 48 hours and 72 hours for analysis of mRNA level and harvested at from day 4 to day8 for analysis of protein level. Contrary to the knockdown experiments that mRNA expression of TYR was downregulated by MEF2A siRNA, mRNA expression of TYR was increased by MEF2A overexpression (Fig. 8a). Protein expression level was analyzed by comparing with pCDH-control viral overexpression and alpha-tubulin (α tubulin) was used as a loading control and quantification. After the MEF2A viral overexpression, protein expression of MEF2A was considerably increased at day 4, 6, 8 (Fig. 8b). According to MEF2A expression, protein expression of TYR was also increased consistently. Furthermore, protein expression of TRP-1 was slightly increased but not consistent (Fig. 8b,c). These data suggest that the increase of melanin synthesis after overexpression of MEF2A is due to mainly upregulation of tyrosinase expression.

Figure 8

a


TYR (48h)
**
Relative mRNA level
1.5
1.0
0.5
0.0
con
MEF2A
TYR (72h)
Relative mRNA level
1.5
1.0
0.5
0.0
con
MEF2A

b


day4
day6
day8
con MEF2A con MEF2A con MEF2A
MEF2A
TYR
TRP−1
α tubulin

c

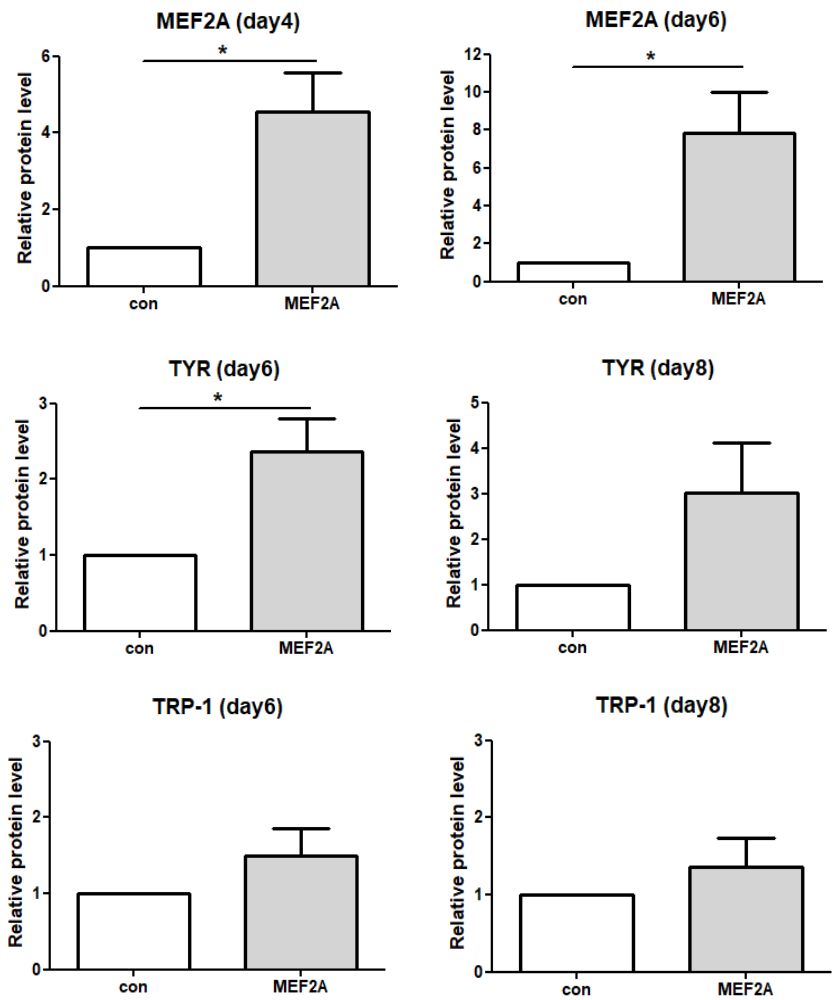

MEF2A (day4)
Relative protein level
6
4
2
0
*
con
MEF2A
MEF2A (day6)
Relative protein level
12
10
8
6
4
2
0
*
con
MEF2A
TYR (day6)
Relative protein level
3
2
1
0
*
con
MEF2A
TYR (day8)
Relative protein level
5
4
3
2
1
0
con
MEF2A
TRP-1 (day6)
Relative protein level
3
2
1
0
con
MEF2A
TRP-1 (day8)
Relative protein level
3
2
1
0
con
MEF2A

Figure 8. Overexpression of MEF2A upregulates both mRNA and protein expression of tyrosinase.

HEMn cells were treated with the pCDH-Control lentivirus (con) or pCDH-MEF2A lentivirus (MEF2A) for 8 hours with polybrene (6ug/ml) and harvested at 48, 72 hours after the transfection for the analysis of mRNA expression and harvested at day4, 6, 8 for the analysis of protein expression levels. **(a)** Tyrosinase (TYR) mRNA expression levels were obtained using RT-qPCR and normalized by 36B4 control. (*$p<0.05$ versus pCDH-control viral overexpression, n=4, paired t-test) **(b)** Western blot assay was performed to examine MEF2A, tyrosinase (TYR) and tyrosinase-related protein-1(TRP-1) protein levels. Alpha tubulin(α tubulin) was used as a loading control. **(c)** Relative band density was normalized by α tubulin and analyzed using Image J software. Graphs show mean ±SEM. (*$p<0.05$ versus pCDH-control viral overexpression, n=4, paired t-test)

Figure 9

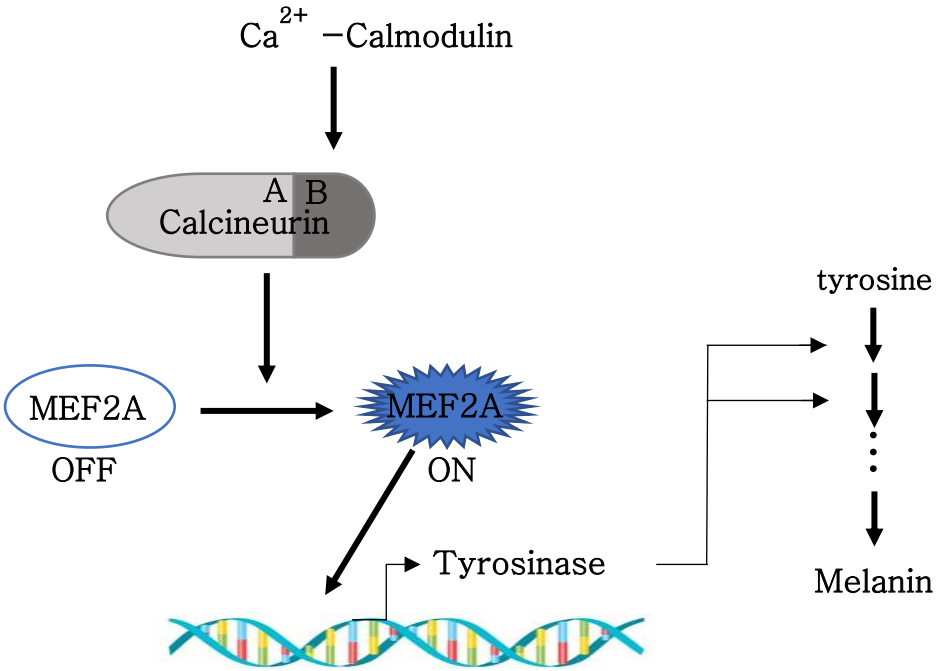


Figure 9. Schematic diagram showing the effects of calcineurin and MEF2A signaling on melanin synthesis.

The Ca^{2+} and calmodulin dependent calcineurin activates transcriptional activity of MEF2A and then MEF2A increase mRNA and protein level of tyrosinase which is most important enzyme of melanin synthesis from tyrosine amino acids in human epidermal melanocytes. However, unlike knockdown of calcineurin expression, solely overexpressed calcineurin has no effect on melanin synthesis.

DISCUSSION

Calcium (Ca^{2+}) has been proposed in many aspects of melanocyte biology as well as melanin synthesis and Ca^{2+} influx induced by UVA or UVB is reported to contribute to the consecutive stimulation of melanosome transfer [34-36].

Although the various Ca^{2+} channels like calcium release-activated calcium channel protein 1 (Orai1), endoplasmic reticulum stromal interaction molecule (STIM), and transient receptor potential melastatin 1 (TRPM1) have been reported to affect melanin synthesis in human primary melanocytes, studies about the intracellular signals affected and activated by Ca^{2+} like calcineurin have not been fully elucidated [37-39].

For the treatment of Vitiligo, atopic dermatitis and psoriasis in skin, calcineurin immunosuppressive inhibitors such as cyclosporin A, FK506 and pimecrolimus have been used to prevent the transcription of proinflammatory cytokines [25, 26]. Although these calcineurin inhibitors were known to regulate melanocyte migration, growth and melanin transfer [30, 31], the role of calcineurin itself in human epidermal melanocytes has not been reported in human skin. There are also conflicting results about the effects of calcineurin inhibitors on

melanin synthesis. In this study, our results show that calcineurin itself affects melanin synthesis through regulation of tyrosinase expression. This correlation between calcineurin itself and tyrosinase is the new finding in human epidermal melanocytes.

Myocyte enhancer factor 2 (MEF2) is known as a transcription factor responsive to calcium and calcineurin in human skeletal muscle and cardiomyocytes [40, 41], but the correlation between calcineurin and MEF2 has not been reported in human skin. Moreover, no research about MEF2 family has been done in human skin including epidermal melanocytes. On the basis that MEF2A and MEF2C are also expressed in the skin and MEF2A is more expressed than MEF2C by transcriptome analysis [32], we assumed that MEF2A may be the one of transcription factors that respond to calcineurin in human skin. Thus, our study places MEF2A as a downstream factor of calcineurin signaling that affects melanin synthesis in human epidermal melanocytes.

Our results show that calcineurin directly activates transcriptional activity of MEF2A and this finding is the first result in human epidermal melanocytes. Knockdown of calcineurin inhibited melanin synthesis through downregulation of both mRNA and protein expression of tyrosinase. The same results were also found in the knockdown of

MEF2A experiments. Decreased level of MEF2A downregulated both mRNA and protein expression of tyrosinase and reduced the total amount of melanin. However, unlike the overexpression results that MEF2A induced the increase of melanin synthesis through upregulation of both mRNA and protein expression of tyrosinase, overexpressed level of calcineurin had no effects on melanin synthesis. Since calcineurin is a phosphatase that depends on the Ca^{2+} and calmodulin levels [42], it seems that both Ca^{2+} and calmodulin is necessary for increase of calcineurin activity together with increase of expression levels. And some studies reported that calcineurin regulates p53 and BAD (Bcl-2 associated death promoter) as well as NFAT [43, 44]. Thus, unlike the overexpression results of downstream factor MEF2A, the overexpression of calcineurin may affect various factors that could suppress melanin overproduction.

Our data support a model whereby calcineurin also functions to regulate tyrosinase expression and melanin synthesis through regulating transcriptional activity of MEF2A in human epidermal melanocytes. Since MEF2A affected the protein expression of tyrosinase as well as the mRNA expression, MEF2A may be transcription factor of tyrosinase promoter directly or indirectly.

Together, our results are new findings that suggest MEF2A's role in

human skin especially in melanocytes through regulating tyrosinase expression which is the most crucial factor in melanogenesis. We suggest that MEF2A could be a new candidate gene that regulates skin pigmentation

References

1. Bonaventure, J., M.J. Domingues, and L. Larue, *Cellular and molecular mechanisms controlling the migration of melanocytes and melanoma cells.* Pigment Cell Melanoma Res, 2013. **26**(3): p. 316-25.
2. Seiberg, M., *Keratinocyte-melanocyte interactions during melanosome transfer.* Pigment Cell Res, 2001. **14**(4): p. 236-42.
3. Lin, J.Y. and D.E. Fisher, *Melanocyte biology and skin pigmentation.* Nature, 2007. **445**: p. 843.
4. D'Mello, S.A., et al., *Signaling Pathways in Melanogenesis.* Int J Mol Sci, 2016. **17**(7).
5. Thody, A.J., et al., *Pheomelanin as well as eumelanin is present in human epidermis.* J Invest Dermatol, 1991. **97**(2): p. 340-4.
6. Slominski, A., et al., *Melanin pigmentation in mammalian skin and its hormonal regulation.* Physiol Rev, 2004. **84**(4): p. 1155-228.
7. Pillaiyar, T., M. Manickam, and S.H. Jung, *Recent development of signaling pathways inhibitors of melanogenesis.* Cell Signal, 2017. **40**: p. 99-115.
8. Rousseau, K., et al., *Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis.* Faseb j, 2007. **21**(8): p. 1844-56.
9. Im, S., et al., *Activation of the cyclic AMP pathway by alpha-melanotropin mediates the response of human melanocytes to ultraviolet B radiation.* Cancer Res, 1998. **58**(1): p. 47-54.
10. Fell, G.L., et al., *Skin beta-endorphin mediates addiction to UV light.* Cell, 2014. **157**(7): p. 1527-34.
11. Kauser, S., et al., *Regulation of human epidermal melanocyte biology by beta-endorphin.* J Invest Dermatol, 2003. **120**(6): p. 1073-80.
12. Yang, G., et al., *Inhibition of PAX3 by TGF-beta modulates melanocyte viability.* Mol Cell, 2008. **32**(4): p. 554-63.
13. Puri, N., et al., *Basic fibroblast growth factor promotes melanin synthesis by melanocytes.* Arch Dermatol Res, 1996. **288**(10): p. 633-5.
14. Imokawa, G., Y. Yada, and M. Miyagishi, *Endothelins secreted from*

human keratinocytes are intrinsic mitogens for human melanocytes. J Biol Chem, 1992. **267**(34): p. 24675-80.

15. Hachiya, A., et al., *The paracrine role of stem cell factor/c-kit signaling in the activation of human melanocytes in ultraviolet-B-induced pigmentation.* J Invest Dermatol, 2001. **116**(4): p. 578-86.
16. Gledhill, K., et al., *Prostaglandin-E2 is produced by adult human epidermal melanocytes in response to UVB in a melanogenesis-independent manner.* Pigment Cell Melanoma Res, 2010. **23**(3): p. 394-403.
17. Yuan, X.H. and Z.H. Jin, *Paracrine regulation of melanogenesis.* Br J Dermatol, 2018. **178**(3): p. 632-639.
18. Rodriguez, C.I. and V. Setaluri, *Cyclic AMP (cAMP) signaling in melanocytes and melanoma.* Arch Biochem Biophys, 2014. **563**: p. 22-7.
19. Singh, S.K., et al., *E-cadherin mediates ultraviolet radiation- and calcium-induced melanin transfer in human skin cells.* Exp Dermatol, 2017. **26**(11): p. 1125-1133.
20. Motiani, R.K., et al., *STIM1 activation of adenylyl cyclase 6 connects Ca(2+) and cAMP signaling during melanogenesis.* Embo j, 2018. **37**(5).
21. Li, H., A. Rao, and P.G. Hogan, *Interaction of calcineurin with substrates and targeting proteins.* Trends Cell Biol, 2011. **21**(2): p. 91-103.
22. Connolly, S., et al., *Calcineurin Regulatory Subunit Calcium-Binding Domains Differentially Contribute to Calcineurin Signaling in Saccharomyces cerevisiae.* Genetics, 2018. **209**(3): p. 801-813.
23. Wakelam, M.J., *The fusion of myoblasts.* Biochem J, 1985. **228**(1): p. 1-12.
24. Mao, Z. and M. Wiedmann, *Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons.* J Biol Chem, 1999. **274**(43): p. 31102-7.
25. Megna, M., et al., *Systemic Treatment of Adult Atopic Dermatitis: A Review.* Dermatol Ther (Heidelb), 2017. **7**(1): p. 1-23.
26. Malecic, N. and H. Young, *Tacrolimus for the management of psoriasis: clinical utility and place in therapy.* Psoriasis (Auckl), 2016. **6**: p. 153-163.

27. Potthoff, M.J. and E.N. Olson, *MEF2: a central regulator of diverse developmental programs.* Development, 2007. **134**(23): p. 4131-40.
28. Edmondson, D.G., et al., *Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis.* Development, 1994. **120**(5): p. 1251-63.
29. Lee, J.Y. and W.H. Kang, *Effect of cyclosporin A on melanogenesis in cultured human melanocytes.* Pigment Cell Res, 2003. **16**(5): p. 504-8.
30. Jung, H., et al., *FK506 regulates pigmentation by maturing the melanosome and facilitating their transfer to keratinocytes.* Pigment Cell Melanoma Res, 2016. **29**(2): p. 199-209.
31. Xu, P., et al., *Pimecrolimus increases the melanogenesis and migration of melanocytes in vitro.* Korean J Physiol Pharmacol, 2017. **21**(3): p. 287-292.
32. Reemann, P., et al., *Melanocytes in the skin--comparative whole transcriptome analysis of main skin cell types.* PLoS One, 2014. **9**(12): p. e115717.
33. Muller, M.R. and A. Rao, *NFAT, immunity and cancer: a transcription factor comes of age.* Nat Rev Immunol, 2010. **10**(9): p. 645-56.
34. Buffey, J.A., M. Edgecombe, and S. Mac Neil, *Calcium plays a complex role in the regulation of melanogenesis in murine B16 melanoma cells.* Pigment Cell Res, 1993. **6**(6): p. 385-93.
35. Hu, Q.M., et al., *Induction of retinal-dependent calcium influx in human melanocytes by UVA or UVB radiation contributes to the stimulation of melanosome transfer.* Cell Prolif, 2017. **50**(6).
36. Buffey, J.A., et al., *Evidence for a calcium/calmodulin involvement in density-dependent melanogenesis in murine B16 melanoma cells.* Pigment Cell Res, 1991. **4**(3): p. 112-9.
37. Stanisz, H., et al., *The role of Orai-STIM calcium channels in melanocytes and melanoma.* J Physiol, 2016. **594**(11): p. 2825-35.
38. Stanisz, H., et al., *ORAI1 Ca(2+) channels control endothelin-1-induced mitogenesis and melanogenesis in primary human melanocytes.* J Invest Dermatol, 2012. **132**(5): p. 1443-51.
39. Devi, S., et al., *Calcium homeostasis in human melanocytes: role of transient receptor potential melastatin 1 (TRPM1) and its regulation by ultraviolet light.* Am J Physiol Cell Physiol, 2009. **297**(3): p. C679-

87.

40. Friday, B.B., et al., *Calcineurin initiates skeletal muscle differentiation by activating MEF2 and MyoD.* Differentiation, 2003. **71**(3): p. 217-27.

41. Ye, J., et al., *Translation of Myocyte Enhancer Factor-2 is induced by hypertrophic stimuli in cardiomyocytes through a Calcineurin-dependent pathway.* J Mol Cell Cardiol, 2012. **53**(4): p. 578-87.

42. Shi, X., et al., *The interaction between calcineurin and alpha-synuclein is regulated by calcium and calmodulin.* Biochem Biophys Res Commun, 2018. **496**(4): p. 1109-1114.

43. Yang, Y., et al., *Downregulated TRPV1 Expression Contributes to Melanoma Growth via the Calcineurin-ATF3-p53 Pathway.* J Invest Dermatol, 2018. **138**(10): p. 2205-2215.

44. Spurney, R.F., *Non-immunologic actions of calcineurin inhibitors in proteinuric kidney diseases.* Front Endocrinol (Lausanne), 2014. **5**: p. 181.

국문 초록

칼시뉴린(calcineurin)은 칼슘과 칼모듈린(calmodulin)에 의존적으로 활성화되는 인산가수분해효소로 칼슘 신호전달 경로에 중요한 인자이다. T 림프구 매개 면역반응 활성화에 중요한 인자이기 때문에 사이클로스포린 A(cyclosporin A)와 타크로리무스(tacrolimus, FK506) 등의 국소 칼시뉴린 억제제는 백반증과 같은 자가 면역 질환에 국소 면역 억제제로 가장 흔하게 사용되고 있다. 하지만, 이러한 국소 칼시뉴린 억제제들이 피부 표피 멜라닌세포에서 멜라닌 합성에 미치는 영향은 다르게 보고되어 있어 논란의 여지가 있다. 따라서 본 연구에서는 칼시뉴린 본연의 단백질이 멜라닌 합성 과정에 미치는 영향을 알아보고자 하였다. 칼시뉴린에 의해 조절을 받는 하위 인자로 NFAT와 MEF2 단백질이 잘 알려져 있지만, 사람 피부 조직에서 칼시뉴린-NFAT의 신호 전달 경로는 잘 알려진 반면에 칼시뉴린-MEF2의 경로는 아직 보고된 바 없다. MEF2는 근육 세포 분화 및 발달에 중요한 전사 조절 인자로 근육 이외에도 심장, 신경 및 면역세포에서 활발하게 연구되었지만 피부에서의 역할은 아직 알려져 있지 않다. 본 연구에서는 피부 표피의 각질형성세포와 멜라닌세포 그리고 피부 진피의 섬유아세포에서 MEF2A의 발현을 비교해본 결과, 멜라닌세포에서 가장 발현이 높게 나타남을 확인하였다. 또한, luciferase reporter 실험을 통하여 멜라닌세포에서도 칼시뉴린에 의해 MEF2A의 전사 활성이 증가함을 확인하였다. 이후 칼시뉴린과 MEF2A가 멜라닌 합성 과정에 미치는

영향을 확인해 보고자 유전자 간섭 실험을 통해 멜라닌세포에서 발현을 각각 억제한 결과, 칼시뉴린과 MEF2A 발현 감소에 따라 멜라닌 합성량이 감소함을 관찰하였다. 이러한 멜라닌 합성의 감소가 타이로시네이즈(tyrosinase), TRP-1, DCT, MITF 등과 같은 멜라닌 합성 핵심 효소들의 변화에서 기인한 것인지 확인해보았다. 그 결과, 가장 첫 단계에 관여하는 핵심 산화 효소인 타이로시네이즈의 mRNA와 단백질 발현이 모두 감소한 것을 확인하였다. 이후 칼시뉴린과 MEF2A의 역할을 한번 더 검증해보기 위해 바이러스 매개 과발현 실험을 진행하였다. 멜라닌세포에서 칼시뉴린을 과발현 시켰을 때는 멜라닌 합성량에 변화가 없었지만, MEF2A를 과발현 시킨 결과 유전자 간섭 실험과 반대로 타이로시네이즈의 mRNA와 단백질 발현이 증가하여 최종 멜라닌 합성량이 증가함을 관찰하였다.

결과를 종합해 보았을 때, 칼슘 신호에 의존적인 칼시뉴린은 MEF2A 전사 조절 인자의 활성을 증가시켜 멜라닌 합성에 핵심 효소인 타이로시네이즈의 발현을 증가시킴으로써 멜라닌 합성에 관여하고 있음을 새롭게 규명하였다. 본 연구에서는 사람 피부에서 처음으로 MEF2의 역할을 밝혔고 특히 멜라닌세포에서 멜라닌 합성을 조절하는데 새로운 표적 인자가 될 수 있음을 새롭게 제시하였다.

주요어

멜라닌세포, 피부 색소, 멜라닌, 칼슘, 칼시뉴린, MEF2, 타이로시네이즈

학번 : 2017-20815