# Mechanism of *Mitf* inhibition and morphological differentiation effects of hirsein A on B16 melanoma cells revealed by DNA microarray

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## Abstract

### Background

We have previously reported that hirsein A inhibits melanogenesis in B16 melanoma cells by downregulating the *Mitf* gene expression.

### Objective

In this study, microarray was employed to determined the transcriptional response of B16 cells to hirsein A (HA) treatment and to find out the mechanism underlying *Mitf* downregulation.

### Methods

DNA microarray, spotted with 265 genes for melanogenesis and signal transduction, was performed using the total RNA isolated from B16 cells treated with HA. Validation of the results was done using real-time PCR. In addition, real-time PCR using primers for *Mda-7* gene and F-actin staining were performed. Transfection experiments were performed to knockdown the expression of the *Mc1r* gene to evaluate its role in the cell morphological change observed.

### Results

As expected, the expressions of the *Mitf*-regulated melanosome transport genes and the *Mc1r* gene were downregulated. Furthermore, the expressions of the MAPK pathway intermediates were either up- or downregulated. Genes associated with cell differentiation, such as *Gadd45b*, were upregulated and prompted us to determine the expression of the *Il-24* (*Mda-7*) gene using real-time PCR. There was an increase in the *Mda-7* mRNA expression in B16 and HMV-II melanoma cells, and in human melanocytes. To better visualize the cell morphology, F-actin

staining was performed and the results showed an increase in the dendrite outgrowth in HA-treated cells. Silencing the *Mc1r* gene did not cause a change in the B16 cell morphology observed in cells treated with HA.

**Conclusion**

This study demonstrated that HA downregulates *Mitf* gene expression by regulating the expressions of the MAPK signaling pathway intermediates. In addition, the inhibited *Mc1r* gene expression also contributed to the overall *Mitf* downregulation but do not play a role in the observed change in B16 cell morphology. HA surprisingly can regulate genes associated with differentiating cells (*Mda-7*) suggesting a role for HA in the melanoma cell differentiation induction. While the exact molecular mechanism by which HA promotes cell differentiation remain to be determined, it is clear that HA can downregulate *Mitf* expression and promote cell differentiation and has the potential to be used in the development of therapy for melanoma.

**Keywords:** hirsein A, *Mitf*, *Mc1r*, B16 murine melanoma cells, differentiation

**Abbreviations:** HA, hirsein A; B16 cells, B16 murine melanoma cells; HMV-II, HMV-II human melanoma cells; HEMs, human epithelial melanocyte; α- MSH, alpha- melanocyte stimulating hormone; Rp-cAMPS, adenosine-3,5'-cyclic monophosphorothioate, Rp-isomer; *Mitf, m*icrophthalmia-associated transcription factor; *Mc1r, m*elanocortin 1 receptor; *Rab27a*, RAB27A, member RAS oncogene family; *Sorbs3*, sorbin and SH3 domain containing 3; *Mlph*, melanophilin; *Ldb1*, LIM domain binding 1; *Myo5a* , myosin VA; *Ppap2b*, phosphatidic acid phosphatase type 2B; *Gadd45b*, growth arrest and DNA-damage-inducible 45 beta; *Pxn*, Paxillin; *Map2k3*, *m*itogen-activated protein kinase; *Wisp1*,WNT1 inducible signaling pathway protein 1; *Prkx, p*rotein kinase, X-linked; siRNA, short interfering RNA.

## Introduction

Pigmentation in humans depends on the amount and deposition of melanin in the skin, which relies on the biosynthesis of melanin or melanogenesis within the membrane-bound organelles termed melanosomes [1-2]. Melanogenesis involves the conversion of tyrosine to melanin through a series of enzymatic and spontaneous reactions mediated by the enzymes tyrosinase (Tyr), tyrosinase-related protein 1 (Trp1) and tyrosinase-related protein 2 (Trp2) [3-6], the expression of which is regulated by the microphthalmia- associated transcription factor (*Mitf)* gene [7]. Melanin is necessary for the prevention of cancer caused by UV irradiation and its regulation is important in addressing a number of diseases and cosmetic problems [8].

In controlling melanogenesis, knowledge of the function and regulation of the *Mitf* gene is very important because it is also the gene responsible for the development, function, and survival of melanocytes [9] and melanoma cells [10]. On the other hand, *Mitf* promotes malignant behavior as observed in some human melanomas and human clear cell sarcoma wherein *Mitf* has been demonstrated as an amplified oncogene and an understanding of its function and associated pathways have been suggested to help shed light on improving therapeutic approaches for melanoma [10]. More importantly, *Mitf* has become a molecular target for the management of melanoma [11].

The cAMP/protein kinase A (PKA) pathway is one of the most important signaling pathways that can induce melanogenesis [12-13]. Cyclic AMP, through the activation of PKA and cAMP-responsive element binding protein 1 transcription factors, upregulates the expression of *Mitf* [14]. The production of the melanogenic enzymes (tyrosinase, TRP-1, and DCT) is controlled by *Mitf* at the mRNA level [15]. cAMP can promote the transcription of *Mitf* through a classical cAMP response element and this has been demonstrated by using a dominant-negative mutant of

*Mitf* which showed that *Mitf* is required for the cAMP effect on tyrosinase promoter [14].

In melanocytes, the expressions of the *Tyr, Trp1, Dct* genes, and the melanosome transport associated genes *Rab27a*, *Myo5a*, and *Mlph* are regulated by the *Mitf* [16-20]. Melanosomes are transported first within the melanocyte, from around the nucleus and from the tips of the dendrites of the melanocytes to keratinocytes for effective pigmentation[21]. Pigment cell dendricity or cell differentiation is usually associated with an increase in melanin content to facilitate the transfer of melanin inside the melanosome, from melanocytes to neighboring keratinocytes [21]. However, *Mitf* which regulates melanogenesis is found to be highly expressed in most primary melanomas [22] and reinforces the idea that the origin of malignant melanoma is linked to the melanocyte lineage, thus making *Mitf* important in melanoma cell survival [6]. The signaling pathways that regulate *Mitf* are the same pathways that are relevant in melanoma tumorigenesis and progression [23, 24]. The use of cell differentiation agents has been suggested as a potentially less toxic approach to cancer therapy or 'differentiation therapy' [25, 26]. Recently, several groups of researchers focus on the anticancer gene therapeutic potential of the melanoma differentiation-associated gene-7 (*mda-7*) [27]. Mda-7 belongs to the IL-10 family of cytokines and has therefore been re-designated IL-24. Mda-7 is a molecule whose expression is elevated in terminally differentiated human melanoma cells and can differentiate between cancer cells and normal cells [27].

Daphnane-type diterpenes have been used to investigate processes such as apoptosis, neutrophism and tumor promotion [28], and have shown significant MET tyrosine kinase inhibition activity [29]. Recently, we reported that hirsein A (HA) from *Thymelaea hirsuta* L. can inhibit melanogenesis by decreasing the tyrosinase enzyme mRNA expression through the downregulation of the *Mitf* gene expression [30]. Furthermore, HA treatment also induced an

increase in the dendrite outgrowth of B16 cells. Although the effect of HA on *Mitf* and melanogenic enzymes' mRNA expressions have been reported, the effect of HA on other genes related to melanogenesis, the underlying cause of *Mitf* gene inhibition and B16 cell morphological change are yet unknown.

In this study, microarray analysis was used and the results helped identify the targets of HA in B16 melanoma cells to understand the underlying reason for the inhibition of the *Mitf* gene expression. In addition, the data generated in this study will give an insight into the morphological change observed.

## Materials and Methods

### Cell culture and treatment method

B16 murine melanoma cells were purchased from the Riken Cell Bank in Tsukuba, Japan, and maintained as a monolayer culture in DMEM (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum or FBS (Sigma), 50 U⁄ ml penicillin and 50 µg/ml streptomycin (Cambrex, East Rutherford, NJ, USA). Human melanoma HMV-II cells were maintained in Ham F12 (Gibco) supplemented with 10% FBS. Both cell lines were incubated at 37$^{o}$C in a 95% air and 5% $CO_2$ incubator. Human epidermal melanocytes or HEM (Cryopreserved HEM, Cat. 104-05n), melanocyte growth medium (Cat. 135-5001) and trypsin/EDTA solution (Subculture Reagent Kit, Cat. 070-100) were purchased from Cell Applications, Inc.$^{TM}$ (San Diego, CA, USA). Cells were subcultured in 75 cm flasks (Corning, Inc, New York, NY, USA) at 37$^{o}$C in a 5% $CO_2$ humidified incubator, and expanded for at least five passages. The medium was changed twice a week and subcultured when they are about 80% confluent. For total RNA extraction of B16 or HMV-II cells, cells at a density of $3 \times 10^6$ cells per petri dish were seeded onto 100-mm dishes and cultivated using the method described above. After overnight incubation, the medium was then replaced with 1 µM hirsein A (HA)-containing medium followed by incubation for 4 h prior to the extraction of total protein or total RNA. For RNA isolation from HEM, cells were seeded into 10-cm culture plates and incubated for 24 hours, after which, HEM were treated with or without HA and incubated for an additional 4 h prior to total RNA isolation. HA was isolated as described previously [31] while Rp-cAMPS (adenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer) was obtained from SantaCruz Biotechnology (Santa Cruz, CA) and the α-MSH (alpha melanocyte-stimulating hormone) from Sigma,( St Louis, MO, USA ). Rp-cAMPS was used as a positive control for the inhibition of

the cAMP-dependent protein kinase (PKA) [32]while α-MSH was used to activate the cAMP pathway [ 33].

**Total RNA Extraction**

Total RNA was extracted using ISOGEN kit (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions and quantified using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies). Reverse transcription polymerase chain reaction (RT-PCR) was carried out with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, using 1 μg of total RNA. Three different RNA sample sets were used to ensure that the confirmation of the gene expression patterns by real-time PCR and employed a different sample set than those used for DNA microarray.

**DNA Microarrays**

To determine the gene expression changes in B16 cells treated with HA, DNA chips loaded with 265 genes prepared by GenoPal™ microarray (Mitsubishi Rayon Co., Ltd, Tokyo, Japan), were used to determine the effect on genes significant for melanogenesis, membrane-bound receptors, tyrosine kinase regulation, melanosome transport, and other cell signal regulation-related genes (including the housekeeping and negative control genes). aRNA was synthesized from total RNA previously extracted from B16 cells following the GenoPal protocol. Briefly, RNA was amplified using the MessageAmpII biotin-enhanced amplification kit (Applied Biosystems Japan, Tokyo, Japan), according to the manufacturer's instructions, and column purified. Biotinylated aRNA (5 μg) was fragmented using fragmentation reagents (Applied Biosystems Japan) and then incubated at 94°C for 7.5 min. Hybridization was carried out in 150

µL of hybridization buffer [0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20] and 5 µg of fragmented biotinylated aRNA at 65°C overnight. After hybridization, the DNA microarray was washed twice in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at 65 °C for 20 min followed by washing in 0.12 M Tris-HCl/0.12 M NaCl for 10 min. The DNA microarray was then labeled with streptavidin-Cy5 (GE Healthcare Bio-Science KK, Tokyo, Japan). The fluorescent-labeled DNA microarray was washed for 5 min four times in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at room temperature. Hybridization signal acquisition was performed using a DNA microarray reader adopting multibeam excitation technology (Yokogawa Electric Co., Tokyo, Japan). The DNA microarrays were scanned at multiple exposure times ranging from 0.1 to 40 s. Then, the intensity values with the best exposure condition for each spot were selected.

**Analysis of the microarray data**

Detected hybridization signals that were higher than the background intensity (BI) were regarded as 'positive' and the relative amount of each transcript was normalized to the amount of the *Actb*, *Arbp*, *GAPDH*, *GUSB*, and *PGK1* transcripts in each sample. Transcripts were considered significantly altered when the average log of the ratio (between treated and untreated control) calculated from 3 replicates per treatment was $\leq$ -0.5 for the downregulated genes and $\geq$ 0.5 for the upregulated genes. The log of the ratio between the treated sample and control was then subjected to hierarchical clustering using Euclidean distance and average linkage algorithm of the TIGR Mev version 3.0.3 software (The Institute for Genomic Research, MD, USA) [33] for visualization of the response to HA, Rp-cAMPS or α-MSH treatment. Gene expression ratios were displayed in green and red for decreased and increased gene expression, respectively, compared to untreated control.

**Quantitative real-time PCR analysis**

Quantitative real-time polymerase chain reaction (rt-PCR) analysis was performed with a 7500 Fast Real-Time PCR system using TaqMan Universal PCR mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA) to quantify the expression of the following genes: *Mitf*, *Mc1r*, *Myo5a*, *Myo7a*, *Rab27a*, *Mlph*, *Sorbs3*, *Ppap2b*, *Gadd45b*, *Map2k3*, *Wisp1*, and *Prkx*. *Gapdh* was used as a housekeeping gene. Rt-PCR reactions were run on ABI 7500 Fast (Applied Biosystems) with the following thermal cycling protocol: 95 $^{o}$C for 10 min followed by 40 cycles of 95 $^{o}$C for 15 s and 60 $^{o}$C for 1 min. The Assay ID of the primers (Applied Biosystems) used for Rt-PCR are the following : *Mitf* – Mm00434954_m1, *Mc1r* - Mm00434851_s1, *Rab27a*- Mm00469997_m1, *Sorbs3*- Mm00488174_m1, *MyoVa* - Mm00487823_m1, MyoVIIa - Mm01274015_m1, *Mlph* – Mm00453498_m1, *Ppap2b*- Mm00504516_m1 , *Gadd45b* - Mm00435123_m1, *Map2k3* – Mm00435950_m1, Prkx – Mm00457493_m1, *Wisp1*- Mm00457574_m1, *Pxn* – Mm00448533_m1, *Il24* – Mm00474102_m, 1 and *Gapdh* – Mm99999915_g1. Data were analyzed using 7500 Fast System SDS Software 1.3.1. (Applied Biosystems).

**F-actin staining**

The distribution of F-actin was visualized by staining B16 cells with phalloidin-rhodamine. B16 cells were subcultured onto four-well chamber slides (Nunc, Rochester, NY, USA) at a density of $4 \times 10^4$ cells per well. After overnight incubation, the medium was replaced with 1 μM HA- or 0.2 μM α-MSH-containing medium and incubated for an additional 24 h. After removing the medium, the cells were washed with PBS and fixed with 3.7% formaldehyde for 5 min, washed and permeabilized with 0.2% Triton X-100 in PBS for 5 min,

and then washed with PBS thrice. The cells were then stained with rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO, USA) (70 nM) and incubated for 30 min at room temperature in the dark, after which the cells were again washed three times with PBS. Mounting reagent containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was added and fluorescent signals were detected and photographed using a Leica DMI-4000B fluorescence microscope and a Leica DFC300FX CCD camera (Leica, Wetzlar, Germany). The images were analyzed using a light microscope system (Leica).

**Transfection experiments**

To determine if the observed change in cell morphology was due to the inhibition of the Mc1r gene expression, transfection experiments using Lipofectamine RNAiMAX (Invitrogen) complexed with sequence-specific Silencer Select siRNA that targets murine Mc1r mRNA purchased from Ambion (Austin, TX) (sense: 5'-CCCUCUUUAUCACCUACUAtt-3', anti-sense: 5'-UAGUAGGUGAUAAAGAGGGtg-3') was performed following the manufacturer's instructions. B16 cells were seeded into 6-well plates at a density of $1\times10^5$ cells per well, incubated for 24 prior to transfection with Negative control siRNA or Mc1r siRNA (at a final concentration of 5 nM). After 24 h of incubation, control cells were left untreated or treated with 200 nM α-MSH or 1 μM hirsein A (HA) and incubated further for 4 h prior to RNA extraction.

**Statistical evaluation**

Mean values ± SEM were calculated; statistical analyses of the results were performed using Student's t-test to determine the significance of results versus that of the control. A value

of $p< 0.05$ was considered significant.

**Results**

**Transcriptional profile of the hirsein A-treated B16 cells**

To shed light on the molecular mechanism of the effect of hirsein (HA) treatment on the genes that are important for pigmentation and intracellular signaling pathways, the gene expression profile of B16 melanoma cells, cultured in the presence or absence of HA, Rp-cAMPS or α-MSH, was analyzed using DNA microarray. Gene expressions, expressed as $\log_2$, were decreased for *Mitf* and *Mc1r*, by about 2- to 3-fold, respectively, while the expressions of the melanosome transport-associated genes (*Rab27a* and *Mlph*), were decreased 2-fold (Table 1). In addition, *Sorbs3*, a gene involved in cell adhesion, negative regulation of transcription from RNA polymerase II promoter, and positive regulation of MAPKKK cascade, was also significantly downregulated by HA treatment but not with Rp-cAMPS or α-MSH treatment. Rp-cAMPS or α-MSH caused a slight increase in *Sorbs3* gene expression. Furthermore, HA treatment downregulated the expressions of the following genes: *Mreg* (functions in developmental or melanocyte differentiation), *Agt* (functions in G-protein coupled receptor protein signaling pathway, and activation of phospholipase C activity), *Map2k5* (functions in MAPKKK cascade, positive regulation of cell growth and epithelial cell proliferation), *Prkar2b* (functions in cell proliferation, regulation of protein kinase activity and protein phosphorylation, signal transduction). The downregulated (11) and upregulated (22) genes were subjected to hierarchical clustering to determine the relationship between genes (Figure 1). From among these 33 genes modulated by HA, α-MSH and Rp-cAMPS treatments had an influence on the expressions of 22 and 25 genes, respectively. Treatment with HA, but not with Rp-cAMPS or α-MSH, caused a two- or three-fold increase in the expressions of the following genes: *Ppap2b*,

*Pxn*, *Gadd45b*, *Wisp1*, *Mapkapk3* and *Ppm1*. The gene tree generated by hierarchical clustering, yielded two main groups that represent the HA-upregulated and the HA-downregulated genes, with two and three subgroups each, respectively.

**Differentially expressed genes validated using real-time PCR**

The relative changes in the expression of genes, initially determined by microarray as expressed differentially following hirsein treatment, were validated using the TaqMan gene expression system and presented in Figures 2a (*Mitf* and *Mc1r* genes), 2b (*Rab27a*, *Mlph*, and *MyoVa* or melanosome transport-associated genes), Figure 2c (*Sorbs3*), and Figure 2d (six of the upregulated genes). Results show that the mRNA levels for *Mitf* and *Mc1r* gene expressions were decreased significantly ($p<0.05$). In the same manner, the expressions of the melanosome transport associated genes were reduced by 50% compared to that of the control. Highly significant increases in the expressions of the *Ppap2b*, *Gadd45b*, and the *Map2k3* genes were observed. *Ppap2b* and *Gadd45b* genes were increased by 800% and 200%, respectively. The results of the real-time PCR analysis showed changes in genes' expressions which are consistent with the microarray data.

**Enhanced *Mda-7* gene expression confirmed the cell differentiation effects of HA**

The increase in the expressions of the cell differentiation-associated genes, *Ppap2b* and *Gadd45b* prompted us to determine if the observed change in morphology may be due to differentiation by determining the mRNA expression of the melanoma differentiation-associated gene 7 (*Mda-7)* gene in B16 cells. The expression of the *Mda-7* gene in HA-treated murine melanoma cells, human melanoma, and human melanocytes were compared by determining the

expression of the *Mda-7* gene in B16 cells, HMV-II cells, and HEMs using real-time PCR (Fig. 3). Compared with the control, results show that treatment with HA or α-MSH treatment promoted a significant increase in *Mda-7* gene expression in B16 cells. In HMV-II cells however, only HA was able to increase the *Mda-7* gene expression. Both HA and α-MSH significantly increased the expression of *Mda-7* gene in human melanocytes.

**Staining with rhodamine-phalloidin revealed an increase in F-actin polymerization**

To determine if the increase in dendricity after HA treatment is due to an increase in the F-actin polymerization, we determined the expression of F-actin by staining B16 cells, treated with HA or α-MSH, with rhodamine-phalloidin. Results showed that HA-treated cells had more phalloidin-labeled F-actin (Fig. 4) than the control or α-MSH-treated cells. HA-treated cells had dendritic extensions not observed in control or MSH-treated cells due to a changed F-actin polymerization. It can also be noted that like melanocytes, HA-treated cells appeared to be scattered, and not clumped together as observed in control cells, similar to that of the melanocytes in the skin.

**Silencing the Mc1r gene expression did not cause the change in cell morphology**

To determine if the change in morphology was caused by the inhibition of the *Mc1r* gene expression, transfection experiments were performed. Cells that were transfected with Mc1r siRNA and negative control siRNA exhibited a change in cell morphology (Fig. 5a). Verifying the gene expressions using real-time PCR revealed a significant increase and decrease in expression of Mc1r genes in Negative Control-transfected cells treated with α-MSH and HA respectively. Trasfection with *Mc1r* siRNA silenced the *Mc1r* gene expression and subsequent

treatment with α-MSH did not cause any increase in expression. Treatment with HA, however, further decreased the expression of Mc1r gene (Fig. 5b). To determine the effect of Mc1r knowckdown on *Mitf* mRNA expression, real-time PCR was also performed using specific primers for *Mitf*. Results showed that in the same manner, negative control siRNA-transfected cells had significant increase in *Mitf* expression when treated with α-MSH and significant inhibition of expression when treated with HA (Figure 5c). Although transfection with *Mc1r* siRNA caused a decrease in *Mitf* expression, its expression was not completely inhibited (25%). Treatment with α-MSH did not increase the *Mitf* expression more than that of the control. Treatment with HA further decreased (by 95%) the *Mitf* gene expression.

**Discussion**

Our data showed that HA, a daphnane diterpene that has a melanogenesis inhibitory effect, elicited a unique transcriptional response in B16 melanoma cells. A few of the differentially expressed genes are those that have been reported to be regulated by the *Mitf* gene such as the melanosome transport genes *Rab27a*, MyoVa, and the Min Table 1), as well as genes associated with the MAPK pathway, MAPKKK cascade, phospholipid metabolic process, Wnt signaling pathway, and cell differentiation (Table 2).

Hierarchical clustering comparing HA with α-MSH or Rp-cAMPS revealed two main clusters representing the HA-downregulated and the HA-upregulated genes (Fig. 1). The downregulated genes formed three subclusters – with the highly downregulated genes in the two subclusters (*Mitf* gene in one cluster and *Mc1r*, *Mlph*, *Rab27a*, and *Sorbs3* genes in the second). *Mitf* is known to regulate the expressions of *Rab27a*, *Mc1r* [16, 35-36]. Clusters based on gene expression data are enriched for genes known to be involved in similar biological processes, implying that genes of unknown function may be involved in those same processes [37-38]. The obtained results, therefore, imply that since Sorbs3 was clustered with the *Mitf*-regulated genes, the *Mitf* gene might have an influence on the expression of the *Sorbs3* gene. Although more tests are yet to be done to determine this relationship between *Mitf* and *Sorbs3*, it has been established that co-expression of genes of known function with poorly characterized or novel genes may provide a simple means of gaining leads to the functions of many genes for which information is not currently available [37]. Both *Mc1r* and *Sorbs3* genes contain NF-kappaB1 and NF-kappaB in their upstream promoter region **(**www.genecards.org/, accessed 1 December 2010). Reports on *Sorbs3* gene associated it with cell adhesion and regulation of transcription from RNA polymerase II promoter, and its gene product, vinexin, functions to enhance actin cytoskeletal

organization [39].

Genes that were downregulated by HA, such as *Mitf and Mc1r*, are significant for the inhibition of overall pigmentation. One of the regulators of *Mitf* gene expression is the α-MSH. α-MSH activates melanocortin-1 receptor (Mc1r), resulting in a cAMP-mediated phosphorylation/activation of the CREB/ATF family of transcription factors to induce *Mitf* expression and regulate pigmentation in melanocytes [40]. Moreover, one of the most important signaling pathways found to induce melanogenesis is the cAMP/protein kinase A (PKA) pathway. *Mc1r* promoter activity was induced approximately 5-fold in the presence of Mitf [41]. Although it has been reported that the *MC1R* promoter may also be regulated by *Mitf*, raising the possibility that *Mitf* and *Mc1R* participate in a feedback loop [42], it has also been observed that in the presence of Mitf, the *Mc1r* promoter activity is induced 5-fold. Cyclic AMP, through the activation of PKA and cAMP-responsive element binding protein 1 transcription factors, upregulates the expression of *Mitf*. Elevated cAMP levels induce and increase Mitf levels, and therefore suggests that in principle, *Mitf* expression should be regulated by *Mc1r*. [43]. The time-dependent decrease in the cellular cAMP content (data not shown) means that the decrease in cAMP content occurred in a later stage while the inhibition of *Mitf* gene expression occurred 4 h after treatment with HA suggesting that *Mitf* was inhibited not just by the decrease in the *Mc1r* gene expression but by also other signaling pathway such as the MAPK pathway.

The four genes that were downregulated are *Rab27a*, M*yoVA*, *Mlph*, and *Myo7a* and these genes play an important role in melanosome transport. Melanosomes develop and mature within melanocytes and their transport from the cell body of melanocytes to the tip of their dendrites requires two distinct motors, Rab27a [16-17] and myosinVa [17], while Slac2-a/Melanophilin binds Rab27a and myoVa [18-19].

For the upregulated genes, the hierarchical clustering analysis produced two main clusters with the four highly expressed genes (*Ppap2b, Gadd45b, Pxn, Map2k3)* in one cluster, and other 19 genes in the second cluster (Fig.1). *Ppap2b* codes for protein that has lipid phosphatase activity and is involved in lipid phosphorylation, blood vessel development, and the Wnt signaling pathway among others. The expression of *Ppap2b* gene is found to be enhanced by epidermal growth factor in Hela cells. [www.genecards.org/,accessed 1 Dec 2010]. *Gadd45b* gene is associated with the regulation of growth, apoptosis, and the cell cycle regulation. *Gadd45b* gene expression is known to be induced by NFκB (its promoter has three NFκB-binding sites) [44].

Although most of the genes from same pathways were regulated in similar manner, MAPK signaling pathway genes were were up- or down-regulated. *Map2k5* gene was downregulated but the *Map2k3* gene was upregulated. *Map2k5* acts as a scaffold for the formation of a ternary MAP3K2/MAP3K3-MAP3K5-MAPK7 signaling complex (http://www.genecards.org/cgi-bin/carddisp.pl?gene=MAP2K5). The *Map2k3* gene has a MAP kinase kinase activity [45-46] and involved in the regulation of the cytokine biosynthetic process [47]. Map2k3 phosphorylates, and thus activates, MAPK14/p38-MAPK. This kinase can be stimulated by insulin [48] and by cytokine treatment in human islets and in a mouse beta cell line [49]. Several genes that activate the MAPK activity and the MAPKKK cascade were also upregulated by HA: *Gadd45b*, *Pxn*, *Map2k3*, *Met*, *Mapkapk3*, *Avp1*, *Spag9*, and *Ppm1* (Table2). MAPK signaling cascades, in mammals, regulate important cellular processes such as gene expression, cell proliferation, cell survival and death. Transcription factors are important MAPK targets and that the genes that are regulated in response to MAPKs may be defined by DNA microarray [50]. HA-treatment caused a 3-fold increase in the expression of Map2k3 gene compared to the control. P38 MAPK

signaling cascade has been suggested to activate melanogenesis by activating Mitf [51-52] The decrease in the expression of *Map2k5* gene, revealed by the microarray results, suggests the inhibition of the mitogen-activated protein kinase (MAPK) activity (Table 1). Results of the determination of the expression of the activation of pERK1/2 revealed an activation of ERK1/2 (data not shown). We have previously reported that pERK1/2 expression was increased by *T. hirsuta* extract treatment [53] verifying our previous findings.

The effect of HA on B16 cells was compared with that of the untreated cells (control), α-MSH and Rp-cAMPS. α-MSH stimulates melanogenesis by activating the MC1 receptor (MC1-R) on melanocytes and causes an increase in the dendricity of melanocytes [54-55], while Rp-cAMPS inhibits melanogenesis through its cAMP antagonistic action [32]. The observed effect of α-MSH was not as significant as HA but this could be attributed to the presence of the serum in the medium which could have had a masking effect, as the addition of 200 nm α-MSH was enough to increase the cAMP level significantly. Validation of the DNA microarray results using quantitative rt-PCR showed an observed high correlation between quantitative gene expression values and microarray platform results (Figure 2a-2d). Rt-PCR supports the use of microarray platforms for the quantitative characterization of gene expression [56].

The increase in the expression of the *Gadd45b* gene prompted us to determine the expression its upstream, the melanoma differentiation-associated 7 (*Mda-7*) gene. Recently, the *Mda-7* or interleukin 24 (*mda-7*/*IL-24)* gene has been recognized to have a potential as an anticancer gene, and has been the focus of some groups doing cancer research. We determined the effect of HA on the expression of the *Mda-7* gene in both mouse and human melanoma cells and compared it with its effect on the human epidermal melanocyte. Results show that HA significantly increased the expression of *Mda-7* gene in both mouse and human melanoma cells

and in human epidermal melanocytes (Figure 3). It has been reported that a noteworthy aspect of *mda*-7/IL-24 as a cancer therapeutic is its ability to selectively kill cancer cells without harming normal cells [57].

The increase in the expression of the *Mda-7* gene has been reported to occur after the induction of irreversible growth arrest and terminal differentiation in human melanoma cells treated with recombinant human fibroblast interferon [26, 58]. Here, HA-treated cells exhibited dendricity (Fig. 4), which is a distinctive feature of cell differentiation. Dendrite formation in melanocytes requires actin polymerization in the newly forming dendrite, and dendrite formation in melanocyte is stimulated by hormones and ultraviolet light [53]. Dendricity has been established as a hallmark of cell differentiation in melanocytes [21,59]. HA treatment caused the B16 melanoma cell differentiation, as shown by the F-actin stained cells (Figure 4). HA-treated cells had more dendritic extensions than the control or MSH-treated cells due to a changed F-actin polymerization. Moreover, like melanocytes, HA-treated cells appeared to be scattered, and not clumped together as observed in control cells. The appearance of the HA-treated cells is similar to that of the melanocytes in the skin. In a related study, Edgar and Bennett [60]reported that inhibition of Myosin VA inhibited the formation of dendrites by melanocytes. Here, Myosin VA was inhibited (Table 1) but dendricity was stimulated in the B16 cells. Melanocytes are known to be dendritic and are scattered in the basal layer of the epidermis [61]. It is also interesting to note that while dendricity is usually observed in α-MSH-treated cells due to cAMP upregulation of dendrite formation, HA decreased the expression of the *Mc1r* gene, the cAMP pathway receptor, but still was able to cause an increase in dendrite formation (Figure 5a and 5b). Usually, dendrite formation is a rapid process preceding tyrosinase protein expression and melanosome relocalization [14]. In stationary spread cells, expanding circular F-actin-containing

structures were found to be associated with cell shape changes and reorganization of the actin cytoskeleton [62]. Our data shows that the observed dendritic morphology following HA treatment, was most likely due to the enhanced expressions of genes associated with the cytoskeleton (*Pxn)* and cell differentiation *(Gadd45b).* In general, the extent of dendricity of melanocytes correlates directly with their melanogenic activity. Melanocytic dendrites are microtubule-containing structures and their extension is regulated by a number of growth factors, including α-MSH, ET-1, and NGF, as well as by UVR [63]. Indirectly, *Mc1r* inhibition will cause a decrease in melanocyte dendricity as the inhibition of the Mc1r function induces melanocytes to switched into their pheomelanogenic mode which means an inhibition of the expression of all known melanosomal proteins to basal levels, except for tyrosinase [64,65]. Decreased melanogenesis would mean less melanosome to transport through the cell dendritic extensions.

HA activated the genes that are intermediates of the MAPK cascade and promoted *Il-24/Mda-7* gene expression leading to decreased *Mitf* gene expression and increased cell dendricity, respectively. The downregulation of the *Mitf* gene expression was through the inhibition of the two signaling pathways involved in melanogenesis – the MAPK pathway, and the cAMP pathway, through the downregulation of the *Mc1r* gene expression. This study demonstrated that HA causes *Mitf* inhibition by reducing the expressions of MAPKK intermediates. As expected, the downregulation of *Mitf* expression caused a decrease in the expressions of *Mc1r* and melanosome transport genes. It was also observed that the inhibition of the Mc1r gene only caused a partial decrease in *Mitf* gene expression (Fig. 5c), which means that the inhibition of the MAPK pathway also contributed to the overall downregulation of the *Mitf*

gene following HA treatment. For the change in the cell morphology, genes that are observed to be expressed by differentiating cells, as well as the melanoma differentiation-associated gene (*Mda-7*), were upregulated, and confirms that the cell differentiation effects of HA is independent of its melanogenesis regulatory effects. HA, a daphnane-type diterpene like known cell differentiation agenes -retinoic acid and mezerein, is not just a novel melanogenesis inhibitor but also as a cell differentiation agent that also has potential for use in therapy or management of melanoma.

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Table 1. List of differentially regulated genes in in hirsein A (HA)-, α –MSH- and Rp-cAMPS-treated B16 murine melanoma cells ( ≤ - 0.5 log) as determined by DNA microarray.

| Gene symbol | Gene Name | Biological Process | Log gene expression[a] (Treated/Control) | | |
|---|---|---|---|---|---|
| | | | α -MSH | Rp-cAMPS | HA |
| *Mitf* | microphthalmia-associated transcription factor | Cell differentiation, melanocyte differentiation, negative regulation of apoptosis, pigmentation, regulation of transcription, Wnt receptor signaling pathway | 0.40 | -0.03 | -3.68 |
| *Rab27a* | RAB27A, member RAS oncogene family | Melanosome transport, melanocyte differentiation, | -0.18 | -0.12 | -2.2 |
| *Sorbs3* | sorbin and SH3 domain containing 3 | Cell adhesion, negative regulation of transcription from RNA polymerase II promoter, positive regulation of MAPKKK cascade | 0.26 | 0.18 | -2.08 |
| *Mc1r* | Melanocortin 1 receptor | Melanin biosynthetic process, intracellular protein kinase cascade, positive regulation of protein kinase A and protein kinase C signaling cascade | 0.27 | -0.03 | -1.76 |
| *Mlph* | Melanophilin | Intracellular protein transport, melanocyte differentiation, melanosome localization, pigmentation | -0.20 | -0.05 | -1.52 |
| *Ldb1* | LIM domain binding 1 | Regulation of transcription, Wnt receptor signaling pathway | 0.09 | 0.10 | -0.62 |
| *Myo7a* | Myosin VIIa | Actin filament-based movement, ATP catabolic process, melanosome transport | 0.25 | 0.17 | -0.86 |
| *Mreg* | melanoregulin | Developmental pigmentation, melanocyte differentiation, pigmentation | 0.00 | -0.16 | -0.65 |
| *Agt* | angiotensinogen (serpin peptidase inhibitor, clade | G-protein coupled receptor protein signaling pathway, Activation of phospholipase C activity | -0.23 | -0.02 | -0.57 |
| *Map2k5* | mitogen-activated protein kinase kinase 5 | MAPKKK cascade, positive regulation of cell growth and epithelial cell proliferation | -0.01 | 0.16 | -0.52 |
| *Prkar2b* | protein kinase, cAMP dependent regulatory, type II beta | Cell proliferation, regulation of protein kinase activity and protein phosphorylation, signal transduction | -0.48 | -0.20 | -0.50 |

[a]Data is shown as signal $\log_2$ ratio to control

Table 2. List of differentially upregulated genes in in hirsein A (HA)-, α –MSH- and Rp-cAMPS- treated B16 murine melanoma cells (≥ 0.5 log) as determined by DNA microarray.

| Gene symbol | Gene Name | Biological Process | Log gene expression[a] (Treated/Control) | | |
|---|---|---|---|---|---|
| | | | α - MSH | Rp-cAMPS | HA |
| *Ppap2b* | phosphatidic acid phosphatase type 2B | Phospholipid metabolism, blood vessel development, Regulation of Wnt receptor signaling pathway, positive regulation of transcription factor activity | nd[b] | nd | 3.65 |
| *Gadd45b* | growth arrest and DNA-damage-inducible 45 beta | Activation of MAPKK activity, apoptosis, cell differentiation, negative regulation of protein kinase activity, regulation of cell cycle | nd | nd | 2..85 |
| *Pxn* | Paxillin | Activation of MAPK activity, cell adhesion, cytoskeleton organization, lamellipodium assembly, | nd | nd | 2.35 |
| *Map2k3* | mitogen-activated protein kinase kinase | , Activation of MAPK activity, MAPKKK cascade, regulation of cytokine biosynthetic process, protein phosphorylation,cell proliferation and differentiation | 0.42 | 0.13 | 2.13 |
| *Wisp1* | WNT1 inducible signaling pathway protein 1 | Cell adhesion, regulation of cell growth, Wnt receptor signaling pathway | nd | nd | 1.26 |
| *Prkx* | protein kinase, X-linked | Protein phosphorylation | 0.4 | 0.22 | 1.49 |
| *Met* | met proto-oncogene (hepatocyte growth factor) | Activation of MAPK activity, positive regulation of dendrite morphogenesis | 1.13 | -0.09 | 1.3 |
| *Gclc* | glutamate-cysteine ligase, catalytic subunit | Positive regulation of protein ubiquitination, glutathione metabolic process and biosynthetic process | 0.23 | 0.00 | 1.42 |
| *Creb3l2* | cAMP-responsive element binding protein 3-like 2 | Regulation of DNA-dependent transcription, regulation of transcription | -0.07 | 0.06 | 1.29 |
| *Shc1* | src homology 2 | Cell-cell adhesion, intracellular | nd | 0.06 | 1.26 |

| | | | | | |
|---|---|---|---|---|---|
| | domain-containing transforming | signaling pathway, neuron differentiation, regulation of growth | | | |
| *Kremen1* | kringle containing transmembrane protein 1 | Wnt receptor signaling pathway | nd | nd | 1.01 |
| *Mapkapk 3* | mitogen-activated protein kinase-activated protein kinase 3 | Protein Phosphorylation | nd | nd | 1.02 |
| *Avpi1* | arginine vasopressin-induced 1 | Activation of MAPK activity, cell cycle | 0.69 | 0.12 | 0.99 |
| *Senp2* | SUMO/sentrin specific peptidase 2 | mRNA transport, Wnt receptor signaling pathway | 0.03 | -0.27 | 0.90 |
| *Spag9* | sperm associated antigen 9 | Activation of JUN kinase activity, MAPK activity | 0.23 | 0.05 | 0.76 |
| *Cno* | cappuccino | Melanosome organization | 0.00 | -0.05 | 0.70 |
| *Kif2a/Kn s2* | kinesin family member 2A | Cell cycle, cell differentiation, cell division, microtubule-based movement, mitosis | nd | 0.10 | 0.67 |
| *Csnk1a1* | casein kinase 1, alpha 1 | Cell cycle, cytoskeleton, cell division, cell morphogenesis, Wnt receptor signaling pathway, mitosis | 0.05 | -0.01 | 0.66 |
| *Atf4* | activating transcription factor 4 | Positive regulation of transcription from RNA polymerase II promoter | 0.35 | 0.07 | 0.64 |
| *Ppm1l* | protein phosphatase 1 (formerly 2C)-like | MAPKKK cascade, Protein dephosphorylation, transmembrane receptor protein serine/threonine kinase signaling pathway, | nd | nd | 0.59 |
| *Adcy7* | adenylate cyclase 7 | Activation of adenylate cyclase activity, cAMP biosynthetic process, intracellular signaling pathway, | nd | nd | 0.57 |
| *Csnk2b* | casein kinase 2, beta polypeptide | Regulation of catalytic activity, regulation of phosphorylation, Wnt receptor signaling pathway | 0.16 | 0.01 | 0.55 |
| *Axin1* | Axin 1 | Apoptosis, Wnt receptor signaling pathway, cytoplasmic microtubule organization | -0.07 | 0.13 | 0.55 |

[a]Data is shown as signal $\log_2$ ratio to control
[b] nd – not detected

**Figure Legends**

**Fig.**1 Gene expression map of 33 genes that were differentially expressed in B16 melanoma cells treated with hirsein A (HA), α-MSH, or Rp-cAMPS. The microarray chip was loaded with 265 genes for melanogenesis, melanosome transport, tyrosine kinase, cell signal transduction. The 33 genes subjected to hierarchical clustering were chosen as having a log of -0.5 or lower (the ratio between HA and untreated control) for the downregulated genes and 0.5 or higher (the ratio between HA and untreated control) for the upregulated genes. Horizontal stripes represent genes and columns show experimental samples. Clustering was performed on genes using the euclidian distance method. Red and green color codes for up- and downregulation, respectively.

**Fig.**2 Effect of hirsein A (HA) on the expression of the genes for (a) melanogenesis regulation - microphthalmia-associated transcription factor (*Mitf*) and melanocortin 1 receptor (*Mc1r*), (b) melanosome transport - *Rab27a*, melanophilin (*Mlph*), and Myosin Va (*Myo5a*), (c) sorbin and SH3 domain containing 3 (*Sorbs3)* gene, and (d) upregulated genes in B16 cells. B16 cells were seeded at a density of $3\times10^6$ cells per 100-mm dish. After overnight incubation, the cells were treated with or without 25 μM Rp-cAMPS, 0.2 μmol/L α–MSH, 1 μM hirsein A (HA) for 4 h, after which, RNA isolation and then reverse transcription PCR was carried out to obtain cDNAs that were used for real-time PCR. Results represent the mean ± S.D. of three independent experiments. * Statistically significant ($P< 0.05$) difference between control and treated cells.

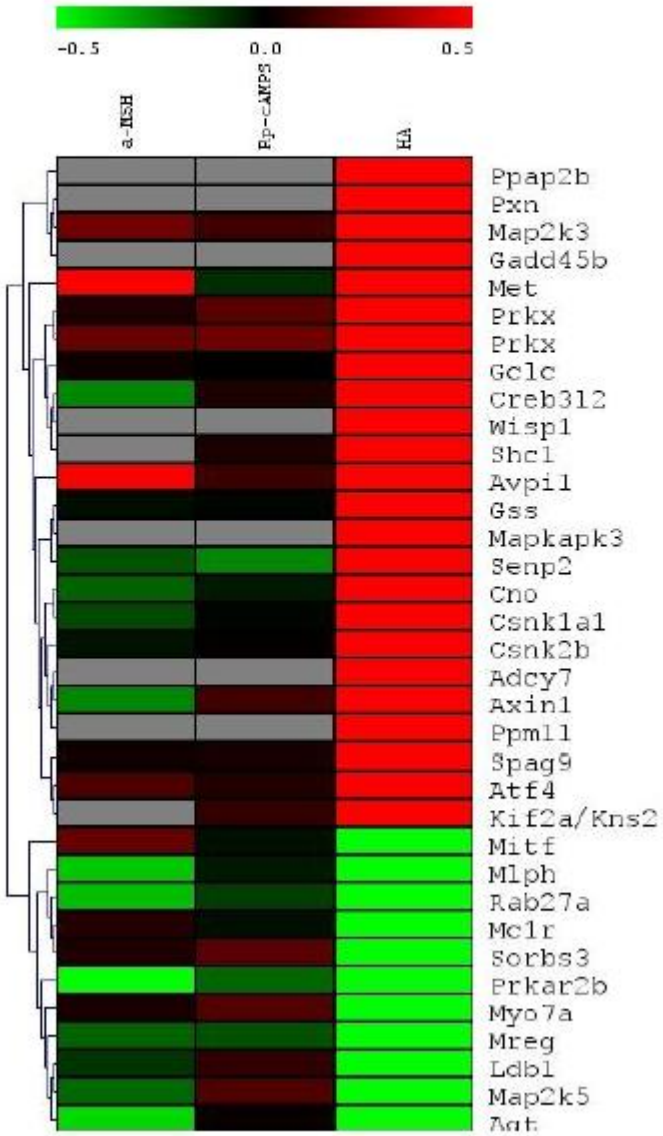
**Fig.** 3. Effect of hirsein A (HA) on the expression of melanoma differentiation gene-7 (*Mda-7*) in B16 melanoma cells (B16), HMV-II melanoma cells (HMV-II), and human epithelial melanocytes (HEM). Cells were seeded at a density of $3\times10^6$ cells per 100-mm dish. After

overnight incubation, the cells were treated with or without 0.2 μM α–MSH or 1 μM hirsein A (HA) for 4 h after which RNA isolation and then reverse transcription PCR was carried out to obtain cDNAs that were used for real-time PCR. Results represent the mean ± S.D. of three independent experiments. * Statistically significant ($P< 0.05$) difference between control and treated cells.

**Fig**. 4. F-actin polymerization in B16 murine melanoma cells. B16 cells were seeded onto four-well chamber slides at $4 \times 10^4$ cells per well. After overnight incubation, the cells were treated with or without 0.2 μM alpha melanocyte stimulating hormone (α–MSH) or 1 μM hirsein A (HA) for 24 h. The cells were stained with rhodamine-phalloidin for the actin cytoskeleton (left panels) and with DAPI for the nuclei (middle panels). Merged images are shown in the right panels. All photographs were taken at 200× magnification. Each bar represents 20 μm.

**Fig.5**. The effect of hirsein A (HA) on the (a) cell morphology (b) *Mc1r* mRNA and (c) *Mitf* mRNA expression of B16 murine melanoma cells transfected with *Mc1r* siRNA. B16 cells were seeded into 6-well plates at a density of $1\times10^5$ cells per well, and the next day cells were transfected with Negative control siRNA or Mc1r siRNA complexed with Lipofectamine RNAiMAX (Invitrogen). After 24 h of incubation, control cells were left untreated (transfection reagent alone), or treated with 200 nM α-MSH or 1 μM hirsein A (HA) and incubated further for 4 h prior to RNA extraction. Images were taken using Leica DM1L microscope camera.

Figure 1


-0.5
0.0
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a-MSH
Rp-cAMPS
HA
Ppap2b
Pxn
Map2k3
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Prkx
Prkx
Gclc
Creb312
Wisp1
Shc1
Avpi1
Gss
Mapkapk3
Senp2
Cno
Csnk1a1
Csnk2b
Adcy7
Axin1
Ppm1l
Spag9
Atf4
Kif2a/Kns2
Mitf
Mlph
Rab27a
Mc1r
Sorbs3
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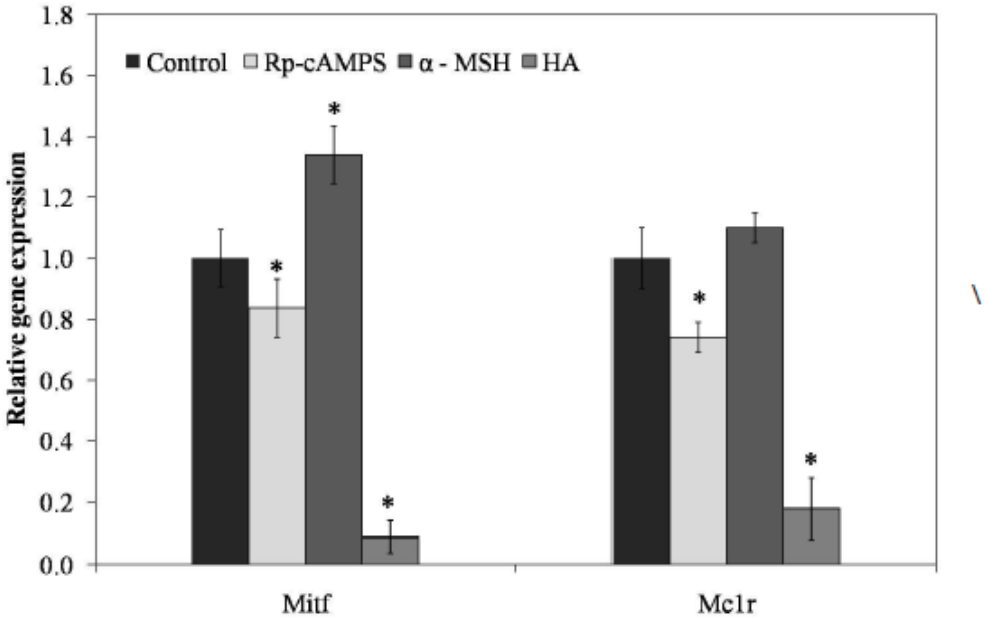
Mreg
Ldb1
Map2k5
Agt

Figure 2.a


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative gene expression
Control
Rp-cAMPS
α - MSH
HA
*
*
*
*
*
*
Mitf
Mc1r

\

Figure 2.b

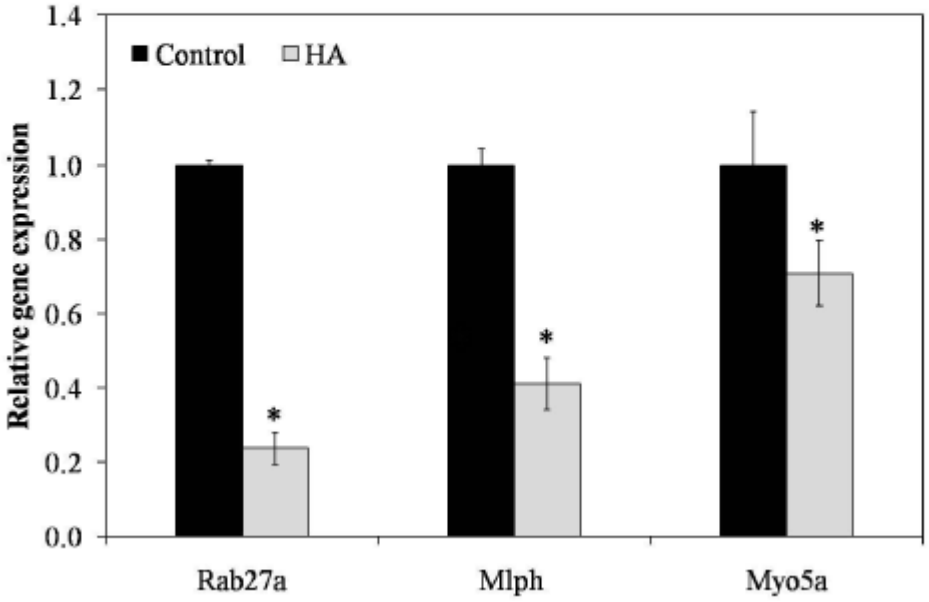

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative gene expression
Control
HA
*
*
*
Rab27a
Mlph
Myo5a

Figure 2.c

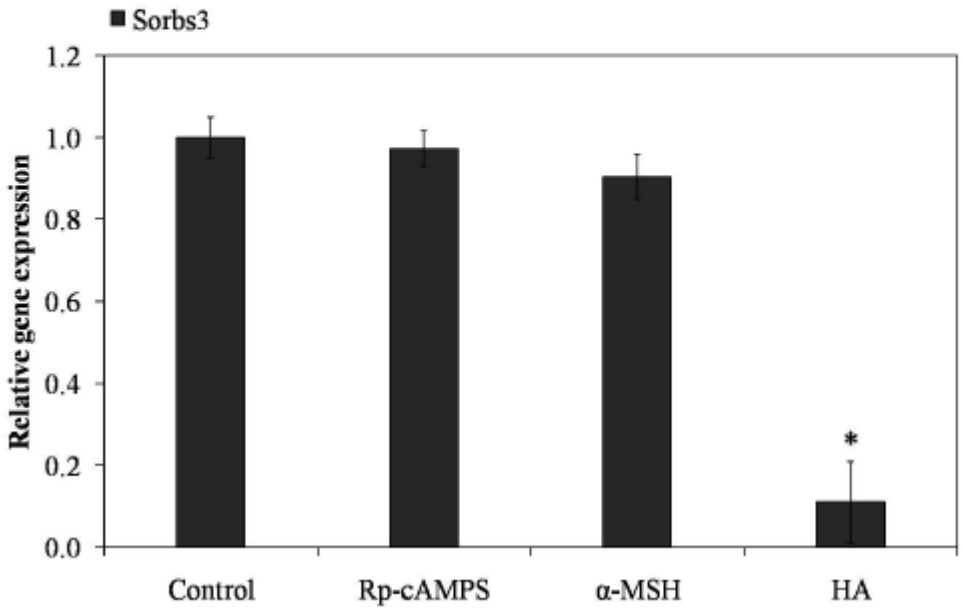

Sorbs3
Relative gene expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Rp-cAMPS
α-MSH
HA

Figure 2.d

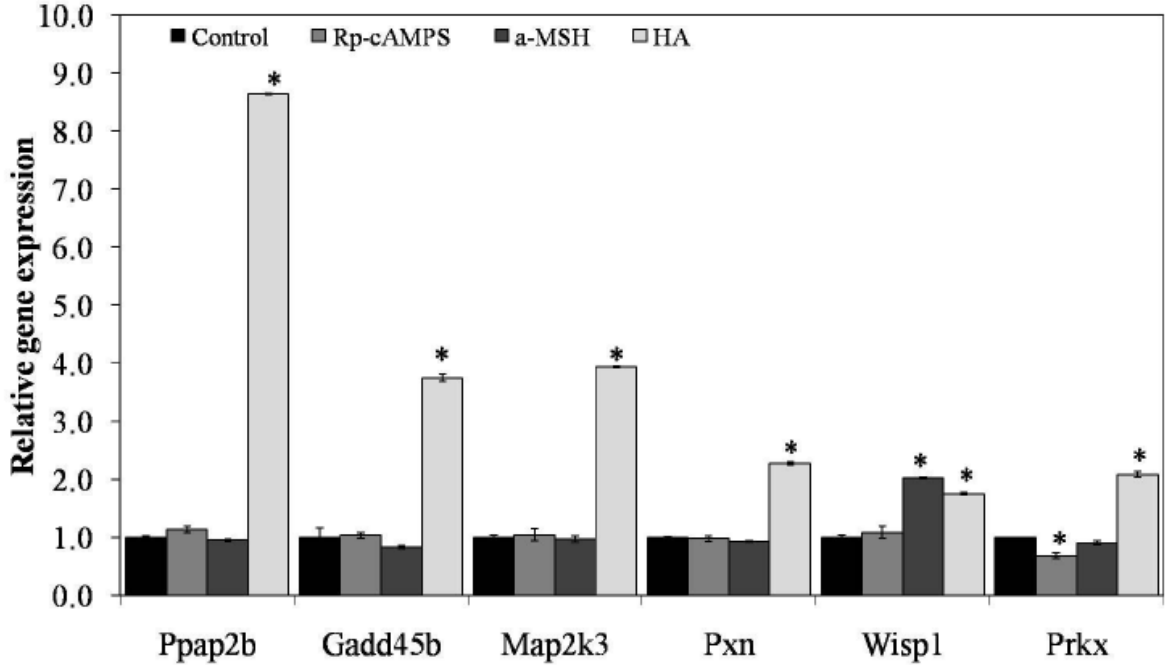

Control
Rp-cAMPS
a-MSH
HA
Relative gene expression
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ppap2b
Gadd45b
Map2k3
Pxn
Wisp1
Prkx

Figure 3

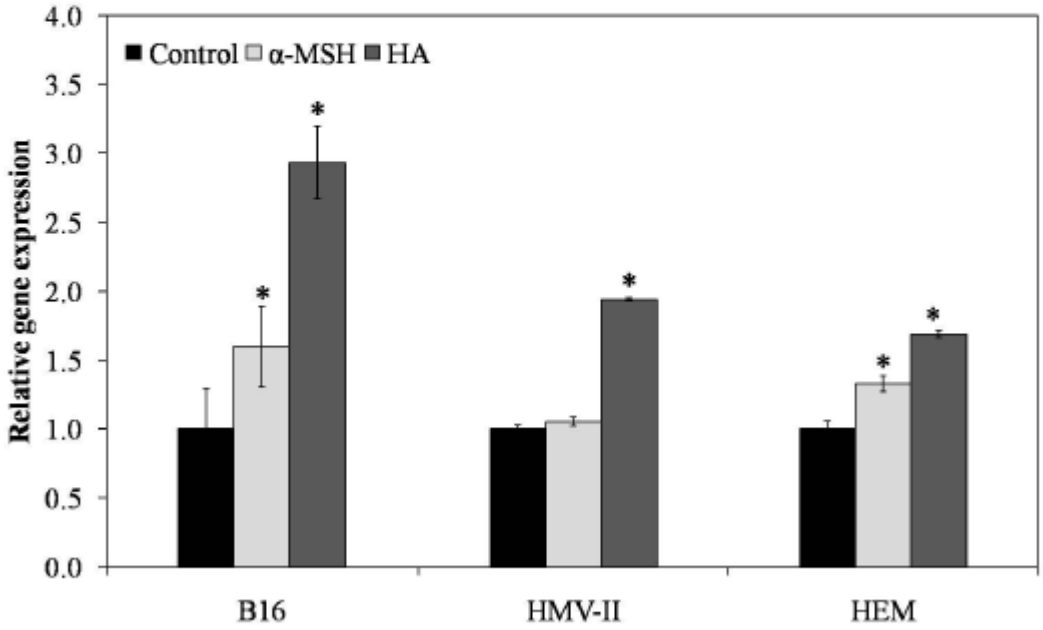

Control
α-MSH
HA
Relative gene expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
B16
HMV-II
HEM

**Figure4**

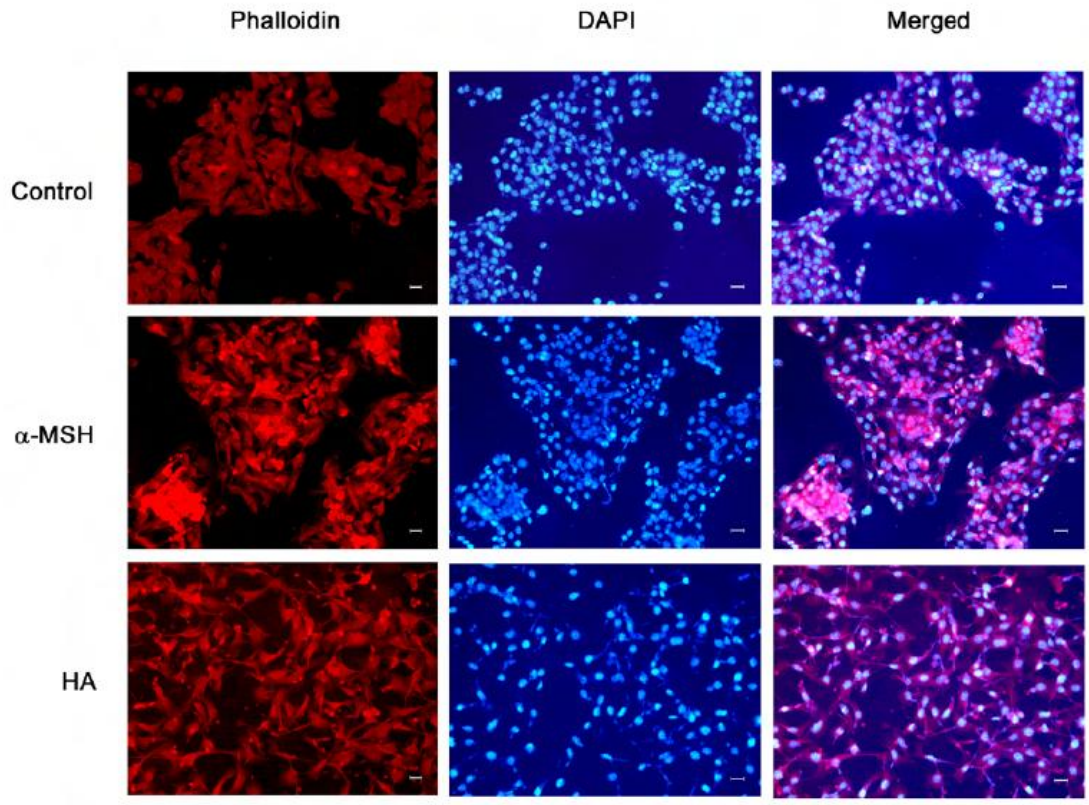


Figure 5a

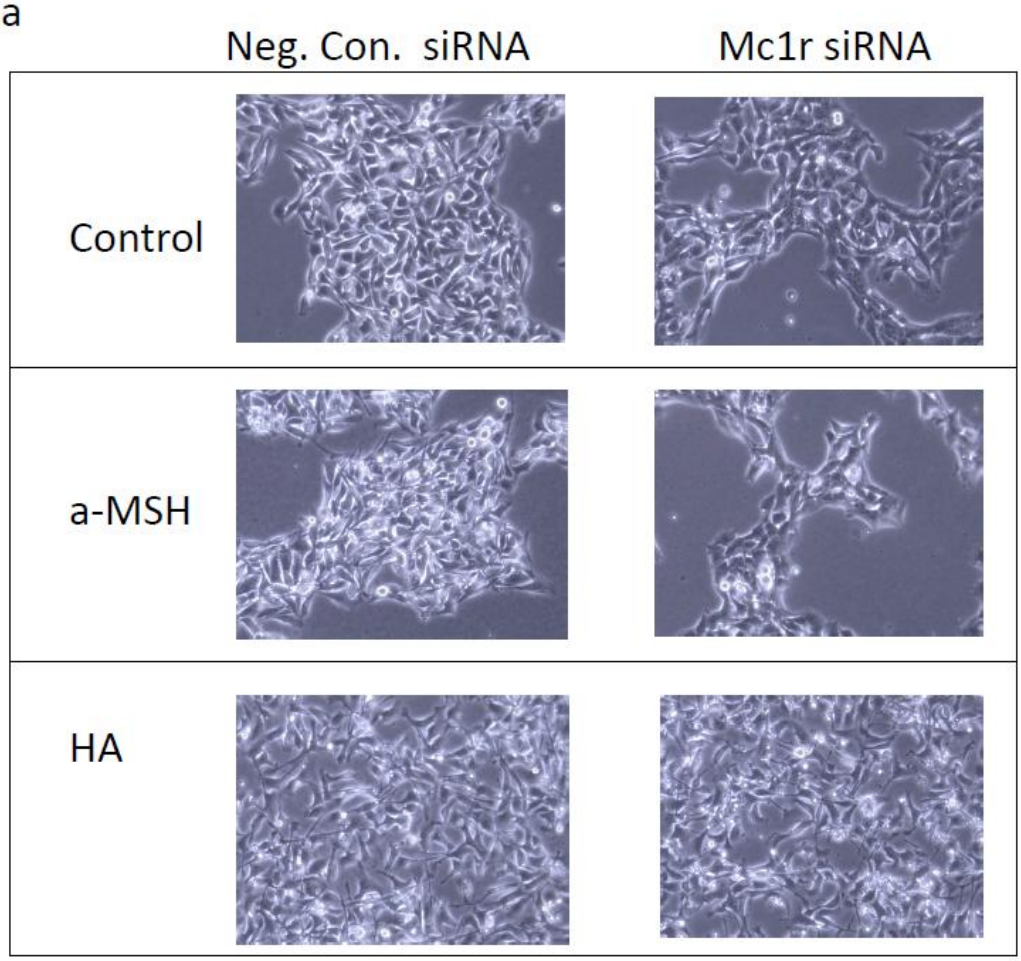


Figure 5b

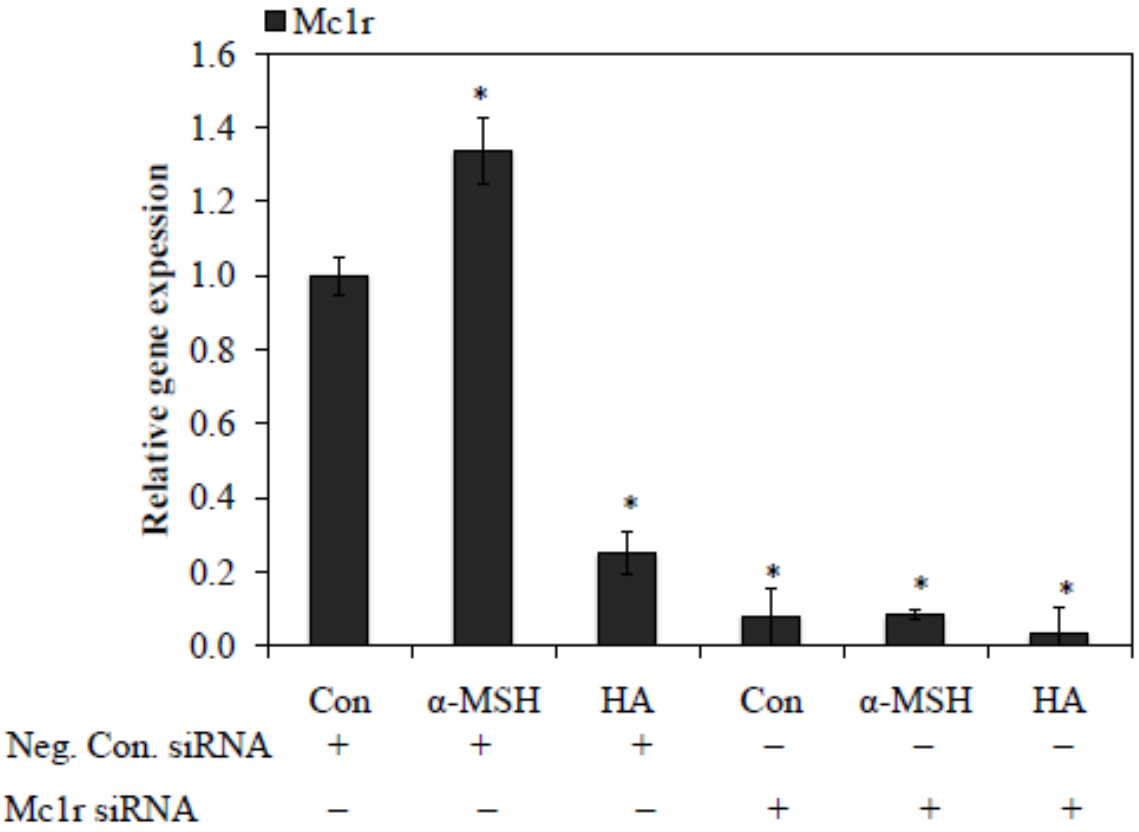
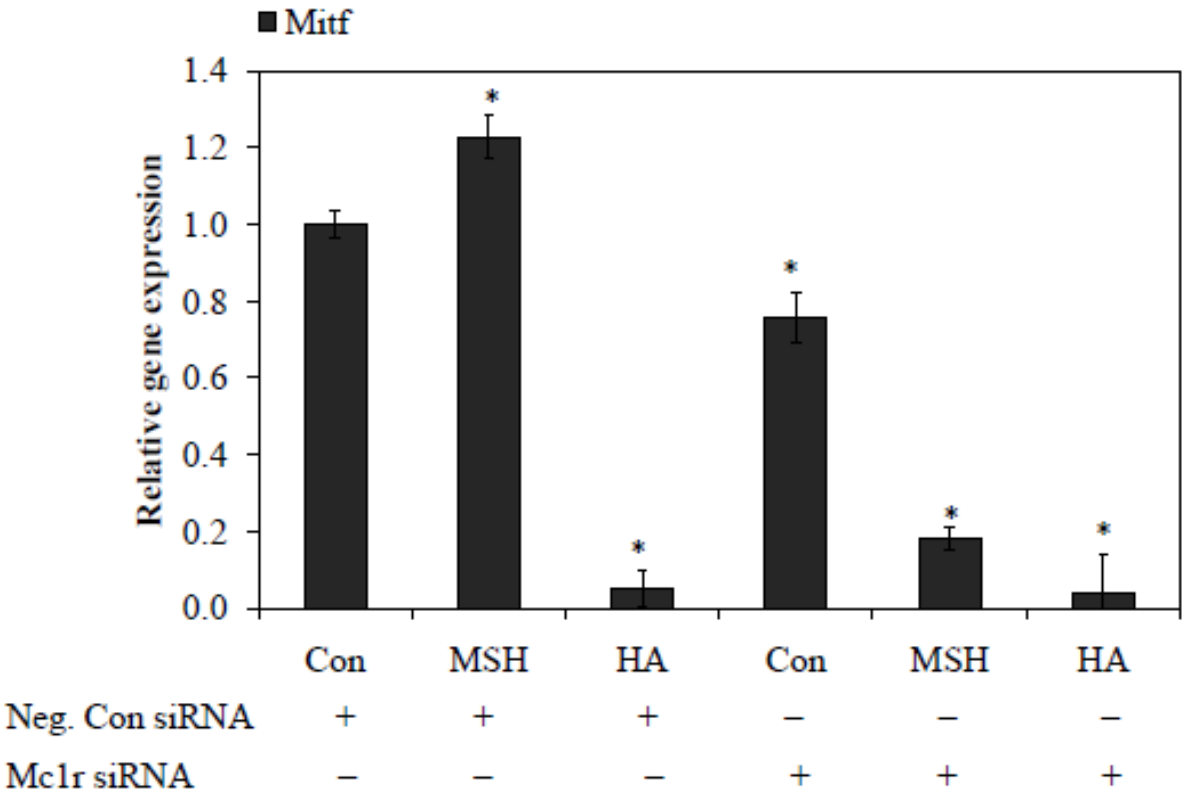

Mc1r
Relative gene expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Con
α-MSH
HA
Con
α-MSH
HA
Neg. Con. siRNA + + + – – –
Mc1r siRNA – – – + + +

Figure 5c


Mitf
Relative gene expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Con
MSH
HA
Con
MSH
HA
Neg. Con siRNA + + + – – –
Mc1r siRNA – – – + + +