Physiological Effect of Organic Acid on Melanocytes and Melanoma Cells

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ZHOU SIQI

Physiological Effect of Organic Acid on Melanocytes and Melanoma Cells

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ZHOU SIQI

Supervisor: Prof. Kazuichi SAKAMOTO

Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai,
Tsukuba City, Ibaraki 305-8572, Japan

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Abbreviations

a-KG, Alpha-ketoglutaric acid

a-MSH, Alpha melanocyte-stimulating hormone

ATP, Adenosine triphosphate

BIO, (2Z,3E)-6-bromoindirubin-3'-oxime

CA, Citric acid

cAMP, Cyclic adenosine monophosphate

CREB, cAMP response element-binding protein

DCFDA, 2',7'-dichlorodihydrofluorescein diacetate

EP, Ethyl pyruvate

ERK, Extracellular signal-regulated kinase

ETC, Electron transport chain

GSK3β, Glycogen synthase kinase 3β

L-DOPA, 3,4-dihydroxy-L-phenylalanine

MA, Malic acid

MAPK, Mitogen-activated protein kinase

MC1R, Melanocortin receptor-1

MITF, Microphthalmia-associated transcription factor

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OA, Oxaloacetic acid

PA, Pyruvic acid

PI3K, Phosphoinositide 3-kinase

PKA, Protein kinase A

ROS, Reactive oxygen species

RPMI-1640, Roswell Park Memorial Institute 1640

TCA cycle, Tricarboxylic acid cycle or citric acid cycle

TRP-1/2, Tyrosinase related protein-1/2

Abstract

Introduction:

Melanin is the pigmentary protein synthesized by melanocytes. The existence of melanin is related to many functions in our body. There are three types of melanin, eumelanin, pheomelanin and neuromelanin. Eumelanin and pheomelanin control the color of our skin and hair, and neuromelanin is in the nerve cytoplasm, providing pigmentation for the grey matter in the brain and central nervous system. In this study I mainly focused on the function of melanocytes in the epidermis.

In human epidermis, the synthesis of melanin is mainly regulated by ultraviolet radiation (UV). UV irradiation induces an increase in reactive oxygen species (ROS) in the keratinocytes in the epidermis, which increase the risk of DNA damage. To avoid this danger, keratinocytes secrete α-melanocyte-stimulating hormone (α-MSH) to stimulate the synthesis of melanin in the surrounding melanocytes. Melanin synthesis process is carried out in melanosome, which is transferred to surrounding keratinocytes after melanin synthesis to help block UV induced damage.

However, the synthesis of melanin is also accompanied by the production of ROS. According to the results of previous studies, ROS is the culprit leading for melanoma, the most dangerous type of skin cancer developing from melanocytes. So far, the amount of specific carcinogenic ROS in the cell has not been determined, and I also have known that too low intracellular ROS content can also cause damage to cells. So, I focus on mitochondria metabolism, one of the sources of intracellular ROS generation organelle. Mitochondria, as I known, is the most important energy supply organelle in the cell. The process of energy (ATP) production is called the citric acid cycle (or tricarboxcylic acid cycle, TCA). The combination of TCA cycle and electron transport chain (ETC) in the mitochondria supply energy and induce ROS generation. TCA cycle also include the formation of a variety of organic acids, which in turn have a feedback

effect on cell energy production and metabolism. Some evidence also proved organic acid can down-regulate the intracellular ROS accumulation.

Objective

I want to investigate the physiological effect of organic acids in the TCA cycle on melanogenesis and anti-melanoma activity.

Result and discussion

1. Pyruvic acid (PA) and Ethyl pyruvate (EP)

Pyruvate, as the final product of glycolysis, joins and enters the TCA cycle. Due to its unstable aqueous solution, I added ethyl pyruvate for comparation. I validated the effect of murine B16F10 melanoma cells on melanin synthesis. First, PA and EP showed an inhibitory effect on melanogenesis, and the same inhibitory effect was also exhibited in the presence of the melanin promoter α-MSH or forskolin (Fsk). Next, I examined the important catalytic enzymes of melanin synthesis tyrosinase and its transcription factors Microphthalmia-associated transcription factor (MITF) and found that their expression was inhibited by PA and EP. Afterwards, ERK, PI3K and GSK3β inhibitors were used to investigate the effect of PA/EP on the upstream factors in melanin synthesis. PI3K/AKT and GSK3β were found to be involved in the pathway of PA/EP inhibition of melanogenesis, whereas the ERK pathway was only present in the EP-regulated melanin reduction process. Therefore, my results suggested that PA and EP were effective melanin inhibitor.

2. Citric acid (CA)

Unlike pyruvate, I clearly found that at the same concentration, CA had a stronger inhibitory effect on the activity and growth of melanoma cancer cells, and the same inhibition effect was found when using other human melanoma cell lines. However, for human normal melanocytes, this inhibition is relatively weak. Thus, it is suggested that CA has stronger inhibitory effect to melanoma cell proliferation. I also examined the effect of CA on melanin synthesis. For murine melanoma cells, CA showed a promoting

effect on melanin synthesis. Inversely in human cells, I found that CA has an inhibitory effect on the synthesis of melanin. These data suggested that CA is a potential effective depigmental agent for humans

General Introduction

1.1 Melanin and Melanocytes

The main factor that causes our skin and hair to turn black is a small molecule called melanin. It is mainly synthesized in cells called melanocytes (Nava, 2008). Melanocytes are widely found in our skin and hair follicles (Slominski et al., 2005; Cichorek et al., 2013). When properly stimulated, melanin is produced, to maintain the body's function and protect our body (Ali, 2017). When melanocytes are deficient, diseases and symptoms are caused, such as vitiligo (Lotti et al., 2015), albinism and adolescent white hair (Yamaguchi and Hearing, 2014).

There are basically three types of melanin in the human body: eumelanin (Meredith and Sarna, 2006), pheomelanin (Hearing and Tsukamoto, 1991) and neuromelanin (Fedorow et al., 2005). Among them, neuromelanin mainly exists in our central nervous system, producing color for the gray matter cells of the brain and spinal cord (Fedorow et al., 2005). The main melanin presenting in hair and skin is eumelanin and pheomelanin (Greco et al., 2011). What I know and focus on is mainly the eumelanin which show a black or brown color, and the melanin referred next in this thesis is eumelanin.

The main role of melanin is to block the genetic damage caused by ultraviolet radiation (UV) (Michaela and Vincent, 2008). Long-term exposure to sunlight causes the skin cells to absorb more ultraviolet light. Experiments have shown that UV arises the reactive oxygen species in the keratinocytes of the skin. Further, raised reactive oxygen species and UV itself cause DNA damage to the keratinocytes (Ryu et al., 2010). In order to avoid this damage, keratinocytes and melanin precursor cells in the skin secrete a large amount of hormone called alpha-Melanocytes stimulating hormone (α-MSH) (Tomohisa H., 2014). α-MSH binds to the Melanocortin-1 receptor (MC1R) on melanocytes membrane, which induces maturation of melanocytes and promotes melanin synthesis and transport (Videira et al., 2013). The melanin after synthesis is

transported into the surrounding keratinocytes (Boissy, 2003), which surround the nucleus and absorb and block ultraviolet rays. Experiments have shown that eumelanin absorbs up to 90% of UV light (Kvam and Tyrrell, 1999), thus reducing the damage of UV rays to skin cells (Fig.1 A page 56).

1.2 Melanin synthesis

The melanin synthesis process takes place in an organelle called melanosome. The amino acid tyrosine is catalyzed by an enzyme to form L-DOPA in the melanosome and L-DOPA is further catalyzed to form DOPAquinone. The most important enzyme that induces catalysis in this process is called tyrosinase (TYR) (Iozumi et al., 1993). There are two additional enzymes called tyrosinase related protein-1 (TRP-1) and tyrosinase related protein - 2 (TRP-2, or DOPAchrome tautomerase, DCT) (Adila et al., 2014), TRP-2 catalyze DOPA quinone to DOPAchrome. Further, TRP-1 catalyze DOPAchrome to melanin. These three enzymes directly determine the efficiency of melanin synthesis and are therefore considered to be the most important regulators of melanin synthesis (Fig.1 B page 56).

The transcription factor that regulates the gene expression of these three enzymes is called Microphthalmia - associated transcription factor (MITF) (Yasumoto et al., 1994). MITF is widely distributed in human cells. It not only promotes the transcription of the three enzymes involved in melanin synthesis, but also plays an important role in regulating the function and activity of cells in many cells (Hershey and Fisher, 2004). In melanocytes, the activity of MITF is also regulated by many upstream factors.

1.2.1 cAMP pathway

First mentioned is the cAMP-PKA-CREB pathway. α-MSH binds to the G Protein-Coupled Receptors MC1R and activates the second messenger Cyclic adenosine monophosphate (cAMP) to be delivered to Protein Kinase A (PKA), which activates phosphorylation of cAMP response element-binding protein (CREB) (Otręba et al., 2012). Phosphorylated CREB enters the nucleus to promote *mitf* transcription (Kim et

al., 2016), thereby promoting melanin synthesis by increasing the protein content of MITF (Fig.2 page 57).

1.2.2 MAP Kinase pathway

Mitogen-activated protein kinase (MAPK or MAP kinase) pathway is a common pathway in eukaryotes. It is associated with many cellular functions, such as cell proliferation, differentiation, gene expression, and apoptosis (Pearson et al., 2001). MAPK usually includes three factors, which are extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and P38, respectively. In other experiments, although JNK and P38 have been described to have a regulatory effect on melanin synthesis through regulating MITF activity (Hoek et al., 2008 ; Mansky et al., 2002), ERK has been recognized as a definite negative regulator of MITF (Peng et al., 2014). In cellular metabolism, the amount of intracellular ROS regulates the degree of phosphorylation of ERK (Kim et al., 2014). Phosphorylated ERK has the ability to catalyze the phosphorylation of MITF at serine 73, and the phosphorylated MITF is degraded by the ubiquitin - proteasome system (Lee et al., 2015). Therefore, ERK pathway has an inhibitory effect on melanin synthesis, and this effect has a certain relationship with the content of ROS (Fig.2 page 57).

1.2.3 AKT pathway

AKT is also called protein kinase B, which is widely distributed in various cells and regulates various physiological functions of cells such as cell proliferation, cell migration, glucose metabolism and apoptosis (Bruce et al., 2002). As an upstream regulator of cell function, AKT also regulates metabolism in melanocytes or melanoma cells. From recent studies, AKT was found to have the ability to inhibit melanin synthesis(Oka et al., 2000).

1.2.4 GSK3β pathway

Glycogen synthase kinase-3 (GSK-3) is involved in energy metabolism, neuronal cell development, and body pattern formation (Ali et al., 2001). GSK3β is downstream

of the AKT pathway, and AKT promotes phosphorylation of GSK3β to regulate its activity (Beurel et al., 2015). Recent experiments have also shown that GSK3β has an inhibitory effect on the function of melanin synthesis (Khaled et al., 2002). Taken together, AKT and GSK3β together regulate the synthesis of melanin(Fig.2 page 57).

1.3 Melanoma

Melanoma cells are usually developed from melanocyte (Bandarchi et al., 2010), which has the characteristics of unlimited proliferation and strong metastatic ability. Melanoma is a cancer that is difficult to cure, and the incidence is increasing year by year. According to statistics, Australia is the region with the highest incidence of melanoma (Owens, 2014). In recent years, the incidence rate in Asia has also gradually increased. Although melanin has a very effective role in protecting against UV irradiation, the synthesis of melanin is accompanied by the production of reactive oxygen species (Uraiwan, 2011). The experiment also shows that melanoma cells contain higher reactive oxygen species than normal cells. As mentioned in the front, there are two types of melanin in human skin, black-brown eumelanin and red-yellow pheomelanin. Previous studies have shown that eumelanin not only effectively blocks UV irradiation, but also has the ability to reduce intracellular ROS (Noah and Douglas, 2013) Inversely, pheomelanin has been shown not only to have no ability to reduce ROS, but also to be one of the important factors in inducing carcinogenesis of melanocytes (Morgan et al., 2013). Therefore, reducing ROS production and aggregation in melanocytes may be one of the strategies to reduce the risk of melanocyte canceration. At the same time, the regulation of melanin synthesis can be achieved by regulating the production and aggregation of intracellular ROS.

1.4 Organic acid in citric acid cycle

It is well known that eukaryotic cell metabolism requires sufficient energy, and the most important energy-generating organelle is mitochondria. The source of energy in cells is usually derived from the process of glucose breakdown, which is divided into 2

parts : 1, glycolysis in the cytoplasm and 2, citric acid cycle in the mitochondria (also known as the Krebs cycle, TCA cycle). Because the energy produced by the TCA cycle is much larger than the former glycolysis, mitochondria are considered to be the critical organelles for energy producing in cell.

Many organic acids are contained in the TCA cycle (khanac acedemy), such as pyruvic acid, citric acid, succinic acid, malic acid, and so on. These organic acids not only participate in energy metabolism, but some organic acids such as pyruvic acid, which are the final products of glycolysis , enter the mitochondria as the initial source of the TCA cycle, and are involved in lipid and ketone bodies metabolism; also such as, citric acid has the function of regulating cell metabolism (Icard et al., 2012) and negative feedback regulation effect on cell metabolism (Ren et al., 2017). Therefore, the presence of organic acids has important significance for cells.

1.5 Aim for this study.

1. Treatment of melanocytes with organic acids and found their effect on melanin synthesis.

2. Exploring the principle of organic acid regulating melanin synthesis

3. The ultimate goal is to treat human diseases by regulating the content of organic acids.

Chapter I. Scanning the effect of organic acid on melanin synthesis

2.1 Introduction

The metabolic process in cells requires the supply of energy, which the energy source is adenosine triphosphate (ATP). The main source of metabolic energy in the cells is glucose. The process taken place in cells of converting glucose into energy ATP is called cell respiration (Bailey, 2012), which is divided into two parts: glycolysis and TCA cycle.

The process of glycolysis can function with or without the presence of oxygen in the cytosol. In this process, glucose molecules are converted into two molecules of pyruvic acid, and 2 molecules of ATP is synthesized. In aerobic conditions, pyruvic acid molecule then enters the mitochondria, a large amount of ATP is produced by the TCA cycle together with the function of the electron transport chain (ETC). While in anaerobic conditions, pyruvic acid is further converted to lactic acid(Jane et al., 2010). Therefore, mitochondria is considered to be the most important energy producing organelle, and the TCA cycle is the most important energy production process.

Many molecules of organic acids participate in the TCA cycle, which is the product also the substrate for the reaction in TCA cycle. I wondered if the addition of organic acids from the outside cells will affect the activity of the TCA cycle, thereby increasing the supply of energy in the cells, and promoting the metabolic activity of the cells.

Because of the process of melanocytes maturation and melanin synthesis is also a process of consuming energy. I want to know whether the supply of organic acids that is necessary for the TCA cycle producing energy from the outside cells will have a positive effect on the synthesis of melanin, or not. These suspects were not yet researched before. Therefore, I used the mouse B16F10 melanoma cells for initial detection to determine this conjecture.

Objective : Preliminary scanning of the effect of organic acids on melanin synthesis and selection of candidate organic acids.

2.2 Materials and Methods

2.2.1 Materials

B16F10 melanoma cells were obtained from RIKEN Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan). Pyruvic acid (PA), Lactic acid (LA), α-ketoglutalic acid (α-KG) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Roswell Park Memorial Institute (RPMI)-1640 medium, Oxaloacetic acid (OA), Citric acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Cell culture

B16F10 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37 °C and 5 % CO_2. After culturing in 10-cm dishes from frozen stocks, cells were seeded at a density of 3.4×10^4 cells/well in a 6-well-plate. Cells were allowed to attach to the bottom of the plate for 24 h before experiments.

2.2.3 Measurement of melanin content

Melanin content was measured as previously described (Lehraiki and Abbe et al., 2014). B16F10 cells were seeded in a 6-well plate. After treatment for 48 h with organic acid, cells were collected by trypsinization and resuspended in 100 μl of 1 N NaOH. After heating at 80 °C for 1 h, the absorbance at 405 nm was measured on a microplate reader. Melanin content was normalized to total protein content.

2.3 Results

2.3.1 LA had no effect on melanin synthesis

Lactic acid is the main product of glycolysis in anaerobic conditions. It can be converted to pyruvate with specific condition and enzyme catalysis. Therefore, I first examined the effect of lactic acid (LA) on melanin synthesis. After allowing the cells to adhere for 12 hours, I changed the medium containing LA. Since LA is acidic, the color of the medium showed acidity (orange to yellow), and it can be found by pH detection that the addition of LA lowers the pH of the medium to between 6.5 and 6.8. After 48 hours of treatment, the cells were well grown in the culture dish and no cell death was observed. By detecting the intracellular melanin, it was found that lactic acid had no effect on the synthesis of melanin (Fig.3 page 58).

2.3.2 PA, OA, MA, α-KG down-regulated melanogenesis

Pyruvic acid is the product of glycolysis and the first material for the TCA cycle, so the quantity of pyruvic acid is important for regulating cellular metabolic activity. Since pyruvic acid changes the metabolic pathway with the amount of oxygen, I only discuss the case where pyruvic acid is in a sufficient oxygen condition in this assay. At the same time, I also separately added other organic acids in the intermediates of the TCA cycle (Oxaloacetic acid, OA; Malic acid, MA; α-Ketoglutaric acid, α-KG). Same as LA, due to the acidity of these organic acids, the medium is also weakly acidic after the addition of the reagents, and the pH is between 6.0 and 7.0. After 48 hours of treatment, the medium generally returned to normal color (orange) and the medium without treatment was light brown due to the production of melanin. As the concentration increases, the color of the solution itself also tends to be orange (consistent with the color of the normal medium). In the direct observation of cells, high concentration of organic acid treatment reduced the growth ability of cells but did not find a large number of dead cells. This phenomenon indicating that these organic acids are not very toxic to cells, while inhibit cell growth. Detection of melanin in cell

lysates indicated that these organic acids have a dose dependent inhibitory effect on melanin synthesis (Fig.4 A-D page 59).

2.3.3 CA increased melanin content in cells

Citric acid (CA) is the first member in the circle of the TCA cycle. And it is commonly used as an antioxidant agent in our life. As I mentioned above, UV-induced ROS can promote melanocyte maturation and melanin synthesis, while intracellular ROS also can regulate melanin synthesis though the ERK pathway. Therefore, I suspect that CA will also have a certain impact on melanin synthesis. Like other organic acids, I also used 1 mM to 15 mM citric acid to treat B16F10 cells in this experiment. Due to the structure of citric acid itself, the pH of the medium was greatly reduced. In the group at a concentration exceeding 10 mM, the cells were almost completely killed. This result suggested that high concentrations of CA are very toxic to cells. Even in the 5 mM concentration treated group, a small number of floating cells were found. However, contrary to the inhibitory effect on cell viability, CA promoted the synthesis of melanin in cells (Fig.5 page 60). Therefore, these results indicated that CA promotes the synthesis of melanin but inhibits the survival of cells.

2.4 Discussion

In this experiment I investigated that as a member of cellular material and energy metabolism, different organic acids have different physiological effects. Depending on their different regulation of melanin, I divided them into three major categories.

The first is lactic acid that does not affect melanin synthesis. Lactic acid exhibited a more palliative property compared to other organic acids. Even with a concentration of 15 mM, I still found no significant inhibitory effect of lactic acid on cells. I suspected that is why lactic acid had no clear effect on the synthesis of melanin. Lactic acid is the main product of respiration in anerobic conditions, which is mainly produced in muscle cells after strenuous exercise (Goodwin et al., 2007). Experiments have shown that lactate can promote the differentiation of muscle cells (Willkomm et al., 2014). While the main organ for converting lactic acid in the blood into pyruvic acid and producing glucose is liver (Boyle, 2005). Therefore, I speculated that lactic acid has no obvious physiological effect on melanocytes that cannot convert lactic acid into pyruvic acid.

The second group is pyruvic acid, malic acid, α-ketoglutaric acid and oxaloacetic acid, which were able to reduce melanin synthesis. With pyruvic acid as the first, these organic acids reduced the synthesis of melanin and had a weak inhibitory effect on the viability of cells, which limited the cells proliferation. These results were contrary to my speculation that organic acids activate the TCA cycle and promote energy production to promote melanin synthesis. Therefore, I thought it is necessary to carry out a deeper discussion on gene expression and upstream pathways for this phenomenon. So that, I selected the most representative PA for further research.

The third group is citric acid that promoted melanin synthesis. Completely inconsistent with other organic acids, citric acid promoted the synthesis of melanin. From the order of the TCA cycle, the upstream pyruvic acid and the downstream malic acid, Oxaloacetic acid, etc. reduced the melanin synthesis. Therefore, I speculated that CA, and other organic acids, were not directly related to the regulation of melanin through regulating TCA cycle and the synthesis of ATP. If citric acid is assumed to

have antioxidant properties, reduced ROS does promote melanin synthesis by regulating the ERK pathway. However, it is not certain whether CA will reduce UV induced ROS and thus reduce the synthesis of melanin. Therefore, the mechanism of citric acid to promote melanin synthesis still needs to be further improved. At the same time, citric acid exhibited a strong cytotoxicity different from other organic acids for B16F10 cells. Since B16F10 is a murine melanoma cell line, I wondered if this cytotoxicity is restricted to only the mouse cancer cells, to explore whether citric acid has the potential to against melanoma.

In summary, most organic acids have an inhibitory effect on melanin synthesis. Lactic acid has no effect and citric acid promotes melanin synthesis. For the role of organic acids in inhibiting melanin synthesis, I chose pyruvic acid as a representative for further mechanism studies. And citric acid was used to explore whether it has different physiological effects on different cell lines.

Chapter II. Pyruvic acid (PA) / ethyl pyruvate (EP) inhibits melanogenesis in B16F10 melanoma cells through PI3K/AKT, GSK3β, and ROS-ERK signaling pathways

3.1 Introduction

Melanin is the molecule responsible for skin color (Seo and Sharma et al., 2003) that protects skin from ultraviolet (UV) radiation-induced damage (Chen and Tseng et al., 2015). The pigment is synthesized in melanocytes in an organelle known as the melanosome, a process referred to as melanogenesis (Videira and Moura et al., 2013). Tyrosinase is a critical enzyme for melanogenesis, and along with tyrosinase related protein (TRP) - 1/2 catalyzes the rate - limiting steps in melanin synthesis (Jimenez - Cervantes and Martinez - Esparza et al., 2001; Eves and MacNeil et al., 2006). Microphthalmia - associated transcription factor (MITF) is the transcription factor regulating tyrosinase expression and is thus a key regulator of melanogenesis (Chang, 2012). MITF expression and activity are controlled by various upstream factors. Alpha melanocyte-stimulating hormone (α-MSH) released by keratinocytes in response to UV irradiation (Thody and Graham, 1998) binds to melanocortin-1 receptor and stimulates cAMP (Bertolotto and Abbe et al., 1998), which activates protein kinase (PKA) and results in the phosphorylation of cAMP response element-binding protein (CREB). Phosphorylated CREB directly induces the transcription of MITF to promote melanogenesis (Flaherty and Hodi et al., 2012).

In addition to the PKA/CREB signaling pathway, MITF expression and activity are regulated by extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK) family member (Peng and Lin et al., 2014) that phosphorylates MITF at S73 and targets the protein for degradation by the proteasome (Wu and Lin et al., 2011; Lee and Lee et al., 2015). Phosphoinositide 3-kinase (PI3K)/AKT (Hwang and Lee et al., 2016; Chae and Subedi et al., 2017) and glycogen synthase kinase 3 beta (GSK3β) (Shin and Oh et al., 2015) also negatively regulate MITF activity and thereby suppress melanogenesis.

UV radiation induces melanogenesis, which is accompanied by an increase in reactive oxygen species (ROS) production (Mastore and Kohler et al., 2005; Dong and Cao et al., 2010). Pyruvic acid (PA) is a simple three-carbon α-keto monocarboxylic acid that plays a critical role in glycol metabolism and is a component of the tricarboxylic acid cycle (Fink, 2004). Pyruvate is also an effective ROS scavenger and has beneficial effects in diseases caused by increased ROS generation (Salahudeen and Clark et al., 1991; Varma and Devamanoharan et al., 1998). The effect of pyruvic acid on melanogenesis also proved in front. While aqueous solutions of pyruvate have low stability; the ethyl ester form, ethyl pyruvate (EP), is more stable and has anti-inflammatory and coagulant properties in addition to its scavenging function (Fink, 2007).

Objective : In this experiment, I focus on determining and comparing the effects of PA and EP on melanin synthesis and explaining their regulatory mechanism.

3.2 Materials and Methods

3.2.1 Materials

B16F10 melanoma cells were obtained from RIKEN Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan). PA and EP were purchased from Wako Pure Chemical Industries (Osaka, Japan). Roswell Park Memorial Institute (RPMI)-1640 medium, MTT, and l-DOPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-tyrosinase and anti-TRP-1 and -2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against MITF, total GSK3β, p-GSK3β^{Ser9}, AKT, p-AKT^{Thr308}, p-AKT^{Ser473}, and total β-actin were obtained from Cell Signaling Technology (Tokyo, Japan).

3.2.2 Cell culture

B16F10 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5 % CO_2. After culturing in 10-cm dishes from frozen stocks, cells were seeded at a density of 3.4×10^4 cells/well in a 6-well-plate, at 3.0×10^3 cells/well in a 24-well-plate, or 1.0×10^3 cells/well in a 96-well-plate. Cells were allowed to attach to the bottom of the plate for 24 h before experiments.

3.2.3 MTT assay

B16F10 cells were seeded in a 96-well plate. After treatment for 48 h with PA or EP, the medium was replaced with fresh medium containing 500 μg/ml MTT reagent, and the cells were incubated at 37 °C for 6 h. A 10 % SDS solution was added and plates were maintained at room temperature overnight. The absorbance at 570 nm was measured with a microplate reader (Tecan, Kawasaki, Japan).

3.2.4 Measurement of melanin content

Melanin content was measured as previously described (Lehraiki and Abbe et al., 2014). B16F10 cells were seeded in a 6-well plate. After treatment for 48 h with PA or EP, cells were collected by trypsinization and resuspended in 100 μl of 1 N NaOH. After

heating at 80 °C for 1 h, the absorbance at 405 nm was measured on a microplate reader. Melanin content was normalized to total protein content.

3.2.5 Intracellular tyrosinase activity

Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation (Lee and Kim et al., 2013). B16F10 cells were seeded in a 6-well plate and treated with PA and EP for 72 h. The cells were collected by trypsinization, resuspended in phosphate buffer containing 10% Triton X-100, sonicated, and centrifuged at 18,000 g for 20 min. The supernatant was collected and assayed for total protein, and 50 μg were mixed with 2 μl of 10 % (m/v) L-DOPA (Sigma-Aldrich, USA) in phosphate buffer followed by incubation at 37 °C. The absorbance was measured at 475 nm at 0 and 30 minutes. Melanin (Sigma-Aldrich, USA) was used for standard concentration. Tyrosinase activity was calculated with the following formula: Quantity of melanin at 30 min – Quantity of melanin at 0 min.

3.2.6 Detection of intracellular ROS

B16F10 cells were treated with PA and EP for 48 h, then washed twice with PBS and treated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 1 h. The cells were washed with PBS to remove the DCFDA, and fluorescence (excitation/emission = 495/529 nm) was measured on a microplate reader.

3.2.7 Western blotting

B16F10 cells (2.5 × 105) were seeded in a 6-cm dish. After treatment with samples for 48 h, cells were collected in radioimmunoprecipitation assay buffer, sonicated, and centrifuged at 10,000x g for 10 min. The supernatant was collected (protein assay was performed by Pierce^{TM} BCA Protein Assay Kit (Thermo, USA)), and 20 μg protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was blocked for 1 h in 2 % BSA and then incubated overnight at 4 °C with primary antibodies against MITF, ERK, p-ERK, AKT, p-AKT^{Thr308}, p-AKT^{Ser473}, GSK3β, and p-GSK3β^{Ser9}, tyrosinase, and TRP-1 and -2. This was followed

by incubation for 60 min at room temperature with appropriate secondary antibodies. The membrane was stained with LuminoGLO (Cell Signaling Technology) and protein bands were visualized with an AE-9300H EZ-Capture MG imager (ATTO Corporation, Tokyo, Japan).

3.2.8 Real-time PCR

B16F10 cells were seeded in a 6-well plate and after PA or EP treatment for 0, 1, 3, 6, 9, 12, 24, or 48 h, RNA was extracted from the cells using RNAiso Plus (Takara Bio, Otsu, Japan). Next, the RNA was reverse transcribed to cDNA, which was used as a template for real-time PCR amplification of *mitf* (forward: GTGAGATCCAGA GTTGTCGT; reverse: AGTACAGGAGCTGGAGATG) and *tyrosinase* (forward: TGACTCTTGGAGGTAGCTGT; reverse: AACAATGTCCCA AGTACAGG) genes. And GAPDH (forward: TGCCGTTGAATTTGCCGTGAGT; reverse: TGGTGA AGGTCGGTGTGAACGG) was used as internal reference for normalization.

3.2.9 Statistical analysis

Results are reported as mean ± standard deviation. Group means were compared with the Student's *t* test, and differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 PA and EP inhibit melanogenesis

To determine the safe concentrations of PA and EP, the cytotoxicity of the two compounds was evaluated in B16F10 melanoma cells with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with PA and EP concentrations of 0.1–15 mM. A dose-dependent cytotoxicity was observed at concentrations higher than 10 mM (Fig.6 A, B page 61). Although 5 mM of PA and EP significantly decreased cell viability, the cell growth curve showed that there was no big cytotoxicity on cells by PA or EP treatment (Fig.6 C page 61). Therefore, 5 mM of PA and EP were used in next experiment.

Next, I examined the effect of PA and EP on melanin synthesis. Here, I normalized the intracellular melanin content with cell total protein to avoid the effect of samples on cell number decrease and found that intracellular melanin levels were decreased in a dose-dependent manner in the presence of either compound (Fig.6 D, E page 62).

Furthermore, cells treated with the melanogenesis-promoting moleculars α-MSH and forskolin (Lehraiki and Abbe et al., 2014) produced more melanin (Fig.6 F, G page 63). While this increase of melanogenesis was down-regulated by either compound. Therefore, PA and EP were effective molecular that inhibit melanogenesis.

3.3.2 PA and EP inhibit tyrosinase activity and expression

Since tyrosinase is the main enzyme catalyzing melanin synthesis, I measured tyrosinase activity in B16F10 cells based on the catalysis of the tyrosinase substrate 3,4-dihydroxy-L-phenylalanine (L-DOPA) to melanin. Cells treated with 5 mM PA or EP for 48 hours. The melanin production in 30 minutes was regarded as tyrosinase activity. In the results, both PA (Fig.7 A page 64) and EP (Fig.7 B page 64) down-regulated tyrosinase activity, as evidenced by the decrease in melanin levels.

Given our observation that PA and EP treatment decreased tyrosinase activity, I examined tyrosinase expression by western blotting. Cells treated with PA and EP were collected at 24 and 48 hours. I clearly found the reduction effect of PA and EP on

tyrosinase expression at 48 hours (Fig.7 C page 64), while the difference in 24 hours group was not clear (not show). TRP-1 and TRP-2 (also known as dopachrome tautomerase, DCT) along with tyrosinase, regulate melanin synthesis; therefore, I examined the expression of TRP-1 and -2 by western blotting. The expression of both genes was decreased by PA and EP treatment.

To confirm the melanogenesis suppression by PA and EP was caused by down-regulation of tyrosinase gene expression, the mRNA of tyrosinase was measured. The relative quantity of tyrosinase mRNA was decreased in the presence of PA or EP for 24 and 48 hours (Fig.7 D, E page 65). All above, it is implying that PA and EP inhibit melanogenesis by negatively regulating the expression of melanin synthesis-related genes.

3.3.3 PA and EP regulate MITF gene expression

MITF regulates tyrosinase and TRP-1 and -2 expression, indicated that it acts a significant role in melanogenesis. Therefore, I examined MITF expression by westernblot. Cells treated with PA and EP were collected after 48 hours. The tyrosinase expression was same as figure 2C and MITF was significantly decreased by treatment (Fig.8 A page 66).

To investigate whether MITF was involved in PA or EP suppression melanogenesis, I further analyzed *mitf* mRNA quantity. After refreshed medium with PA or EP, which was without any positive molecular like MSH or forskolin, the cells were collected after 0, 1, 3, 6, 9, 12, 24 and 48 hours. The mRNA quantity at 0 hour without any treatment was regarded as 1, the others time points quantity was the relative value compared with 0-hour non-treatment group. The *mitf* mRNA in non-treatment cells was increased at first 3 hours and reduced to normal condition after 6 hours, which phenomenon was never mention previously (Fig.8 B, C page 66). Consistent with my expectations, PA and EP reduced the *mitf* mRNA quantity at almost all the time point, except at 9 hours. These results suggested that MITF was involved in PA and EP induced melanogenesis down-regulation.

3.3.4 ROS-ERK signaling mediates the effects of PA and EP on melanogenesis

ERK phosphorylation has been shown to induce MITF degradation (Song and Balcos et al., 2015) and lead to the inhibition of melanogenesis. Both PA and EP induced ERK phosphorylation without altering ERK gene expression after 24 hours (Fig.9 A page 68). Treatment of cells with the ERK inhibitor U0126 increased melanogenesis (Fig.9 B, C page 68). When U0126 co-treated with PA or EP, melanin synthesis was reduced than inhibitor treatment. On the other hand, PA reduced the melanin content to same level as the group presence of PA, while there was a significant difference between EP and EP/U0126 co-treatment.

PA and EP are both effective intracellular ROS scavengers (Salahudeen and Clark et al., 1991; Varma and Devamanoharan et al., 1998). And ROS is an activator for MAPK signaling pathway (Kim and Park et al., 2014). Therefore, I examined intracellular ROS levels in cells treated with PA and EP. Either compound increased ROS production (Fig.9 D page 69), which consistent with the observed increase in ERK phosphorylation.

These results suggested that the inhibition of melanogenesis by PA was independent of ERK pathway although PA increased ERK phosphorylation and ROS generation, but EP partially targets ERK pathway in inhibiting melanin synthesis

3.3.5 GSK3β is involved in the regulation of melanogenesis by PA and EP

PI3K activates AKT, which then inhibits GSK3β through phosphorylation at Ser9 (Cross and Alessi et al., 1995). Recent studies have shown that GSK3β regulates MITF activity (Shin and Oh et al., 2015) but the detailed mechanisms remain unclear. To determine the contribution of PI3K/AKT and GSK3β signaling to melanogenesis, I treated B16F10 cells with the PI3K inhibitor LY294002 and GSK3β inhibitor (2Z,3E)-6-bromoindirubin-3'-oxime (BIO). Treatment with LY294002 and BIO increased melanin content in a dose-dependent manner (Fig.10 A, B page 70). In addition, tyrosinase and MITF gene expression increased with melanin synthesis (Fig.10 C, D page 70). PI3K inhibitor treatment decreased phosphorylated (p-)AKT level, whereas GSK3β inhibitor reduced GSK3β and p-GSK3β levels. Thus, activated AKT induces GSK3β phosphorylation, which has an inhibitory effect on melanogenesis. I then co-treated cells with PA or EP and the inhibitors and found that PA decreased melanin content to a level similar to the control and PI3K inhibitor groups but not the GSK3β

inhibitor group; the same trend was observed in cells treated with EP (Fig.10 F, G page 71). PA and EP also increased p-GSK3β level and weakly increased p-AKT^{Ser473} (Fig.10 E page 70). These results suggest that PI3K/AKT and GSK3β suppress melanin synthesis and that GSK3β is an important mediator of the inhibitory effects of PA and EP on melanogenesis.

3.4 Discussion

The results of this study demonstrate for the first time that PA and EP inhibit melanogenesis. In cell metabolism, pyruvate is an important intermediate product of glycolysis that is used for ATP synthesis (Fink, 2004). I found that PA, EP, and even sodium pyruvate increased ATP production (Fig.10 page 74), whereas only PA and EP suppressed melanogenesis (Fig.13 A page 75 and Fig.6 D, E page 62), indicating that the latter effect was independent of ATP production. And as the aqueous solution of PA and SP are pyruvate, which means single pyruvate could not regulate melanogenesis. I also used NaOH to neutralize PA and obtained the same results as PA (Fig.13 B page 75), demonstrating that the negative regulation of melanin synthesis by PA is not through the acidification. Therefore, I summarized the effect of PA on down-regulating melanogenesis need pyruvic acid itself.

Tyrosinase is the main enzyme for melanin synthesis and its transcription is regulated by MITF (Jimenez-Cervantes and Martinez-Esparza et al., 2001; Eves and MacNeil et al., 2006; Chang, 2012). In this study, I found that PA and EP treatment suppressed MITF and consequently, tyrosinase gene expression, leading to a decrease in melanin synthesis.

MITF gene expression is regulated by many factors, including CREB, PI3K/AKT (Hwang and Lee et al., 2016; Chae and Subedi et al., 2017), GSK3β (Shin and Oh et al., 2015), and MAPK (ERK, c-Jun N-terminal kinase, and p38) (Peng and Lin et al., 2014). In my study, ERK phosphorylation was increased by PA and EP treatment, which corresponded to a decrease in MITF protein level and an increase in ROS generation. The latter result contradicts the reported role of PA and EP as ROS scavengers (Salahudeen and Clark et al., 1991; Varma and Devamanoharan et al., 1998). ERK inhibitor treatment increased melanin synthesis, indicated the role of ERK pathway in inhibiting the melanogenesis. While the co-administration with PA or EP prevented the melanin synthesis, although there was a significant increase in EP / U0126 co-treatment. It seems that ERK was not involved in PA and EP suppression

melanogenesis, but p-ERK and ROS increase was accordance with melanin synthesis. Thus, I predict that ROS-ERK signaling was not the main regulatory pathway, it is partial involved in PA and EP melanogenesis down-regulation effect.

Inhibition of PI3K increased melanin synthesis, indicating that PI3K/AKT signaling negatively regulates melanogenesis. On the other hand, GSK3β inhibition also increased melanogenesis. PI3K/AKT location at upstream of GSK3β; thus, these results indicated that the PI3K/AKT-GSK3β axis mediates the effects of PA and EP on melanogenesis. The melanin content of PI3K inhibitor / PA or EP co-treatment significantly was not only higher than PA or EP separate treatment, but also lower than inhibitor treatment; while the GSK3β inhibitor/ PA or EP co-treatment showed non-significant difference to inhibitor treatment. And the westernblot result also showed that PA and EP induced GSK3β phosphorylation, which means that GSK3β phosphorylation is the most critical role in PA and EP induced melanogenesis down-regulation.

In recent studies, AKT was also directly induces MITF phosphorylation at Ser510; p-MITF is degraded by protease (Wang and Zhao et al., 2016). This could explain why the PI3K inhibitor induced more melanin synthesis than GSK3β inhibitor.

In conclusion, the results of this study demonstrate that PA and EP negatively regulate melanin production through ERK, PI3K/AKT, and GSK3β signaling pathway (Fig.11 page 73) and they have potential to be used to treat hyperpigmentation disorders.

Chapter III. Citric acid (CA) has different regulatory effects on melanogenesis in different cell lines.

4.1 Introduction

Melanin is wildly included pigmental protein in human skin and hair (Leon and Diane, 2012), which is synthesis in melanocytes organelle melanosome (Slominski et al., 2005) and transported into surrounding keratinocytes (Delevoye, 2014) and hair cells. In human skin, melanin synthesis is related with exposure to UV radiation. Melanin is an effective absorbent of light, which can disipate over 99.9 % of absorbed UV radiation (Meredith and Riesz, 2004). When keratinocytes are exposure in UV radiation, intracellular reactive oxygen species (ROS) generation is increased despite of UVA or UVB (Masaki et al., 2009 ; Henri et al., 2012). At the same time, α-melanocyte stimulating hormone (α-MSH) is secreted by keratinocytes to against UV induced DNA damage (Virador et al., 2002). α-MSH is binding to melanocortin-1 receptor (MC1R) on the melanocyte membrane and activate cyclin AMP (cAMP) secondary messenger system. Protein kinase A (PKA) and cAMP response element binding protein (CREB) are further activated by cAMP and phospho-CREB promote Microphthalmia-associated transcription factor (MITF) gene expression. MITF is the transcription factor for tyrosinase, the most critical enzyme in melanogenesis. Tyrosinase together with tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) catalyze tyrosine to L-DOPA and further to melanin (Hedley et al., 1998 ; Lee, et al., 1972).

Melanomas arise from melanocytes, now it belongs to the most aggressive forms of skin cancer and its incidence is continually rising worldwide (Godar, 2011). Although melanin is necessary for against UV induced cell damage, the process of melanin synthesis is accompanied by ROS accumulation (Smit et al., 2008), which will increase the risk of melanocyte to senescence and melanoma. There are two types of melanin, one is eumelanin with black and brown color, another one is pheomelanin with

red and yellow color (Costin and Hearing, 2007). In recent studies, eumelanin is not only contributing to UV defense, it also has been proved beneficial on suppressing ROS accumulation in melanocytes (Nasti and Timares, 2015). However, many evidences suggested that pheomelanin increased ROS generation and it is contributing to tumorigenesis (Nasti and Timares, 2015 ; Meierjohann, 2014). Another group investigated that mitochondria loss function caused respiratory function disorder and decreased the tumor growth. While, mitochondria function is associated with electron transport chain (ETC), which is accompanied by the ROS generation (Liu et al., 2002). Therefore, I suspected that down-regulated intracellular ROS could contribute to melanoma growth inhibition effect.

Therefore, I attached importance to a metabolism organic molecular, citric acid (CA). Citric acid is a weak organic acid, which occurs naturally in citrus fruits. Intracellular citric acid (CA) or citrate is an essential metabolic intermediary and a key regulator of energy production. CA in mitochondria locate the initial of TCA cycle, the quantity of CA also had feedback effect on pyruvate dehydrogenase activity (Jeremy et al., 2002 ; Taylor and Halperin, 1973). CA out of mitochondria also proved negatively regulating the glycolysis process (Garland et al., 1963). Interesting results suggested that oral administration of citrate may improve thyroid cancer (Halabe Bucay, 2009). Also, some research investigated that citrate is effective on tumor growth suppression (Ren et al., 2017). However, the physiological effect of CA on melanocytes or melanoma is still not understood. Because CA is wildly found in our food to be an anti-oxidant reagent, I am looking forward to its anti-oxidation effect on inhibiting melanoma cells activity. In this experiment, murine melanoma cell (B16F10), normal human epidermal melanocytes (NHEM) and human melanoma (G-361 and HMV-II) were used for CA treatment. The melanoma growth inhibition and apoptosis inducement effect were investigated. And an opposite regulation effect on melanogenesis in human and murine cells was discovered.

Objective : To investigate CA effect on melanogenesis, and compare the effect of on different cell lines

4.2 Materials and Methods

4.2.1 Materials

B16F10, G-361 and HMV-II melanoma cell lines were obtained from RIKEN Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan). Citric acid powder, Roswell Park Memorial Institute (RPMI)-1640 medium, MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-tyrosinase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against MITF, GAPDH and total β-actin were obtained from Cell Signaling Technology (Tokyo, Japan). NHEM cell line and special medium kit were obtained from KURABO (Osaka, Japan)

4.2.2 Cell culture

B16F10, G-361 and HMV-II cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, NHEM cells were cultured in special culture medium at 37 °C and 5 % CO_2. After culturing in 10-cm dishes from frozen stocks, cells were seeded at a density of 1.0×10^5 cells/well in a 6-well-plate, at 3.0×10^3 cells/well in a 24-well-plate, or 1.0×10^3 cells/well in a 96-well-plate. Cells were allowed to attach to the bottom of the plate for 24 h before experiments.

4.2.3 MTT assay

Cells were cultured in 24-well plate. After treatment for 48 h with CA, the medium was replaced with fresh medium containing 500 μg/ml MTT reagent, and the cells were incubated at 37 °C for another 4 h. Isopropanol with 0.04 M HCl solution was added and plates were maintained at room temperature for 5 min. The colored solution was moved into another 96-well assay plate, the absorbance (isopropanol with 0.04 M HCl solution as blank) at 570 nm was measured with a microplate reader (Tecan, Kawasaki, Japan).

4.2.4 Measurement of melanin content

Melanin content was measured as previously described (Lehraiki and Abbe et al., 2014). Cells were seeded in a 6-well plate. After treatment from 1 day to 6 days with CA, cells were collected by trypsinization. After centrifugation at 1000 × g for 5min, supernatant was removed, and cell pellet was resuspended in 100 µl of 1 N NaOH. After heating at 80 °C for 1 h, the absorbance at 405 nm was measured on a microplate reader. Melanin content was normalized to total protein content (by Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific K.K, Tokyo, Japan).

4.2.5 Detection of intracellular ROS

Intracellular ROS detection was following the protocol of Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Tokyo, Japan). Cells were treated with or without CA for 2 or 24 h, then washed twice with washing buffer and treated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 1 h. The cells were washed with washing buffer to remove the DCFDA, and fluorescence (excitation/emission = 495/529 nm) was measured on a microplate reader.

4.2.6 Western blotting

Cells (2.5×10^5) were seeded in a 6-cm dish. After treatment with samples for different times, cells were collected in radioimmunoprecipitation assay buffer, sonicated, and centrifuged at 10,000x g for 10 min. The supernatant was collected (protein assay was performed by Pierce™ BCA Protein Assay Kit (Thermo, USA)), and 20 µg protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was blocked for 1 h in 2 % BSA and then incubated overnight at 4 °C with primary antibodies against MITF, tyrosinase. This was followed by incubation for 60 min at room temperature with appropriate secondary antibodies. The membrane was stained with LuminoGLO (Cell Signaling Technology) and protein bands were visualized with an AE-9300H EZ-Capture MG imager (ATTO Corporation, Tokyo, Japan).

4.2.7 DPPH assay

DPPH was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in EtOH as a concentration of 0.04 mg/ml. Vitamin C and BHT were dissolved in 70 % EtOH to concentration of 1 mg/ml and 2 mg/ml separately (Only in this assay, I prepared CA in 70 % EtOH). Vitamin C was diluted to 10, 20 , 50, 100, 200 μg/ml (Group A), BHT (Group B) and CA (Group C) were diluted to 100, 200, 500, 1000 and 2000 μg/ml. 20 μl of sample from Group A, B, C added into another 96-well assay plate, separately. 180 μl of DPPH solution was added in each well. Incubation at room temperature for 30 min in Dark, then read absorbance at 517 nm wavelength by microplate reader. 20 μl of 70 % EtOH + 180 μl EtOH was considered as blank, 20 μl of 70 % EtOH + 180 μl DPPH was considered as positive control. The raw data was calculated as:

$$\text{scavenging activity} = (1 - \text{Sample OD}/\text{Positive control OD}) \times 100\ \%$$

4.2.8 Statistical analysis

Results are reported as mean ± standard deviation. Group means were compared with the Student's *t* test, and differences were considered significant at $P < 0.05$

4.3 Results

4.3.1. CA decreased intracellular ROS generation in normal human melanocytes.

CA has been proved anti-oxidant effect in many studies (Vinood et al., 2018). Therefore, I firstly measured the scavenging effect of CA out of cells by DPPH assay to investigate the CA effect on free radical procedure. Vitamin C (Vc) and butylated hydroxytoluene (BHT) are widely used anti-oxidant reagent, which was used as positive control in DPPH assay. In the result, the scavenging effect of CA was not found in DPPH assay (Fig.14 A page 76). To assess the intracellular antioxidant effect of CA, I treated cells with CA and intracellular ROS was measured by DCF assay. Murine melanoma cells (B16F10) and human melanocyte (NHEM) were treated with CA for 2 hours and 24 hours, cells after treatment were stained with DCF and the fluorescence was measured. In B16F10 melanoma cells, 2 hours treatment weakly decreased the ROS generation (Fig.14 B page 76), while increased after 24 hours (Fig.14 C page 76). On the other hand, ROS was dose dependently decreased in NHEM cell both in 2- and 24-hours treatment (Fig.14 D, E page 77). These results mean CA had intracellular scavenging effect in normal human cells, which was not directly through reduce the free radical procedure. Furthermore, CA enhanced ROS generation in murine melanoma cells.

4.3.2. CA dose dependently decreased cell viability.

From recent studies, CA has been found that is able to inhibit the tumor growth (Ren et al., 2017 ; Hanai et al., 2012). Therefore, I tested the CA effect on different kinds of cells, including murine melanoma (B16F10), human melanoma (G-361 and HMV-II) and normal human epidermal melanocytes (NHEM). I cultured cells with different concentration of CA, then cell viability was measured by MTT assay. I found cell viability were dose dependently decreased by CA treatment (Fig.15 A-D page 78-79).

4.3.3 CA down-regulated melanogenesis in human cell line, but up-regulated in murine cell line.

At last, I also tested the effect of CA on melanogenesis in different cell lines. Murine melanoma cell line B16F10 was treated with CA for 4 days (after confluence), cells treated with CA produced more melanin than untreatment group (Fig.16 A page 80). Protein analysis for tyrosinase and MITF at day 2 and day 4 by westernblot also proved that CA up-regulated melanogenesis related gene expression in B16F10 melanoma cell line (Fig.16 F page 81).

In human cell lines, when cells treated for 6 days (got confluence), CA inversely decreased melanin synthesis in all three cell lines (Fig.16 C-E page 80). On the other hand, an interesting phenomenon was found that CA dose dependently increased melanin synthesis in 2 days (not confluence yet) treatment (Fig.16 B page 80). I collected the cells at day 2 and day 6, and the gene expression of tyrosinase and MITF show a similar tendency as melanin content in day 2 and day 6 (Fig.16 G page 81). I suspected that the long-term effect of CA on human cell melanogenesis was down-regulation; however, the short-term effect of CA was up-regulation, which was different to murine melanoma cells.

4.4 Discussion

ROS is thought to be a cause of melanocytes carcinogenesis (Meierjohann, 2014 ; Liu-Smith et al., 2014), while glycolysis and the ETC associated with the TCA cycle are accompanied by a large amount of ROS production (Meierjohann, 2014 ; Icard et al., 2012). CA treatment down-regulated these two processes, therefore intracellular ROS accumulation should be decreased. As expectation, ROS accumulation was decreased in normal human cells, but ROS generation was increased in cancer cells over 24 hours treatment. I still had no evidence that the increased ROS is related to inhibition of proliferation and promotion of apoptosis. At least, I confirmed the beneficial effect of CA on human normal cells, although it is not a usual anti-oxidant reagent like Vitamin C.

In this experiment, the inhibition effect of CA on human or mouse melanoma have been confirmed. I also treated normal melanocytes with CA and I investigated that the inhibition effect on NHEM proliferation was weaker than melanoma cells, which was despite human or mouse melanoma. These results indicated that the mechanism of CA on the physiological metabolism of cancer cells as well as normal cells are different. Among cancer cells, they tend to synthesize more of the required intermediate molecules in order to achieve efficient proliferation, rather than providing efficient energy supply through the TCA cycle (Kroemer and Pouyssegur, 2008). Therefore, glycolysis becomes the best way to synthesize several essential metabolic intermediates required for their proliferation and the fastest way to produce energy, although it is inefficient in producing ATP (2 vs 36) (Xu et al., 2005). While, citrate, or citric acid (CA) is the inhibitor of the enzyme of glycolysis first step, Phosphofructokinase 1 (PFK1), which completely blocking the glycolysis when CA is abundant (Ren et al., 2017 ; Lehninger, 1975). Not only glycolysis, TCA cycle downstream, gluconeogenesis and fatty acid synthesis are also regulated by the quantity of CA (Icard et al., 2012 ; Halabe Bucay, 2007). Therefore, I speculated that the ability of CA to inhibit the proliferation of melanoma in this experiment was achieved by inhibiting the supply of intermediate and energy required for cell proliferation in cancer cells. I still need further

experiments to confirm the effect of CA on glycolysis and therefore inhibit the cell proliferation and cell cycle through down-regulating the glycolysis by CA.

Regarding melanin synthesis, I found that CA had different effects on murine cells and human cells. When cells reach confluent, CA promotes melanin synthesis in murine melanoma cells, whereas inhibition has occurred in human cells. For murine melanoma cells, I also shortened or prolonged the treatment time, and the results obtained were almost identical to those of the fourth day. For human cells, it is interesting to note that the results at day 2 are not the same as those for day 6. This phenomenon was also verified by western results for MITF and tyrosinase, two key factors for melanogenesis. Further, I also performed a tracking experiment on normal human melanocytes after CA treatment. I tested the daily melanin production and found that the relative content of melanin in the cell became rhythmic with time, and the treatment of CA affected this rhythm, and the degree of influence increased with the concentration (Fig.17 page 82). As previous studies, MITF expression level will change periodically with time (Drira and Sakamoto, 2015). I also tested the expression levels of MITF and tyrosinase, but unfortunately, I did not get a stable result that was enough to explain the rhythm of melanin synthesis. In previous studies, MITF has been proved to have the function of regulating Hypoxia-inducible factor 1α (HIF-1α) (Buscà et al., 2005), and HIF-1α stimulates many glycolytic enzyme gene which is linked to cancer (Icard et al., 2012). Therefore, I suspect that there is also a link between melanin synthesis and anticancer effects. I will also pay attention to the rhythm of melanin synthesis and its relationship with anticancer effects in the future.

General Discussion

Melanin synthesis is a very complicated process. Usually, due to the exposure of the skin to UV irradiation, a-MSH is secreted to promote maturation of melanocytes and synthesis of melanin (Price et al., 1998). The synthesis of melanin is also affected by many factors, such as ROS, gene expression, and environment.

The synthesis of melanin requires energy as well as cellular metabolism. The TCA cycle is the main source of energy, and I speculated that whether or not the organic acids will accelerate the TCA cycle and produce energy to promote melanin synthesis. Therefore, I discussed the effects of organic acids on melanin synthesis in Chapter I. The same as guessing is citric acid, which promotes the synthesis of melanin. Next is lactic acid, which has no effect on the synthesis of melanin. Finally, pyruvic acid, oxaloacetic acid, malic acid and a-ketoglutaric acid all exhibited inhibition of melanin synthesis. Aside from the interconversion between lactic acid and pyruvate, the location of citric acid in the TCA cycle is between pyruvate and other organic acids. In the chapter II, I also found that after treatment of PA, EP, sodium pyruvate (Fig.12 page 74) or even MA (Fig.18 page 83), ATP was increased to a certain extent. Other members of our laboratory also found elevated ATP when using CA to treat other cell lines. These conclusions demonstrate that the use of organic acids does increase ATP production. However, depending on the fact that different organic acids had a completely opposite effect on the synthesis of melanin, this increased energy production does not appear to be directly related to the synthesis of melanin. From the comparative experiments of pyruvic acid and sodium pyruvate, although the production of ATP was improved, pyruvate reduced the production of melanin, and sodium pyruvate had no effect on the synthesis of melanin. In summary, the regulation effect of melanin by pyruvic acid or other organic acids is independent of ATP synthesis or TCA cycle.

Another point about the discussion of organic acids is acidity. In the Chapter I I also mentioned that the addition of organic acids to the medium caused an increase in the acidity of the solution. According to what I know, too high acidity has a bad effect on cells. Therefore, in order to distinguish the role of acidity, I also carried out neutralization experiments in Chapter II. I added pyruvic acid to the medium, and then adjusted the pH of the solution with NaOH until the same color was returned as the blank medium. The results showed that after neutralizing the acidity of pyruvic acid in the medium, pyruvic acid still has the ability to reduce melanin synthesis. So, I speculated that the ability of organic acids to regulate melanin synthesis is also independent of its own acidity. The ions contained in the previous sodium pyruvate solution are pyruvate ions and sodium ions, and the ions contained in the pyruvic acid solution after neutralization are also pyruvate ions and sodium ions. I concluded that pyruvate ions themselves do not have the ability to modulate melanin synthesis. Therefore, the existence of organic acid molecules themselves is the most important factor regulating melanin synthesis.

α-MSH binds to MC1R on melanocytes, thereby activating the cAMP pathway, which in turn promotes melanin synthesis by promoting mitf transcription (Otręba et al., 2012; Kim et al., 2016). The MITF protein obtained by translation will be regulated by other upstream factors. There are three types of upstream factors that are generally recognized as MAPK, PI3K/AKT and GSK3β. I redefine the effects of the two pathways of AKT and GSK3β on melanin synthesis in Chapter II. Due to the use of PI3K and inhibitors of GSK3β, I found that melanin synthesis was increased by these two inhibitors. This phenomenon indicates that PI3K/AKT and GSK3β have an inhibitory effect on melanin synthesis. And the amount of MITF has increased, indicating that the use of inhibitors reduces the degradation of MITF. Since the known AKT can promote the phosphorylation of GSK3β (Beurel et al., 2015), I improved the original simple PI3K/AKT-GSK3β pathway into AKT to promote GSK3β phosphorylation, while p-GSK3β promoted the degradation of MITF. At the same time, recent experimental results indicate that AKT has a site that directly catalyzes the

phosphorylation of MITF, and phosphorylation of this site also leads to degradation of MITF. Therefore, I summarize the role of the upstream adjustment of MITF as shown in Figure 9. In the presence of inhibitors, PA and EP did not reduce the increase in melanin synthesis caused by BIO, indicating that GSK3β is the most important pathway for PA and EP to regulate melanin synthesis. While the increase in melanin caused by LY294002 was slightly reduced by PA and EP, means AKT cascade is partly involved in PA and EP regulating melanin synthesis. Although PA and EP caused a significant increase in p-ERK, ERK inhibitors could not change the melanin reduction caused by PA. EP also showed only a slight increase. First, the ERK pathway was only partially present in EP-regulated melanin reduction. Second, in the process of PA and EP regulation of melanin synthesis, the phosphorylation of ERK seems to be a result, not a condition that affects melanin synthesis, so the increase in ERK phosphorylation should also be linked to a more upstream site. In summary, I found that PA and EP regulate melanin synthesis not because of altered ATP synthesis or affecting the activity of the TCA cycle. They reduce the synthesis of melanin by regulating the metabolic processes of melanin synthesis.

In the Chapter III, I mainly discussed the role of CA in different cell lines. First, I confirmed that CA has antioxidant properties, but this antioxidant is different from the commonly used antioxidant Vitamin C, which can directly reduce free radicals, but is reduced in the body by metabolic pathways. And in cancer cells of mice, CA exhibits ROS clearance ability that changes with time. As I mentioned before, the synthesis of melanin in the skin is caused by the rise of UV-induced ROS. Therefore, the antioxidant properties of CA should reduce the synthesis of melanin. This result was also verified by a subsequent melanin synthesis experiment. The decrease in ROS caused by antioxidants leads to inhibition of the activity of the ERK pathway, thereby reducing the inhibitory effect on MITF and increasing the synthesis of melanin. This inference was verified in mouse cells, but in mouse cells I found that CA did not have significant ROS clearance during long-term treatment. Since the melanin cancer cells themselves have a high ROS content and the DCFDA method used in this experiment can only

measure the relative ROS content, I still have to determine the specific content of ROS in a single cell. And I need to determine the evaluation criteria for the effect of ROS content on cellular physiology. Second, CA demonstrated that different inhibitory effects on cancer cells and normal cells suggest that CA can be used as a potential anticancer agent. But further experiments have yet to be perfected. Third, I found that CA has different effects on cells from different sources. CA promotes melanin synthesis in mouse-derived cells, but inhibits melanin synthesis in human-derived cells. This is a phenomenon in which melanin synthesis is not identical in human cells and mouse cells. And in the experiments of human normal cells, I found that the relative melanin content of cells changes rhythmically with time. The treatment of CA does not completely hinder this regular rhythm change, but will delay or slow down this rhythm to varying degrees. I speculate that this phenomenon is related to cell proliferation. From the above experimental results, it can be seen that CA has an inhibitory effect on the proliferation of melanocytes, so I will study the relationship between melanin synthesis and cell proliferation.

Future prospects

As small molecule contained in the human body, organic acids play an important role in the physiological metabolism of cells. The effect of regulating the physiology of cells by changing the content of organic acids is the result I expect. In this experiment I found and determined that pyruvic acid can reduce the melanin synthesis, and that citric acid has different effects on different kinds of cells. I am looking forward to further experiments to perfect this theory.

The first is that the role of citric acid remains to be improved. I need to know if citric acid can regulate cell proliferation by regulating the process of intracellular glycolysis. And whether this effect is related to the rhythmic changes in melanin content. Because of the specific inhibitory effect of CA on cancer cells, I expect to find its effect on anticancer and induction of apoptosis.

Secondly, I will continue to explore the remaining organic acids, so as to comprehensively summarize the physiological effects of organic acids on melanin metabolism and melanoma development.

Finally, I plan to use a mouse model to verify the efficacy of the known organic acids in vivo. This proves whether organic acids can be used as an effective agent for regulating melanin synthesis or melanoma development.

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Figure and legends

Fig.1

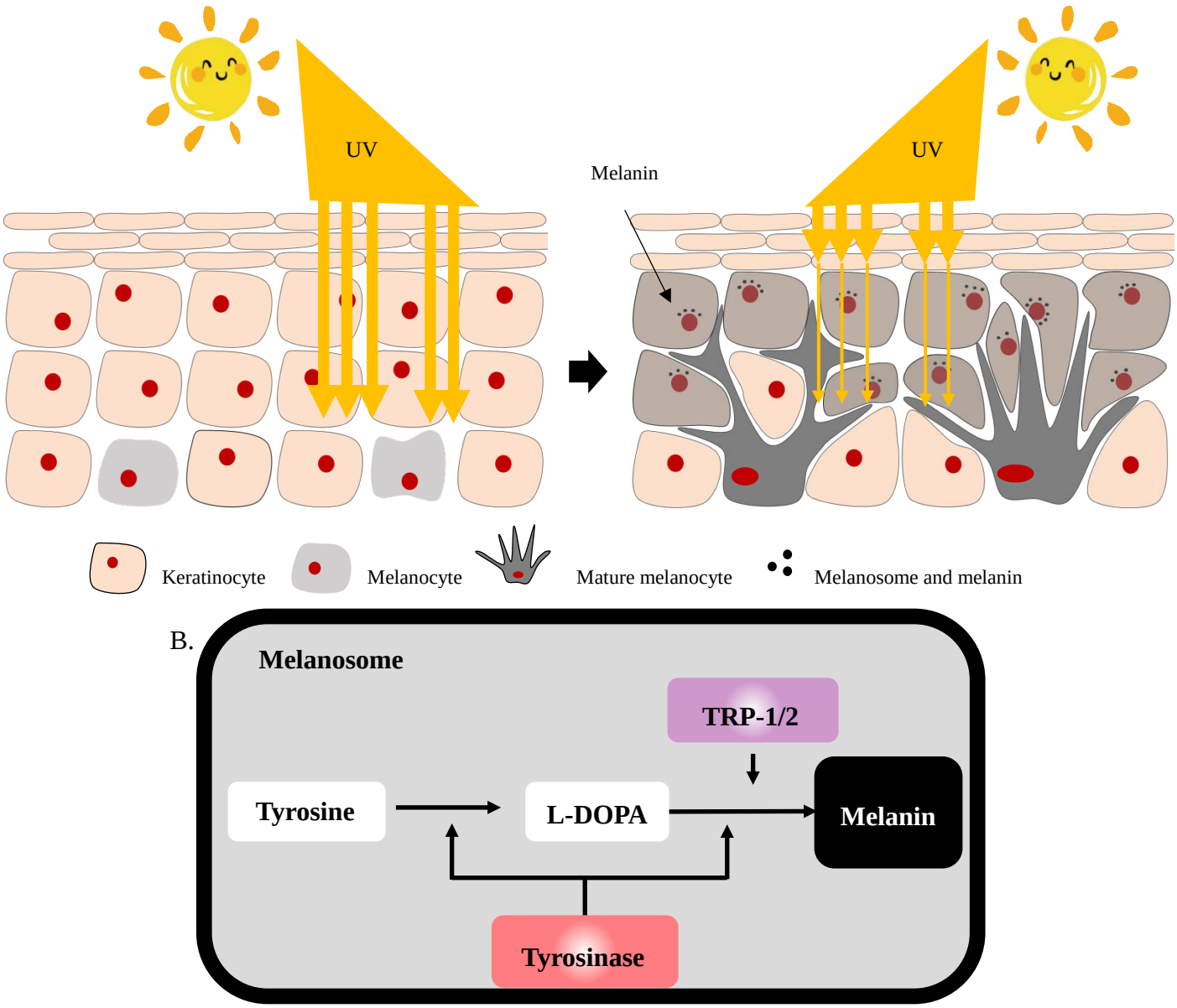


Figure 1. Mechanism of melanin synthesis induced by UV irradiation. A. UV induce melanocytes maturation, produced melanin protect keratinocytes from UV. **B.** Tyrosinase catalyze tyrosine to melanin together with TRP-1 and TRP-2.

Fig.2

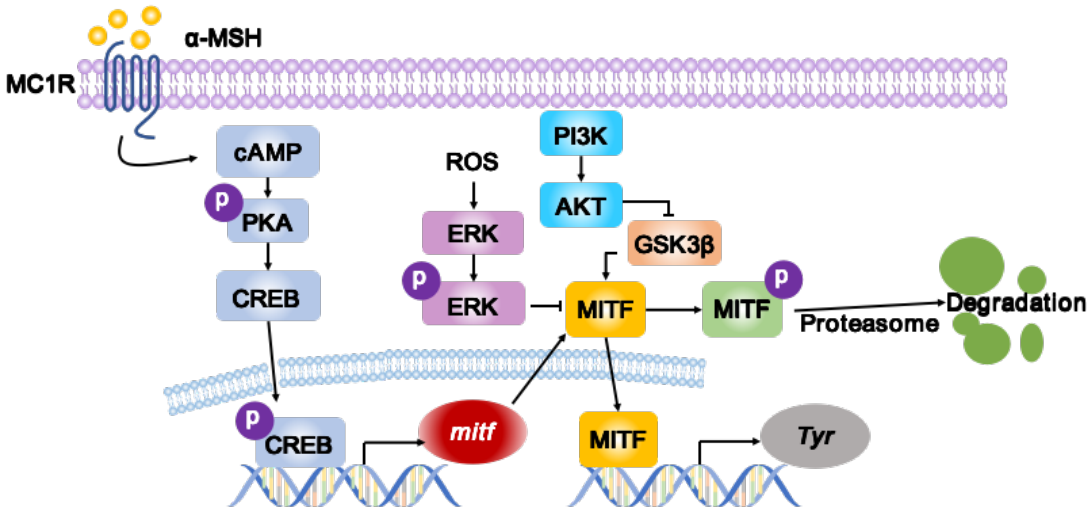


Figure 2. Upstream signaling pathway of MITF. a-MSH binging to MC1R, activates cAMP signaling pathway. cAMP activates PKA and CREB, then promotes *mitf* mRNA transcription. ERK activated by ROS to phosphorylation, p-ERK induced MITF phosphorylation, p-MITF is degraded by proteasome system. AKT and GSK3β had inhibition effect on MITF and melanin synthesis.

Fig.3

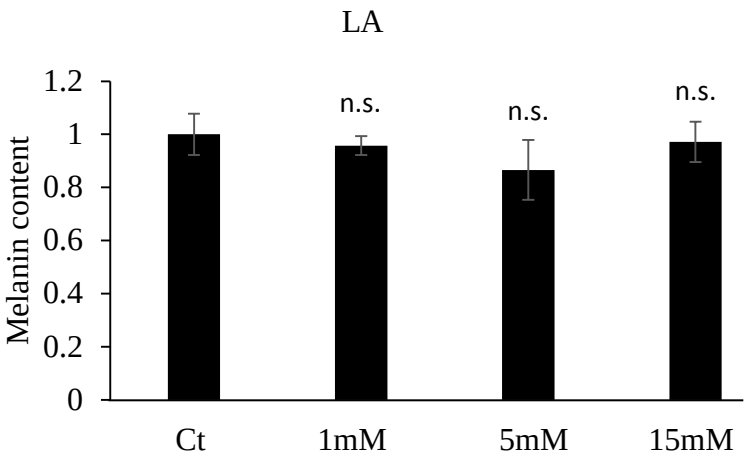


Figure 3. LA had no effect on melanin synthesis. LA treated cells for 48 hours. Cells were collected and breaked by NaOH and heating. The absorbance at 405nm was measured. LA did not regulate the melanin content.

Fig.4

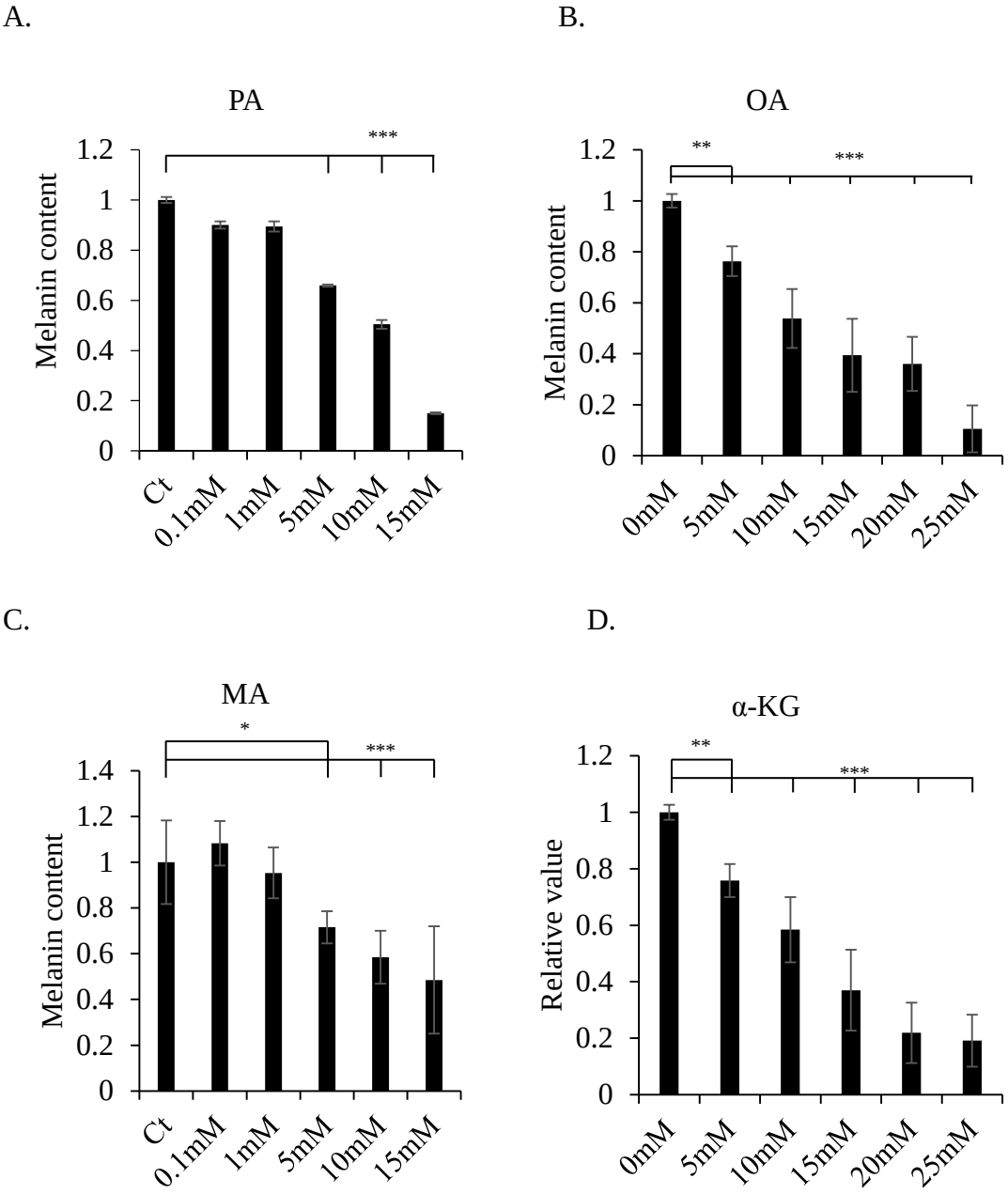


Figure 4. PA, OA, MA, α-KG down-regulated melanogenesis. After treated with PA, OA, MA and a-KG for 48 hours, cells were collected. Cell lysate was used to measure the melanin content. **A-D.** These four moleculor dose dependently down-regulated melanin synthesis in B16F10 cells. Mean ± SD, n⩾3, to compare with control group (Ct) **p* < 0.05; ***p* < 0.01; ****p* < 0.005

Fig.5

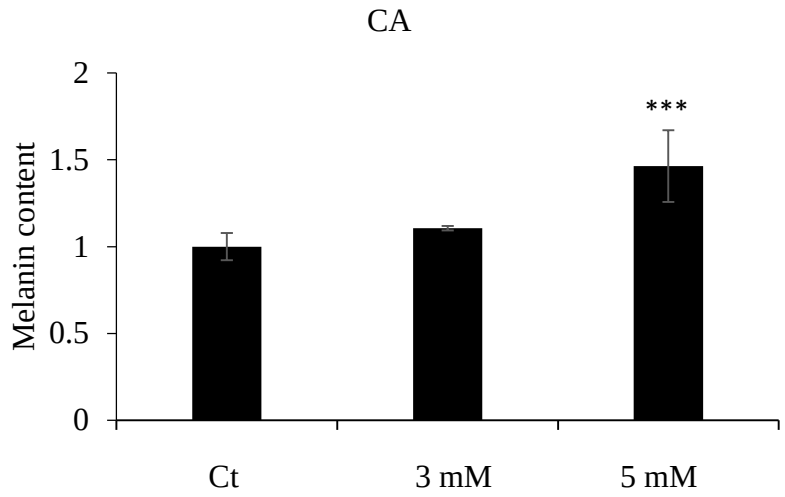
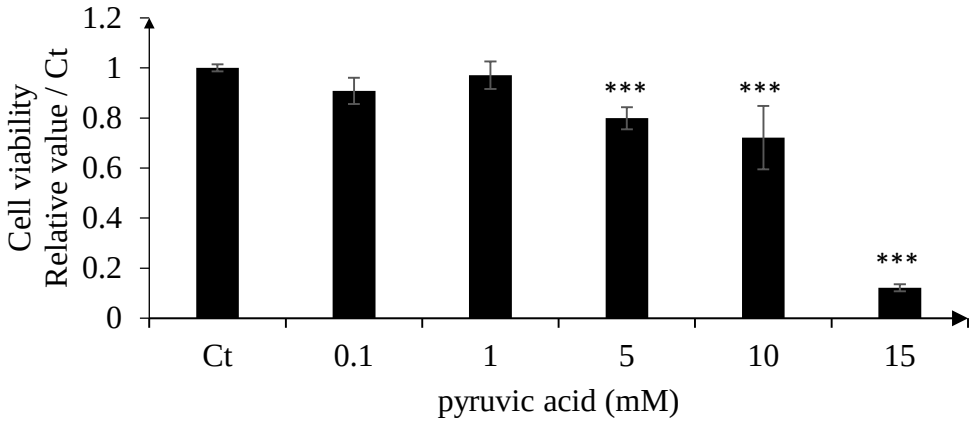


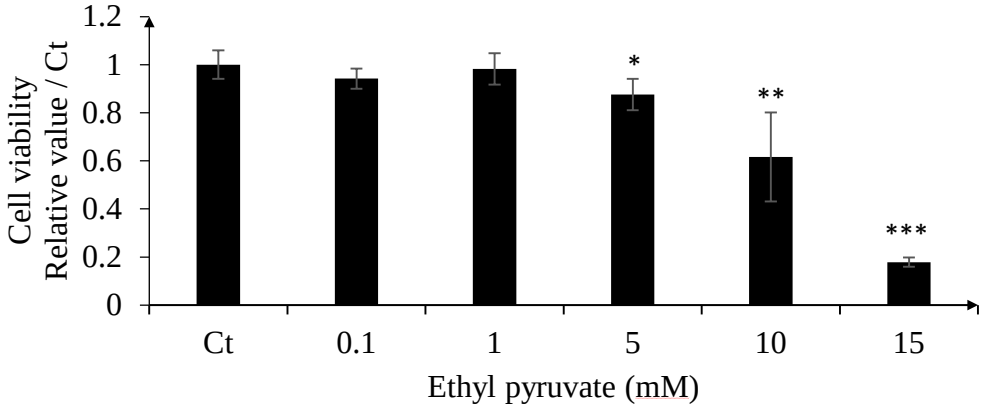
Figure 5. CA increased melanin content in cells. After treated cells with CA for 48 hours, cells were collected. Cell number over 5 mM treatment groups had little cells means the higher toxic of CA on cells. The CA lower 5 mM dose dependently increased melanin synthesis in B16F10 cells. Mean ± SD, n≥3, to compare with control group (Ct) ***$p < 0.005$

Fig.6

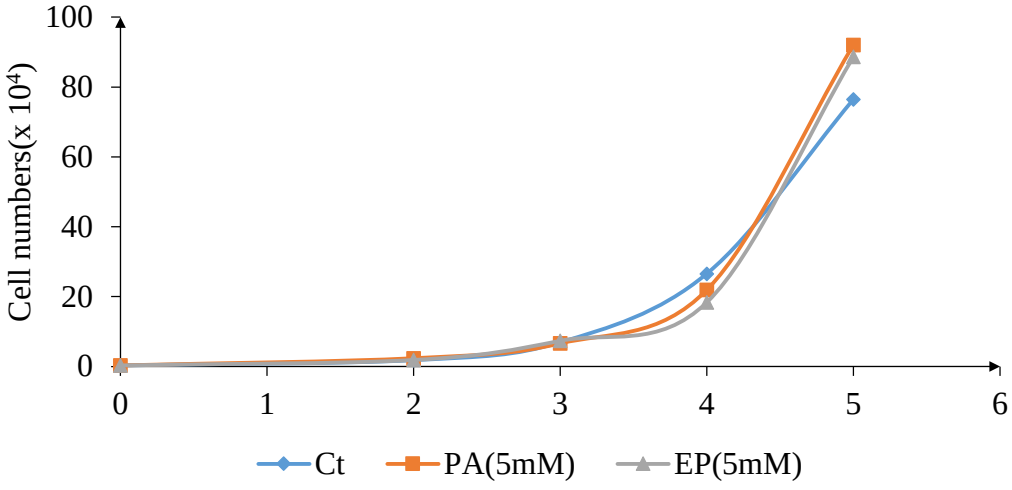
A.


Cell viability
Relative value / Ct
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
Ct
0.1
1
5
10
15
pyruvic acid (mM)

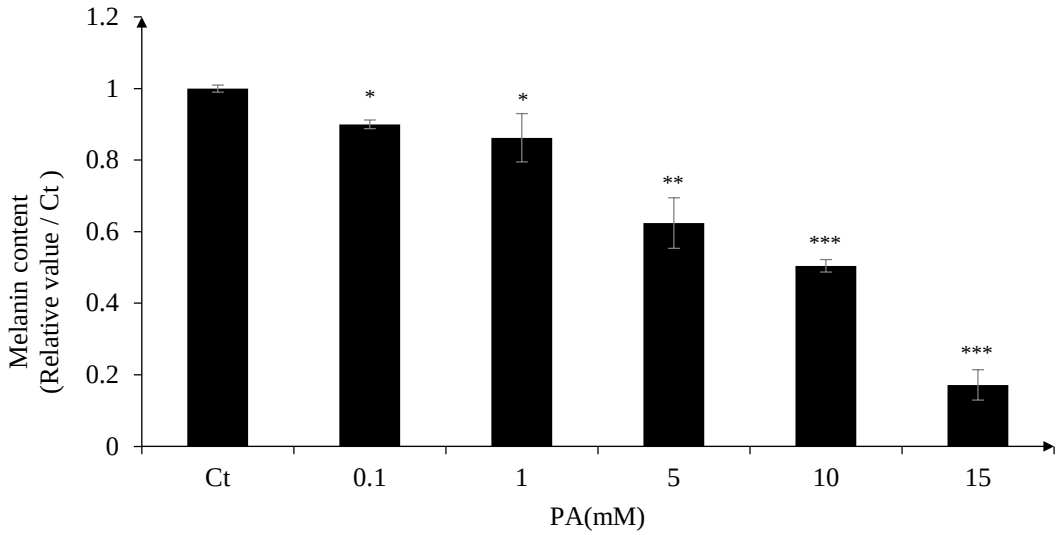
B.


Cell viability
Relative value / Ct
1.2
1
0.8
0.6
0.4
0.2
0
*
**
***
Ct
0.1
1
5
10
15
Ethyl pyruvate (mM)

C.


Cell numbers(x 10⁴)
100
80
60
40
20
0
0
1
2
3
4
5
6
Ct
PA(5mM)
EP(5mM)

D.



E.

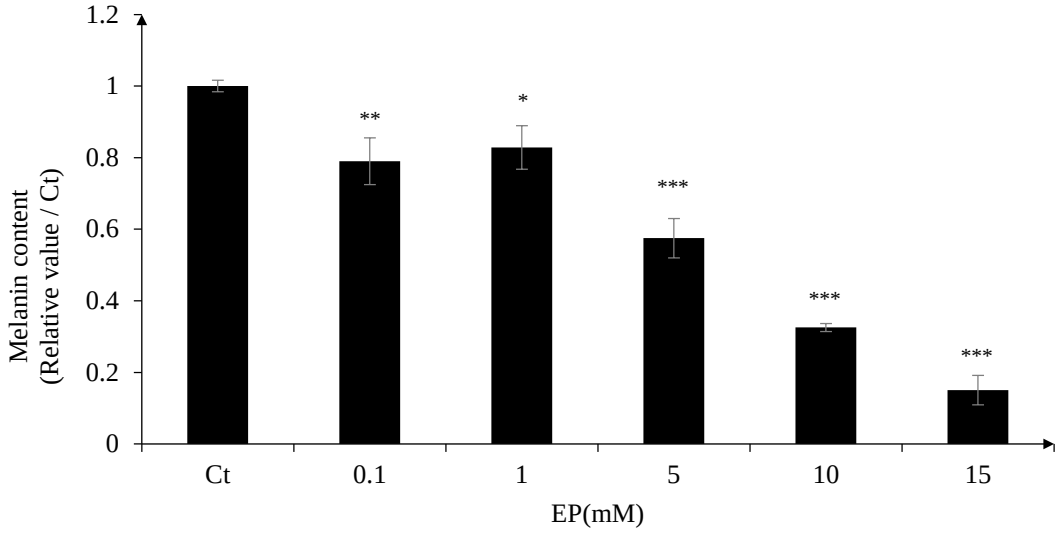
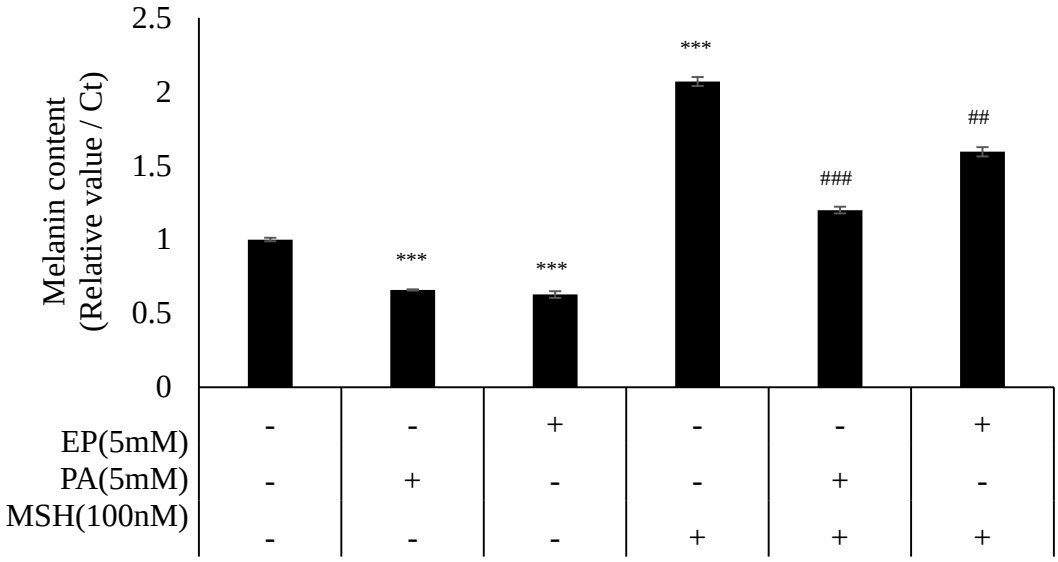


Figure 6. Pyruvic acid (PA) and ethyl pyruvate (EP) dose dependently decreased melanogenesis. B16F10 melanoma cells were treated with different concentration of PA and EP, with or without MSH and forskolin (Fsk). Cells were collected after 48 hours and performed next assays. Control group without any treatment is short for Ct. **A&B**. Effect of PA or EP on B16F10 melanoma cells viability. **C.** B16F10 melanoma cells' growth curve by 5mM PA and EP treatment. **D&E.** PA and EP dose dependently decreased melanin synthesis in B16F10 melanoma cells. Data were normalized to total protein content.

F.



G.

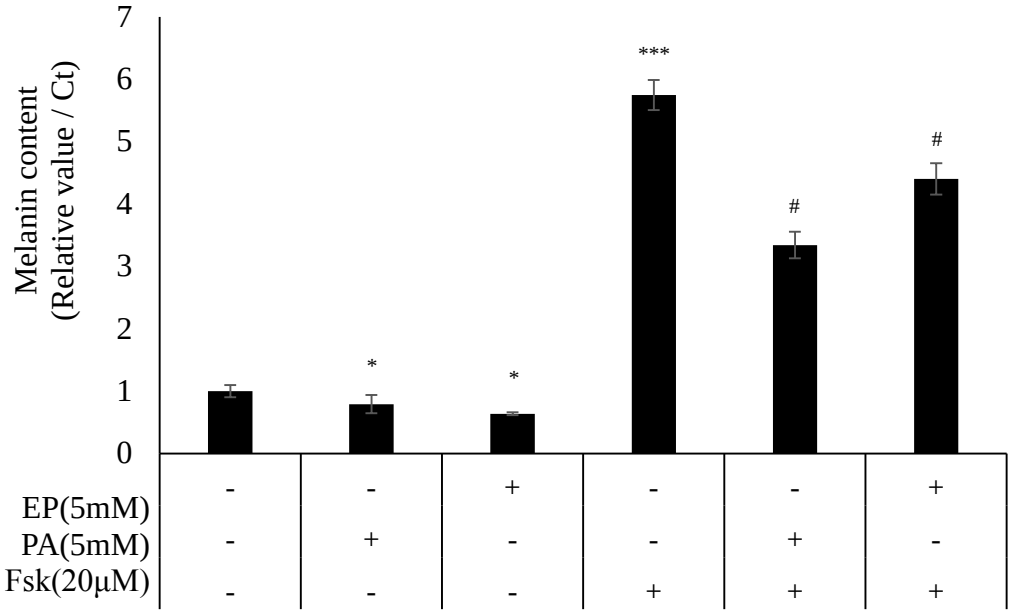
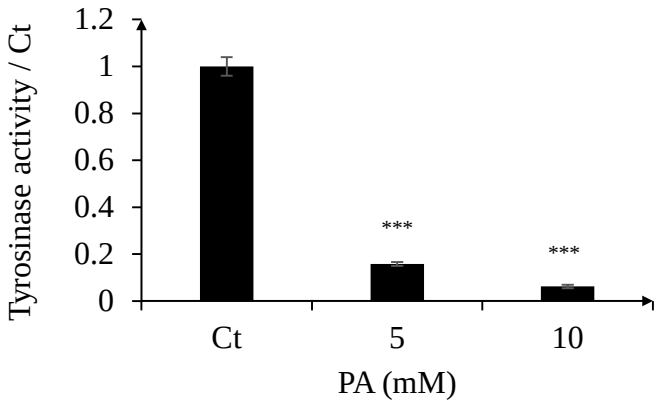


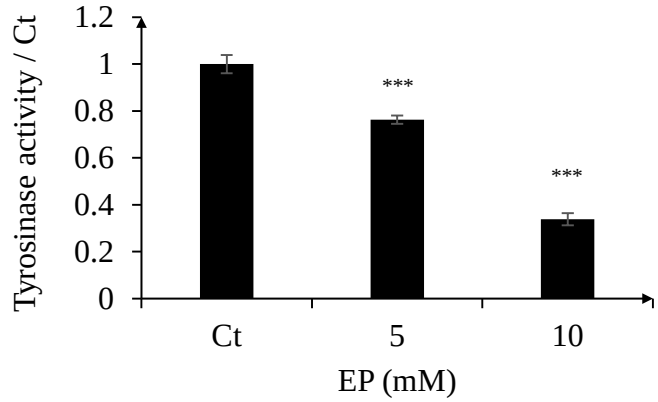
Figure 6. F&G. Melanogenesis promoter α-MSH and Fsk used as positive control, their treatment induced melanin synthesis, which suppressed by 5 mM PA or EP treatment. Data were normalized to total protein content. Mean ± SD, n⩾3, to compare with control group (Ct) *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; to compare with positive control, #$p < 0.05$; ##$p < 0.01$; ###$p < 0.005$.

Fig.7

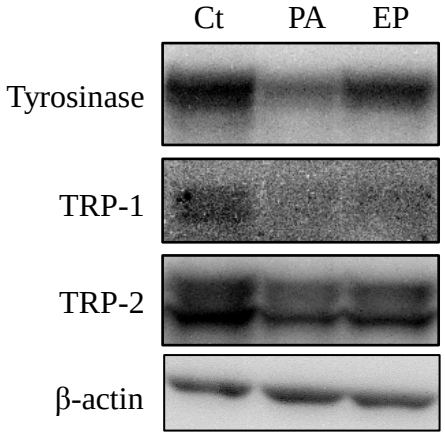
A.


Tyrosinase activity / Ct
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Ct
5
10
PA (mM)

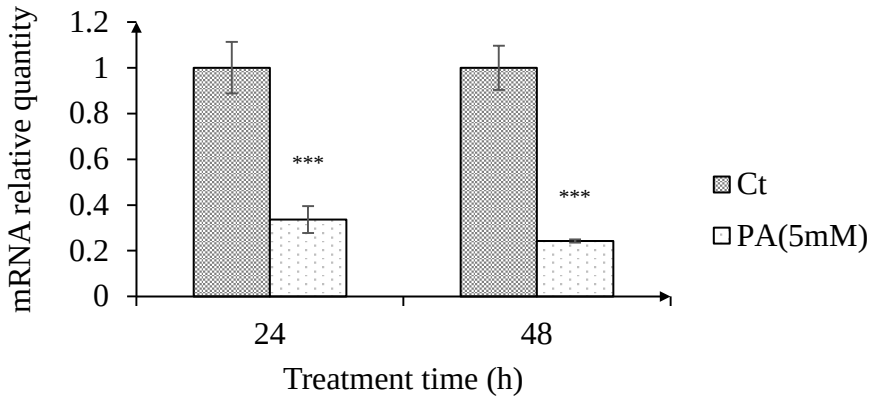
B.


Tyrosinase activity / Ct
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Ct
5
10
EP (mM)

C.


Ct
PA
EP
Tyrosinase
TRP-1
TRP-2
β-actin

D.



E.

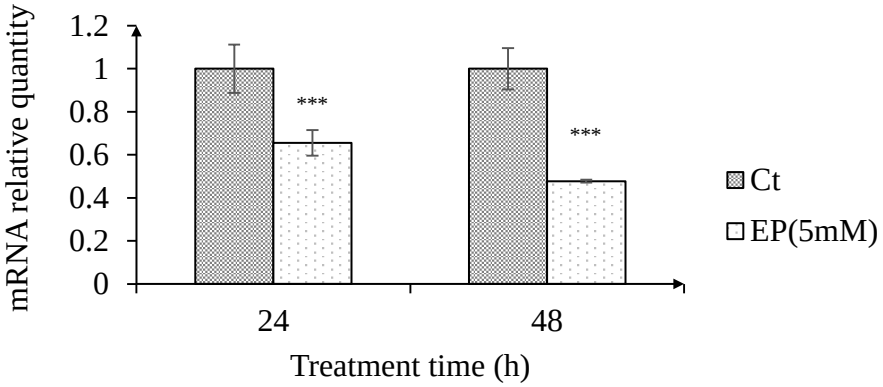
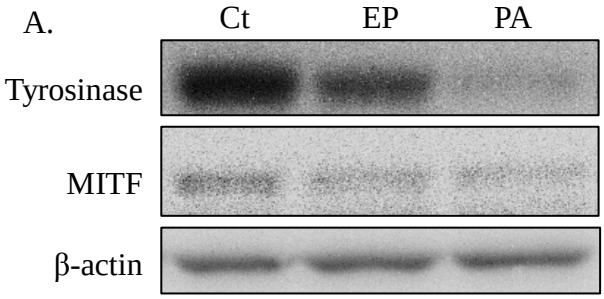


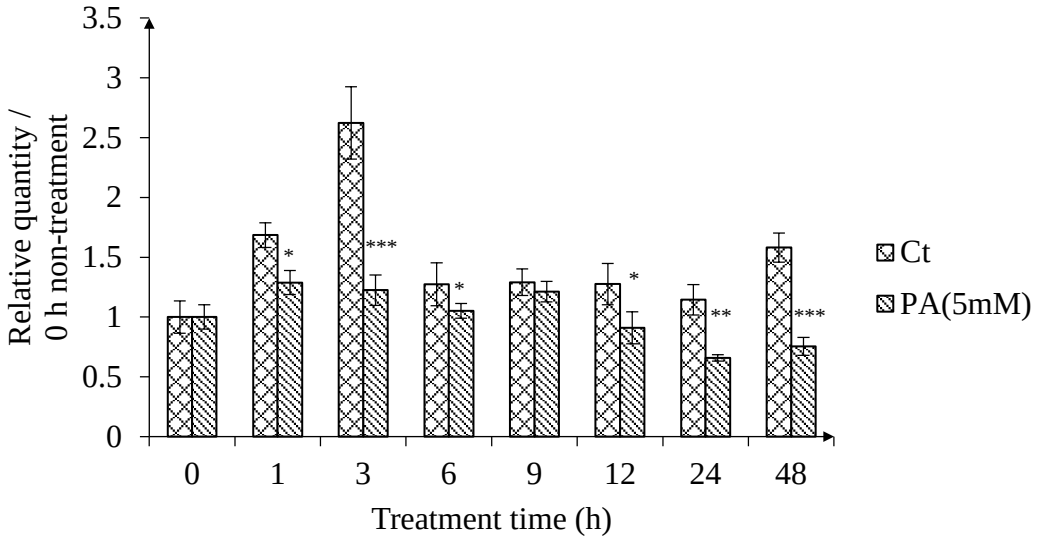
Figure 7. PA and EP decreased tyrosinase activity and gene expression. Cells were treated with PA and EP for 24 or 48 hours. After treatment, cells were collected for next assays. Cells without any treatment was control group, short as Ct. **A&B.** Cells were collected, and the 50 μg of cell lysate were used for tyrosinase activity, the absorbance increase in 30min was measured and calculated by concentrated melanin. The production of melanin in 30 mins was regarded as tyrosinase activity. PA and EP dose dependently decreased the tyrosinase activity. **C.** 48 hours treatment cells were collected for westernblot and tyrosinase, TRP-1, TRP-2 protein quantity was down regulated by 5 mM PA or EP treatment. **D&E.** Tyrosinase mRNA was suppressed after PA and EP, 24 and 48 hours treatment. These mRNA quantities were normalized by GAPDH quantity. Mean ± SD, n⩾3, ***$p < 0.005$

Fig.8

A.


Ct
EP
PA
Tyrosinase
MITF
β-actin

B.


Relative quantity / 0 h non-treatment
3.5
3
2.5
2
1.5
1
0.5
0
0
1
3
6
9
12
24
48
Treatment time (h)
Ct
PA(5mM)

C.

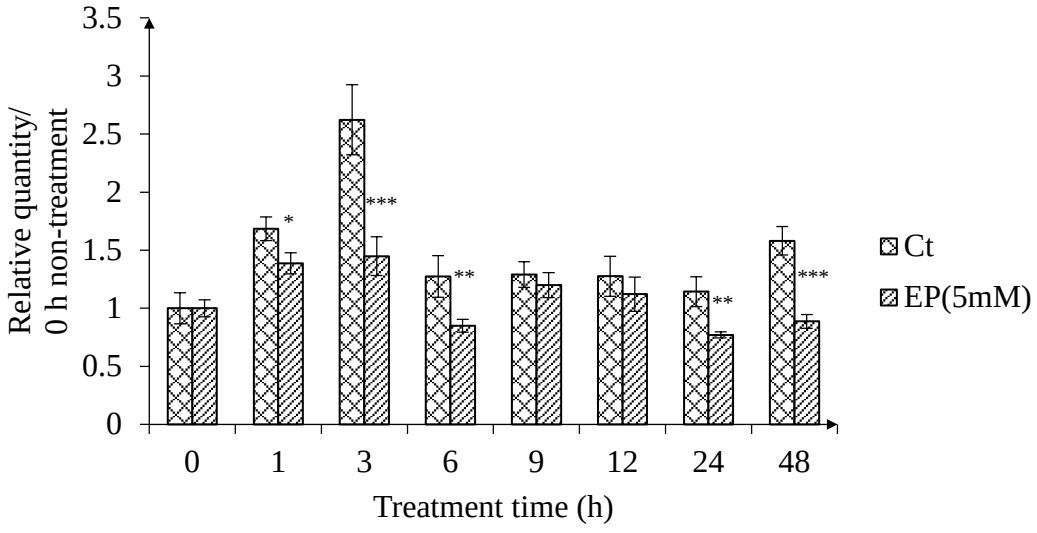
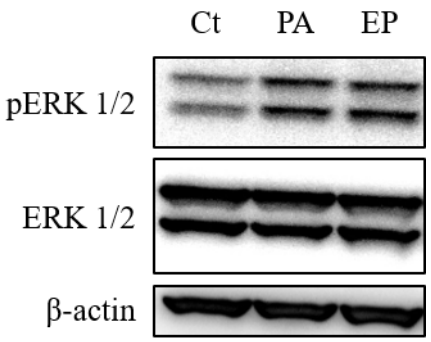

Relative quantity/ 0 h non-treatment
3.5
3
2.5
2
1.5
1
0.5
0
0
1
3
6
9
12
24
48
Treatment time (h)
Ct
EP(5mM)

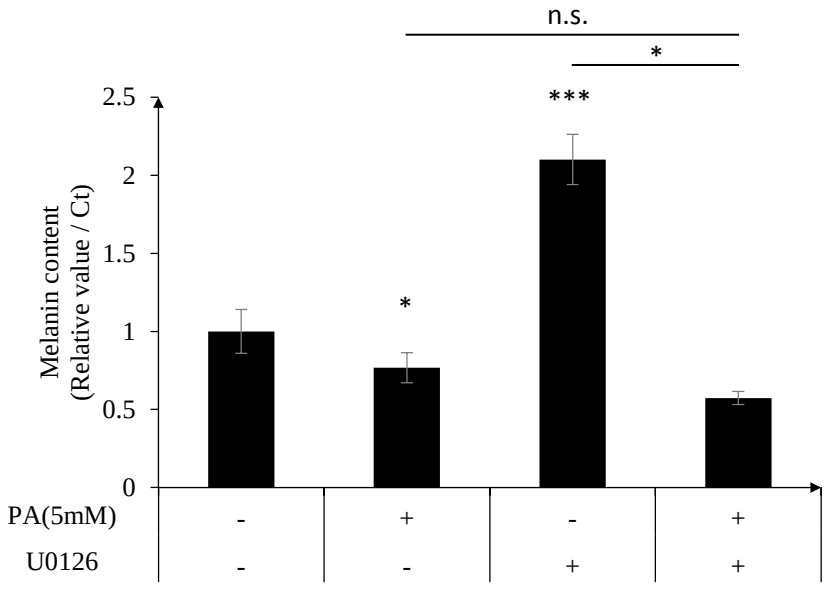
Figure 8. PA or EP regulated MITF time dependently gene expression. B16F10 melanoma cells were treated with PA and EP in 48 hours, cells were collected at 0, 1, 3, 6, 9, 12, 24, 48 hours. **A.** Although tyrosinase was decreased, MITF was non-significant down-regulated by PA and EP treatment at 48 hours. **B&C**. After collected cells, mRNA quantity was measured by RT-PCR. *mitf* mRNA in non-treatment group (Ct) was increased in 3 hours by refeeding fresh medium and return to normal level after 6 hours which without any promoter as MSH or Fsk. Both PA and EP reduced the increased specially at 3 hours *mitf* mRNA, except 9 hours. All these data were normalized by GAPDH mRNA quantity. Mean ± SD, compared with non-treatment groups (Ct) at same time point, n$\geqslant$3, *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

Fig. 9

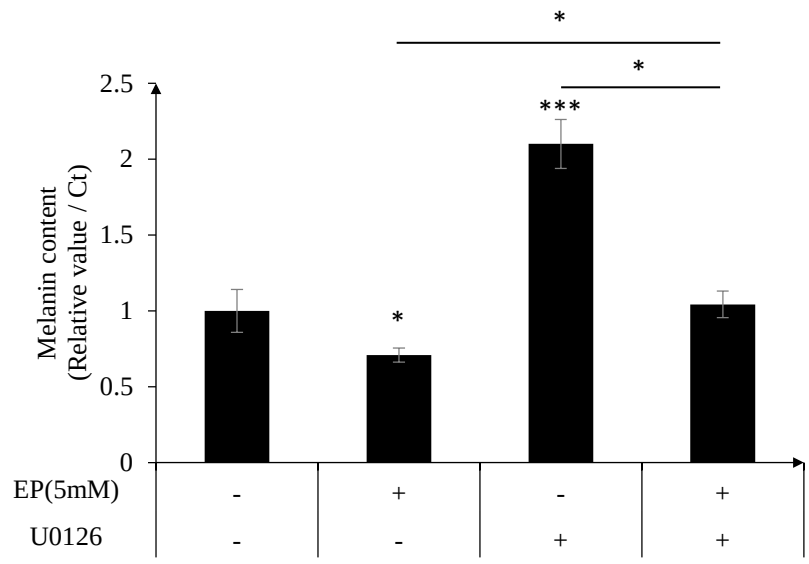
A.


Ct
PA
EP
pERK 1/2
ERK 1/2
β-actin

B.


n.s.
*
***
*
Melanin content
(Relative value / Ct)
2.5
2
1.5
1
0.5
0
PA(5mM)
U0126
-
+
-
+
-
-
+
+

C.


*
*
***
*
Melanin content
(Relative value / Ct)
2.5
2
1.5
1
0.5
0
EP(5mM)
U0126
-
+
-
+
-
-
+
+

D.

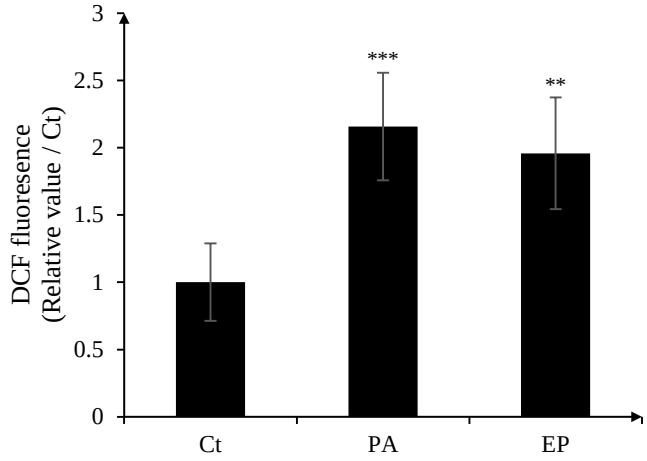
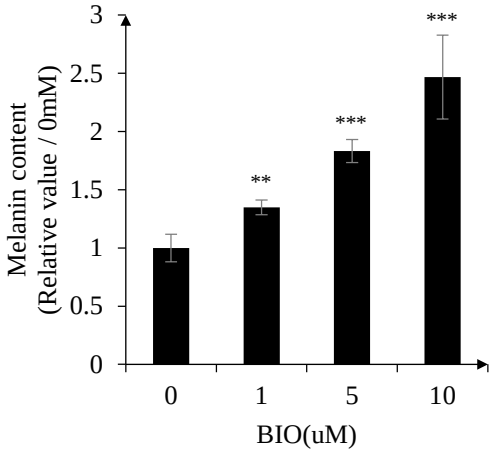


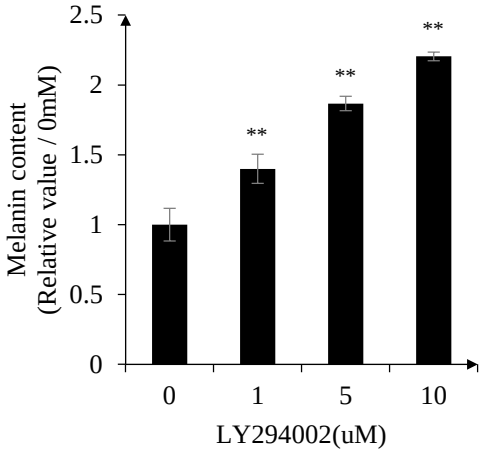
Figure 9. PA and EP regulate melanogenesis through ROS-ERK signaling. A. ERK phosphorylation was increased by PA and EP treatment, whereas ERK gene expression was unaffected. **B, C.** B16F10 melanoma cells were treated with PA, EP, and U0126 for 48 h; melanin content was increased by U0126, but this effect was reversed by PA and EP treatment. **D.** After treatment with PA and EP for 48 h, cells were treated with DCFDA for 1 h and fluorescence was measured (excitation/emission = 495/529 nm). PA and EP increased ROS generation in B16F10 cells. Data represent mean ± standard deviation ($n \geqslant 3$). *P < 0.05, **P < 0.01, ***P < 0.005

Fig. 10

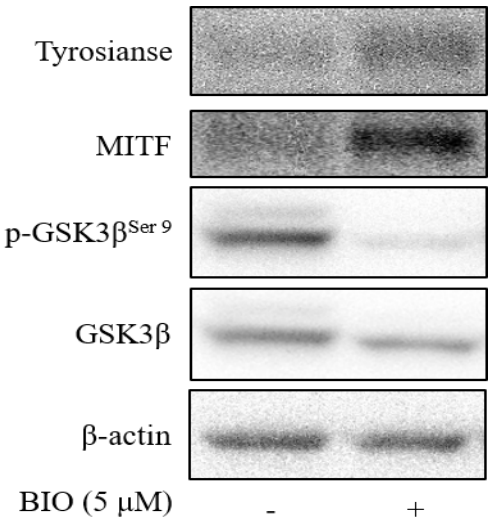
A.


Melanin content
(Relative value / 0mM)
3
2.5
2
1.5
1
0.5
0
**
***
***
0
1
5
10
BIO(uM)

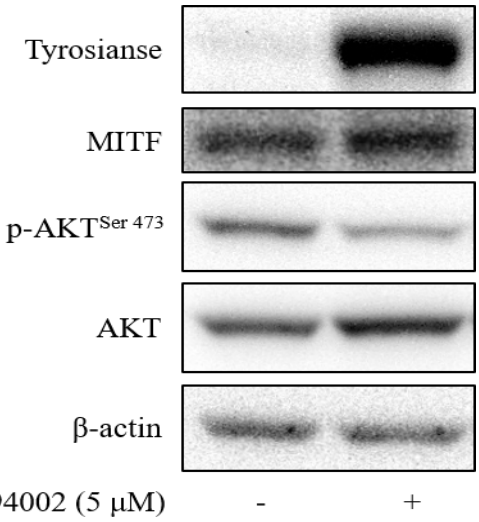
B.


Melanin content
(Relative value / 0mM)
2.5
2
1.5
1
0.5
0
**
**
**
0
1
5
10
LY294002(uM)

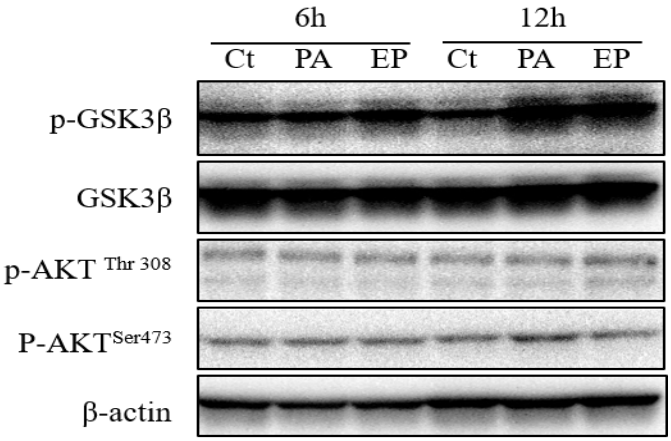
C.


Tyrosianse
MITF
p-GSK3β Ser 9
GSK3β
β-actin
BIO (5 μM)
-
+

D.


Tyrosianse
MITF
p-AKT Ser 473
AKT
β-actin
LY294002 (5 μM)
-
+

E.


6h
12h
Ct
PA
EP
Ct
PA
EP
p-GSK3β
GSK3β
p-AKT Thr 308
P-AKT Ser473
β-actin

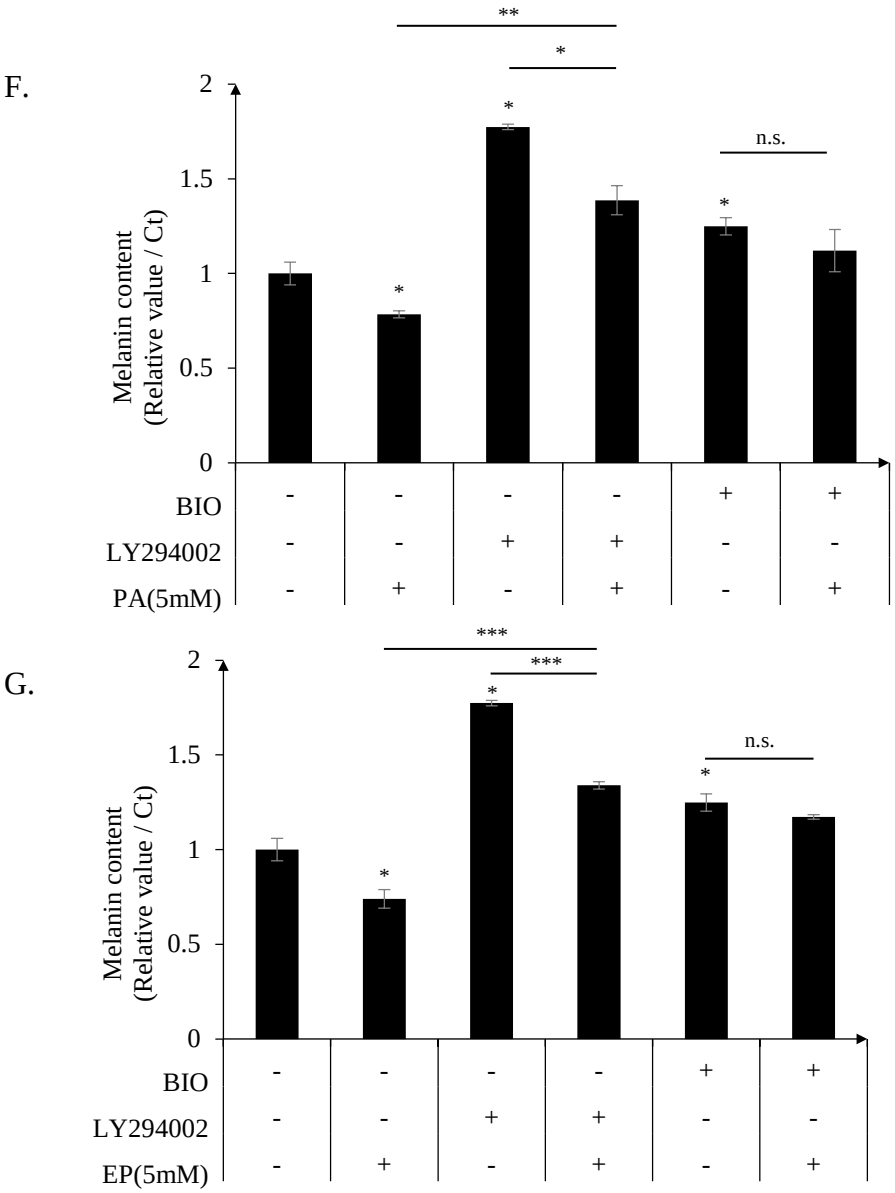


Figure 10. GSK3β plays a key role in the regulation of melanogenesis by PA and EP. Cells were treated with different concentrations of LY294002 and BIO (1, 5, and 10 μM) for 48 h. **A, B.** Inhibition of PI3K and GSK3β (with LY294002 and BIO, respectively) dose-dependently increased melanin synthesis. **C.** Effect of BIO on GSK3β phosphorylation and melanogenesis-related gene expression. **D.** Effect of LY294002 on AKT phosphorylation and melanogenesis-related gene expression. **E.** Cells were treated with PA and EP for 6 and 12 h; lysates were then analyzed by western blotting. GSK3β phosphorylation was increased by PA and EP treatment, whereas AKT phosphorylation at Ser473 was increased by PA treatment. **F,G**. Cells treated with CA were co-treated with BIO or LY294002 for 48 h; the absorbance of the lysates at 405

nm was measured to determine melanin content. Untreated cells served as the control group (Ct). Data represent mean ± standard deviation (n≥3). *P < 0.05, **P < 0.01, ***P < 0.005.

Fig.11

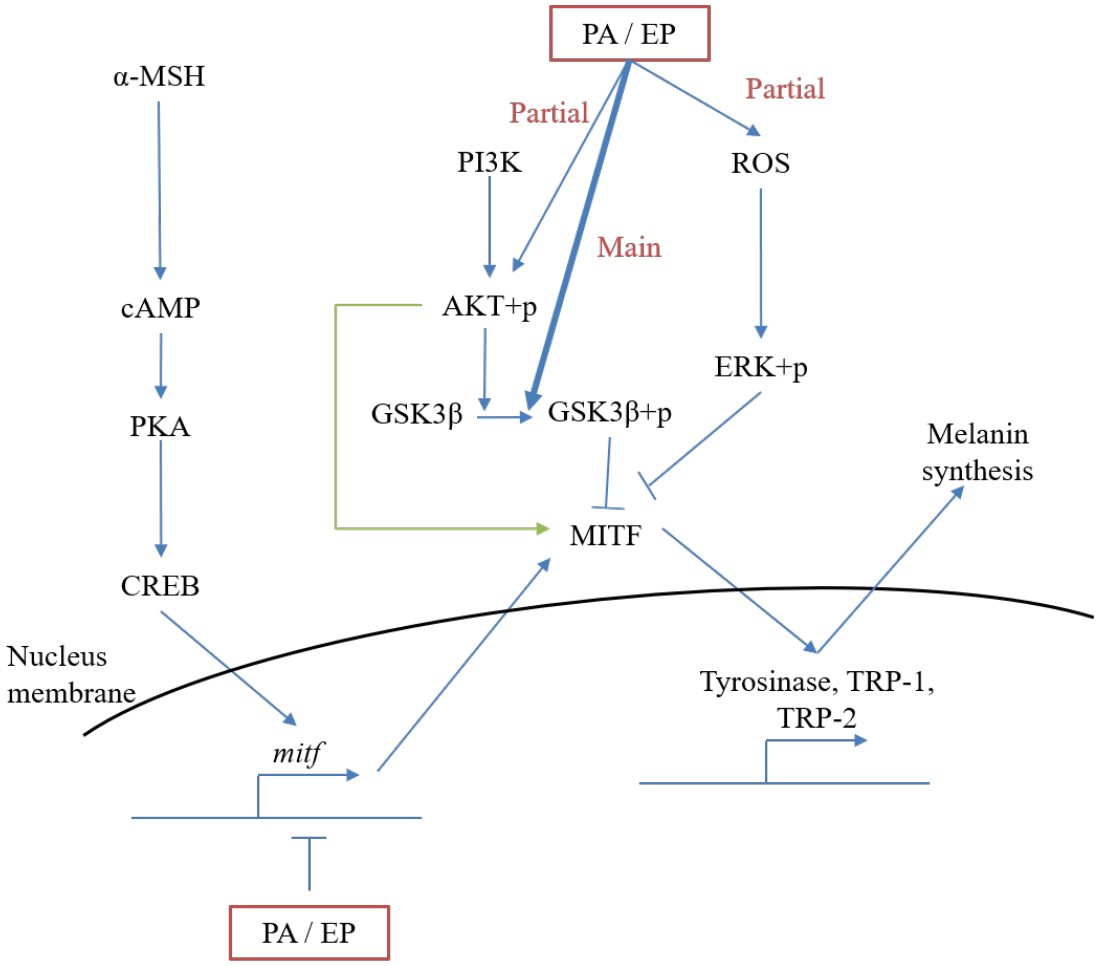


Figure 11. Signaling pathway for PA / EP induced melanogenesis down-regulation in B16F10 melanoma cells.

Fig.12

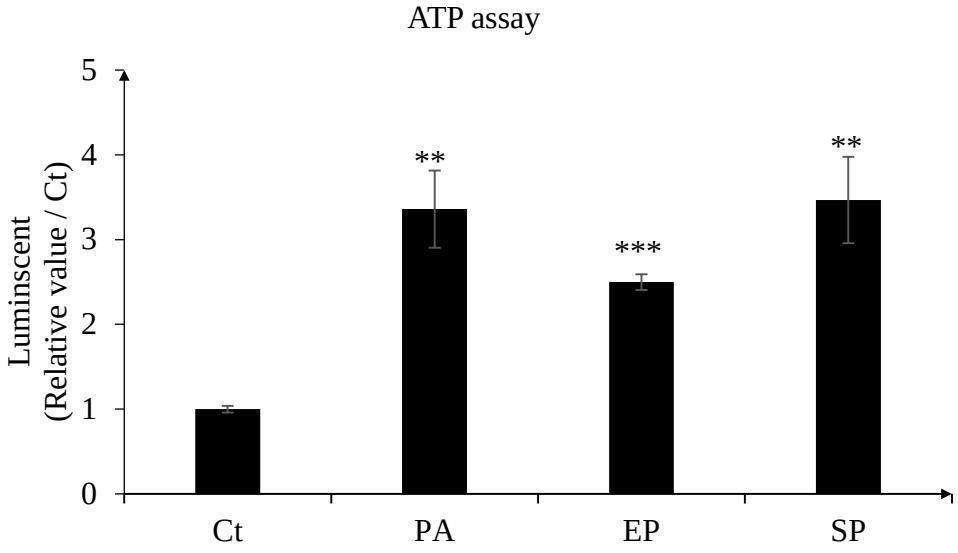
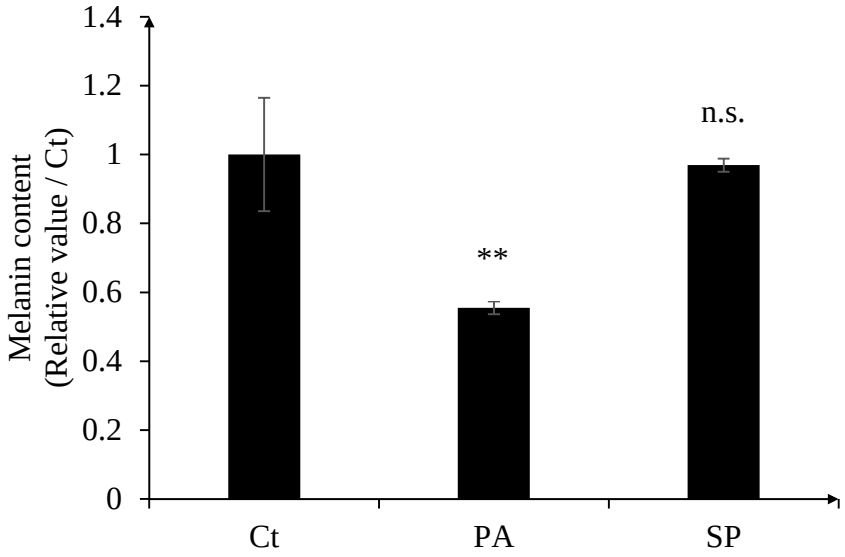


Figure 12. Cells were treated with PA, EP and sodium pyruvate. After 3 hours treatment, medium was removed, and luminance reagent was put in. Luminance in cells were recognized as ATP quantity. Mean ± SD, n⩾3, **p < 0.01; ***p < 0.005

Fig.13

A.



B.

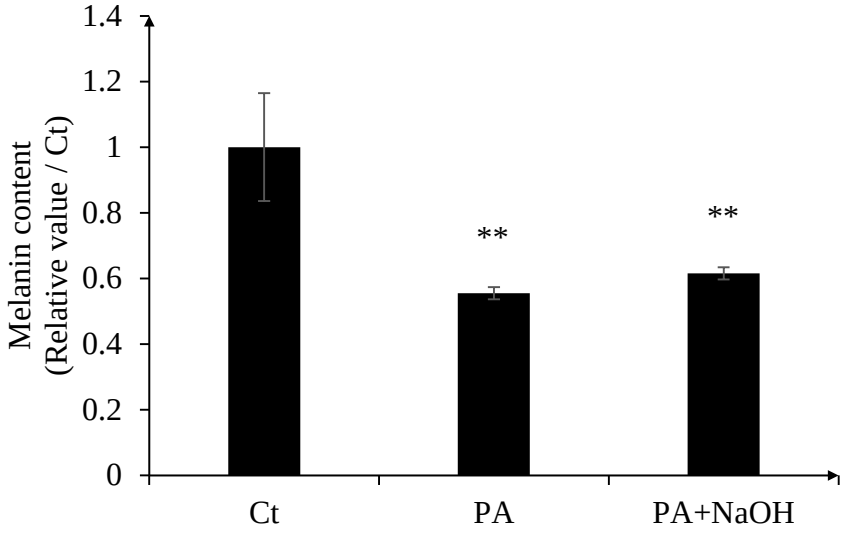
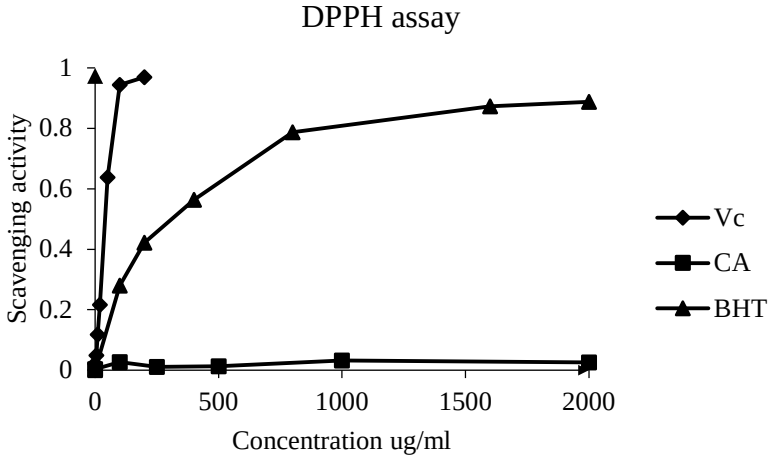


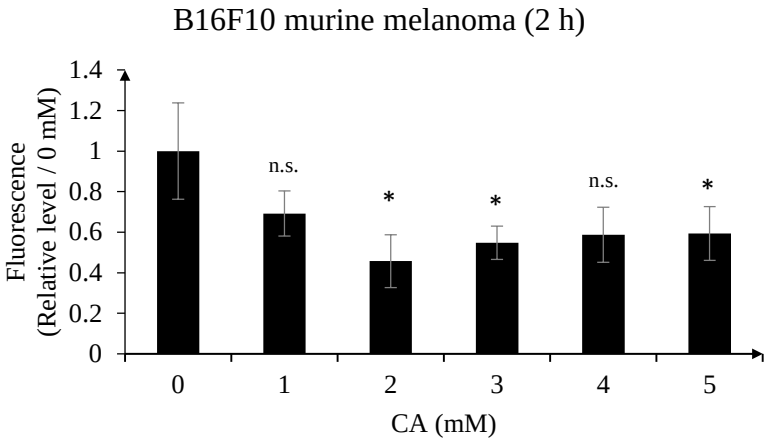
Figure 13. A. Cells were treated with PA, sodium pyruvate (SP). After 48 hours treatment, cells were collected and absorbance at 405nm was measured. **B.** Cells were treated with PA and neutralized PA. After 48 hours treatment, cells were collected and absorbance at 405 nm was measured. Mean ± SD, n⩾3, **p < 0.01.

Fig.14

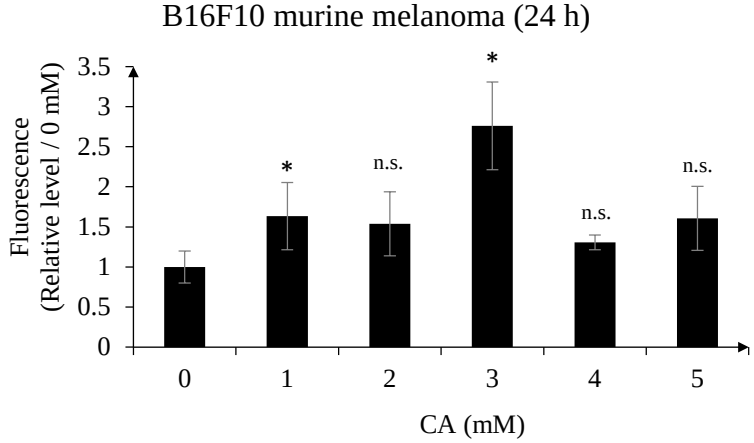
A.


DPPH assay
Scavenging activity
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
2000
Concentration ug/ml
Vc
CA
BHT

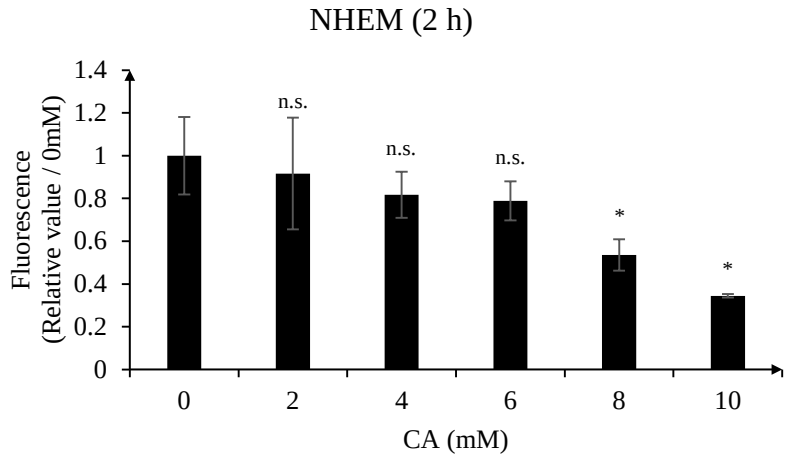
B.


B16F10 murine melanoma (2 h)
Fluorescence
(Relative level / 0 mM)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s.
*
*
n.s.
*
0
1
2
3
4
5
CA (mM)

C.


B16F10 murine melanoma (24 h)
Fluorescence
(Relative level / 0 mM)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
n.s.
n.s.
n.s.
0
1
2
3
4
5
CA (mM)

D.



E.

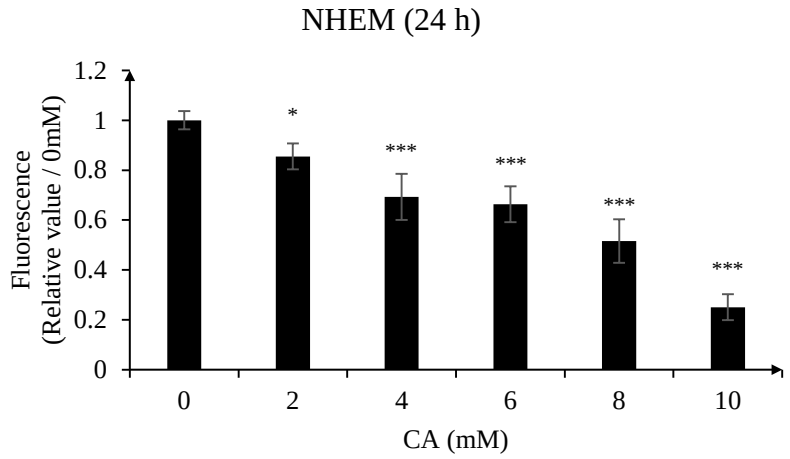
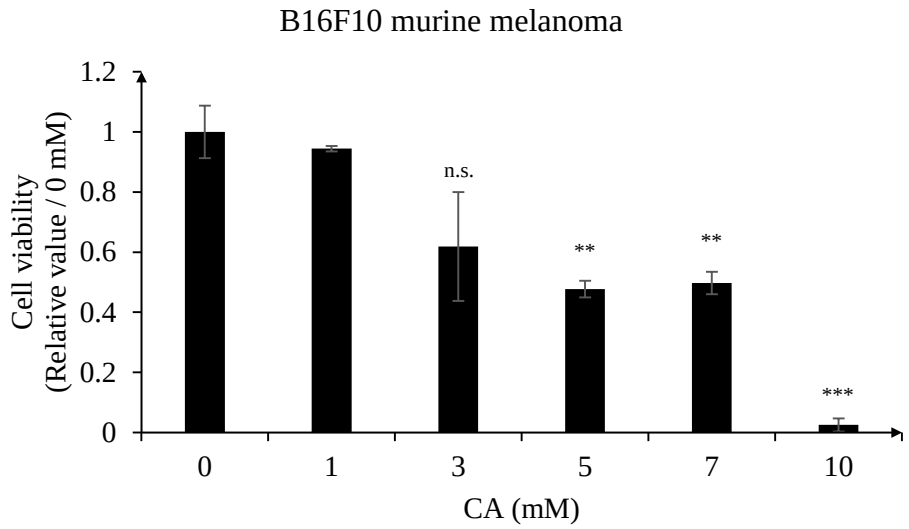


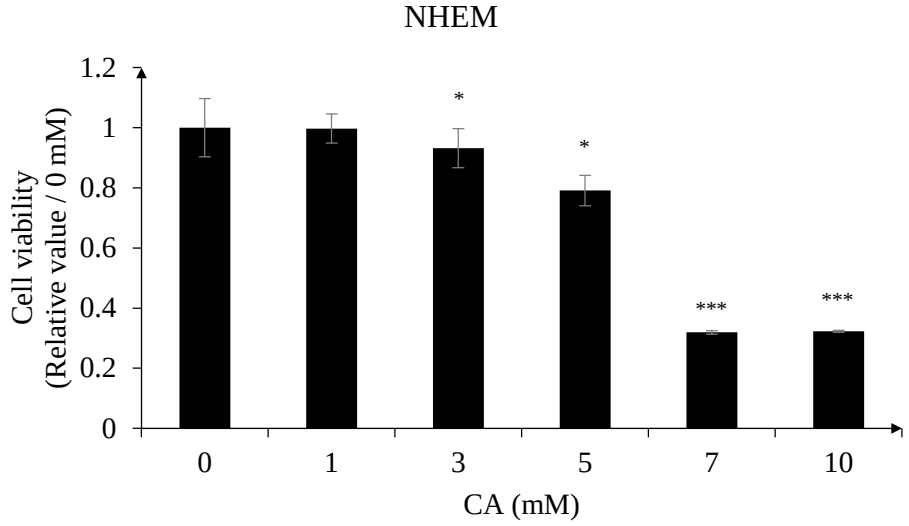
Figure 14. CA decreased intracellular ROS generation in normal human melanocytes. A. Scavenging activity of Vitamin C, BHT and CA on free radical procedure. **B, C**. Murine melanoma cells B16F10 treated with CA for 2 or 24 hours, the intracellular ROS accumulation was decreased in 2 hours but increased after 24 hours. **D, E.** Normal human melanocytes were treated with CA for 2 and 24 hours, the intracellular ROS accumulation was decreased. Mean ± SD, compared with non-treatment groups (0 mM), $n \geqslant 3$, *$p < 0.05$; ***$p < 0.005$, n.s. not significant.

Fig.15

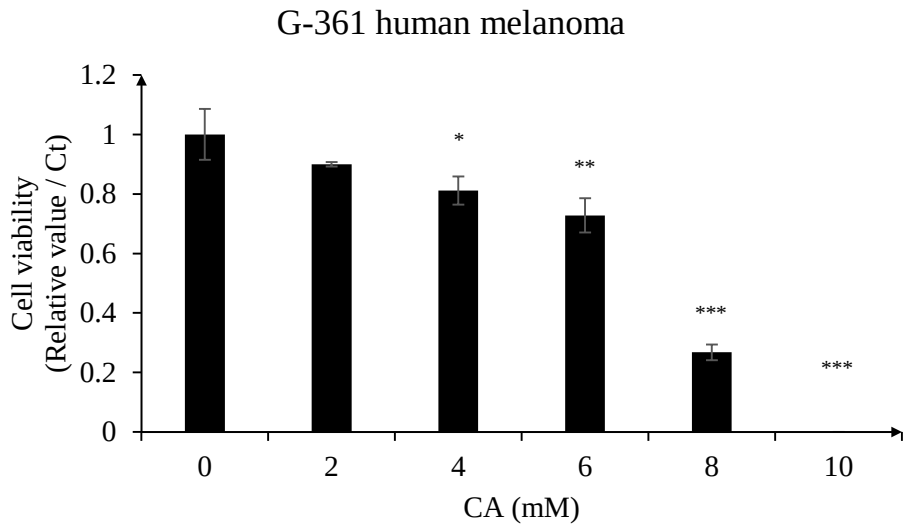
A.


B16F10 murine melanoma
Cell viability
(Relative value / 0 mM)
1.2
1
0.8
0.6
0.4
0.2
0
n.s.
**
**
***
0
1
3
5
7
10
CA (mM)

B.


NHEM
Cell viability
(Relative value / 0 mM)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
***
***
0
1
3
5
7
10
CA (mM)

C.



D.

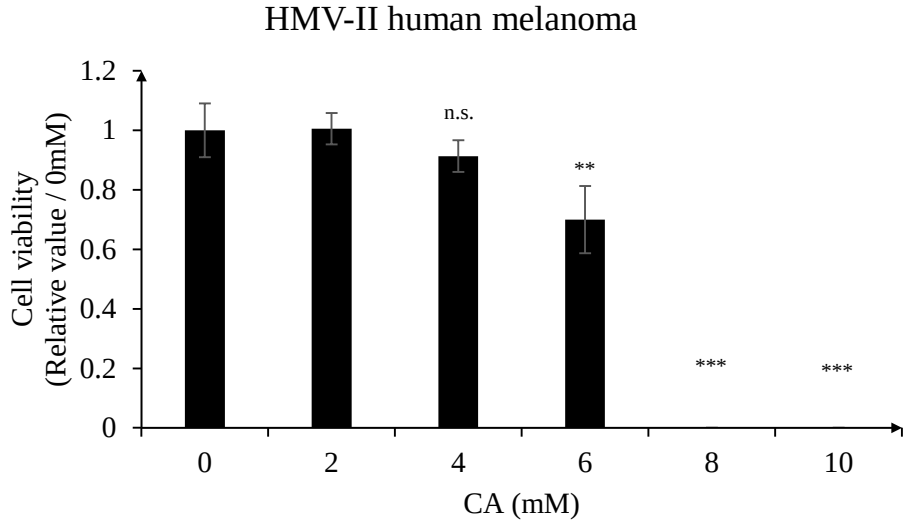
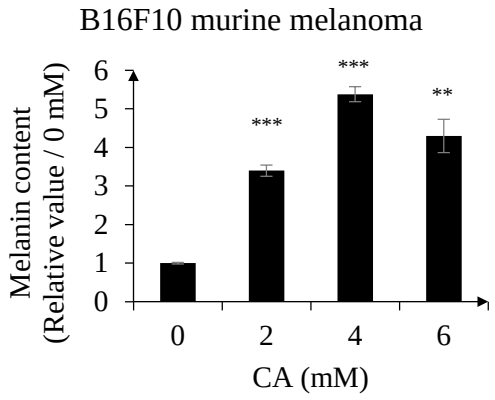


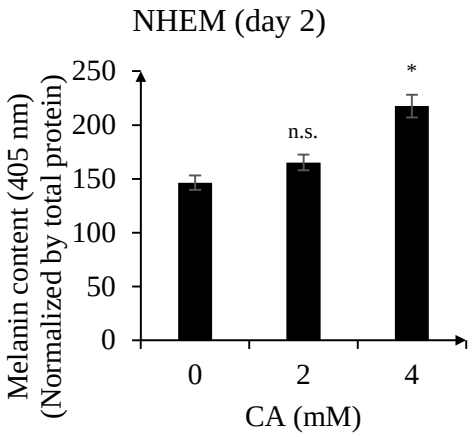
Figure 15. CA dose dependently decreased cell viability and proliferation. Cells were cultured in 96-well-plate, and CA treatment kept 48 hours. MTT assay was performed to assess the cell viability. A similar stronger inhibition effect to melanoma than NHEM has been found. Mean ± SD, compared with non-treatment groups (0 mM), n⩾3, *p < 0.05; **p < 0.01; ***p < 0.005, n.s. not significant.

Fig.16

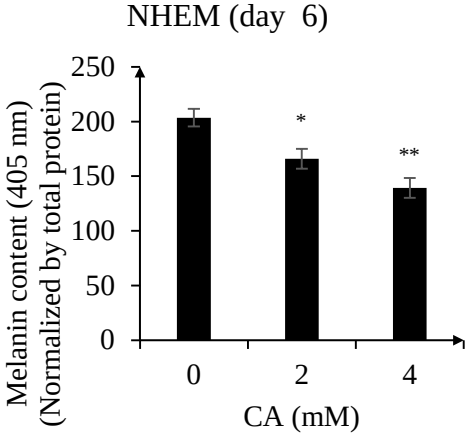
A.


B16F10 murine melanoma
Melanin content
(Relative value / 0 mM)
6
5
4
3
2
1
0
***
***
**
0
2
4
6
CA (mM)

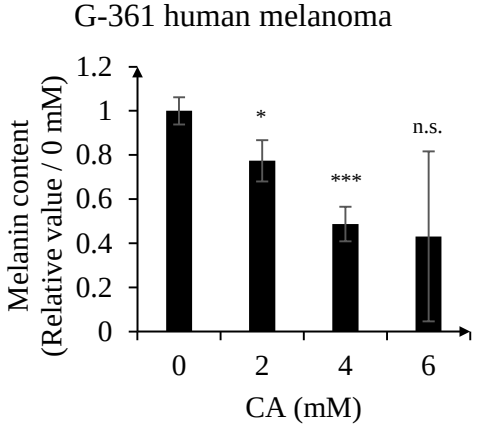
B.


NHEM (day 2)
Melanin content (405 nm)
(Normalized by total protein)
250
200
150
100
50
0
n.s.
*
0
2
4
CA (mM)

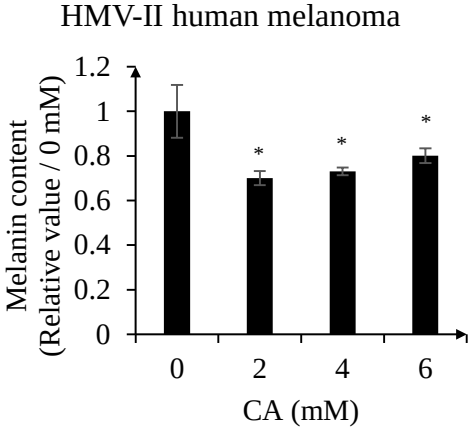
C.


NHEM (day 6)
Melanin content (405 nm)
(Normalized by total protein)
250
200
150
100
50
0
*
**
0
2
4
CA (mM)

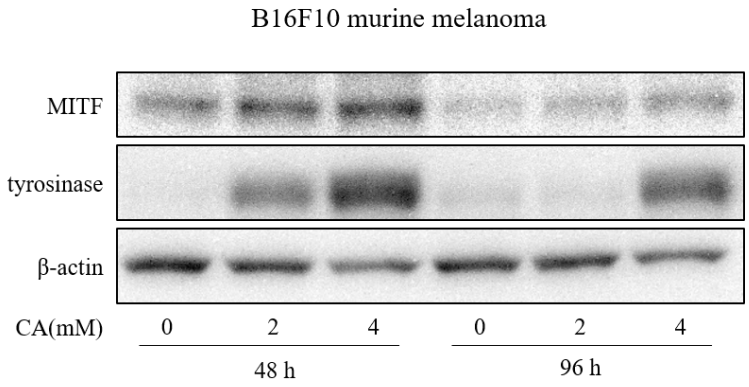
D.


G-361 human melanoma
Melanin content
(Relative value / 0 mM)
1.2
1
0.8
0.6
0.4
0.2
0
*
***
n.s.
0
2
4
6
CA (mM)

E.


HMV-II human melanoma
Melanin content
(Relative value / 0 mM)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
2
4
6
CA (mM)

F.



G.

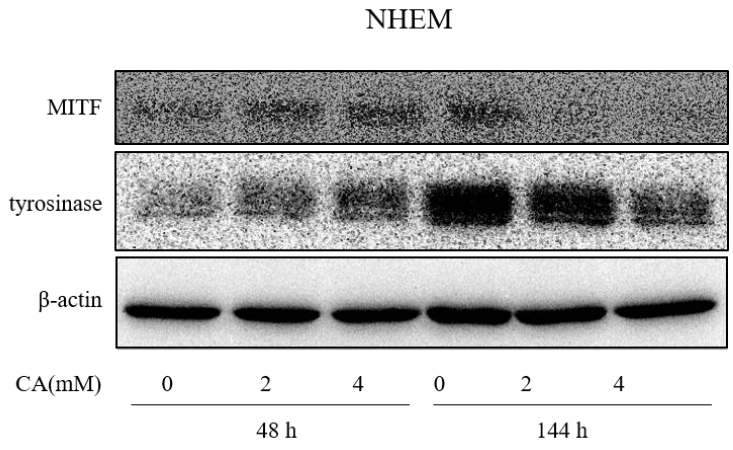


Figure 16. CA down-regulated melanogenesis in human cell line, but up-regulated in murine cell line. Cells were treated with CA until confluence (B16F10 4 days, NHEM 2 and 6 days, G361, HMV-II 6 days). **A**. CA inceased melanin synthesis in murine cells. **B**, **C**. In NHEM cells, CA increased melanin at day 2 but decreased at day 6. **D**, **E**. CA treatment decreased melanin synthesis in human melanoma cell lines. **F**. CA dose dependently increased tyrosinase and MITF gene expression in B16F10. **G**. In NHEM cells, tyrosinase and MITF were increased at day 2 but decreased at day 6, which accordance with melanin synthesis in **B**&**C**. Mean ± SD, compared with non-treatment groups (0 mM), $n \geqslant 3$, *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$, n.s. not significant.

Fig.17

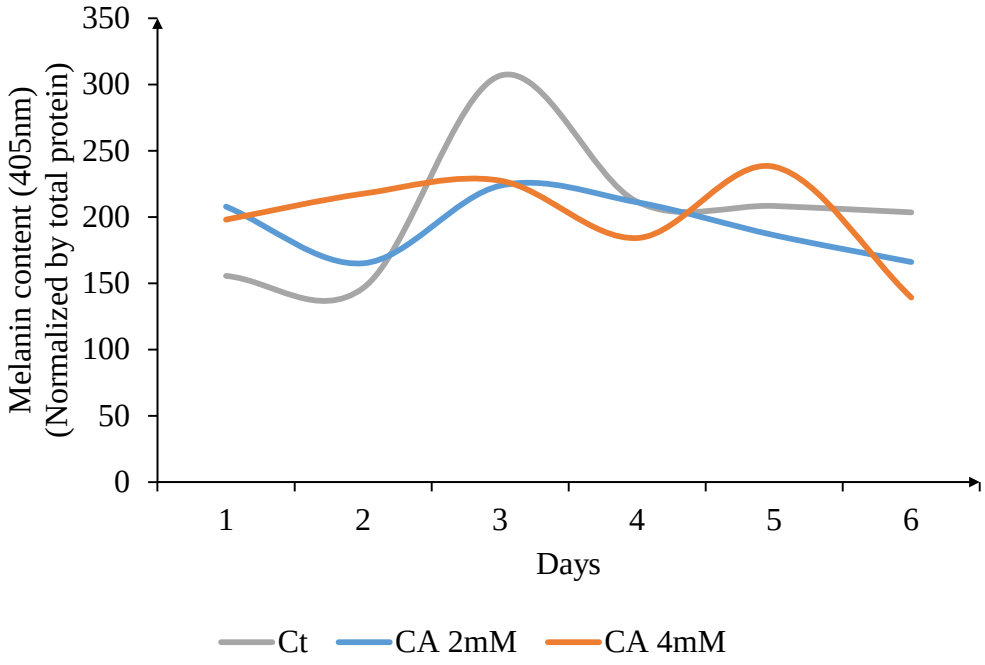


Figure 17. CA regulated rhythm of melanogenesis in normal human melanocytes. Cells were treated with or without CA. Melanin content was measured every day, pure melanin was used to calculate the accurate concentration of melanin. Even without any treatment, melanin quantity was regulated rhythmic. And as the CA treatment, this rhythm was delayed or attenuated.

Fig.18

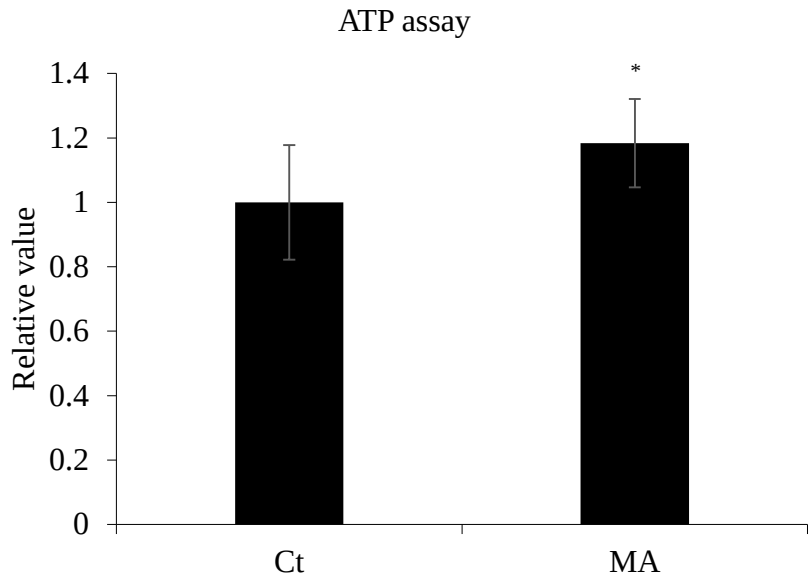


Figure 18. Malic acid (MA) up-regulated ATP production in B16F10 melanoma cells. compared with control groups (Ct), n≥3, *p < 0.05.