学　位　論　文

Studies on the role of α-MSH-MC1R-MITF signaling pathway on TGF-β production in melanoma cells

(メラノーマ細胞における TGF-β 産生に関与する

α-MSH-MC1R-MITF シグナル経路の役割に関する研究)

2016 年 3 月

北　海　道　大　学

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Contents

List of Publications and Presentations

List of Publications

1. **Erika Hayashi**, Kaori Hachiya, Satoshi Kojo, Muhanmmad Baghdadi, Shintaro Takeuchi, Hiroyuki Yamanaka, Hirotake Abe, Haruka Wada, Ken-ichiro Seino. α-MSH stimulation contributes to TGF-β1 production via MC1R-MITF signaling pathway in melanoma cell. Inflammation and Regeneration, November, 2015.

List of Presentations

1. **Erika Hayashi.** α-Melanocyte Stimulating Hormone Contributes to the induction of TGF-β1 expression in melanoma via MC1R-MITF pathway. The 4th IGM Research Workshop, September19, 2013, Sapporo, Japan.
2. **Erika Hayashi**, Testuo Moriguchi, Toru Kondo, Ken-ichiro Seino. Investigation of immunoresponsiveness against cancer initiating cells. The 72nd Annual Meeting of the Japanese Cancer Association, October 3-5, 2013, Yokohama, Japan.
3. **Erika Hayashi,** Hisashi Iizasa, Shan shan Lang, and Jun-ichi Hamada. Development of Kruppel-like factor 4 reporter system using modification of human LEFTY1 gene promoter. The 71st Annual Meeting of the Japanese Cancer Association, September 19-21, 2012, Sapporo, Japan.

Introduction

Melanoma is one of the most aggressive types of skin cancers which show high resistance to standard treatments[1,2]. The incidence of malignant melanoma continues to rise with over 230,000 new cases annually worldwide and more than 55,000 deaths in 2012[3,4]. Patients with thick primary tumors, ulcerated lesions, or lymph node metastases have a high risk of relapse with 5-year mortality rates of 40-80%[5]. Over the past few years, treatment and outcomes for people with metastatic melanoma have been improved considerably with introduction of targeted anticancer drugs[6]. However, some strategies have shown low clinical efficiency in patients with melanoma even in spite of their success in shifting immune systems to antitumor responses in animal models[7]. Programmed death 1 (PD-1) and programmed death ligand (PD-L1) are also being developed. These cumulative responses rates were 28% among patients with melanoma (26 of 94 patients) [8-11].

Very recently, there has been a dramatic increase in interest regarding immunotherapy on targeting Regulatory T cells (Treg) for melanoma treatment[12]. Treg, formerly known as suppressive T cells, are a subpopulation of T cells which modulate the immune system, play roles for maintenance of immune homeostasis and self-tolerance[13,14] and abrogate autoimmune disease[15]. Treg generally suppress or down regulate induction and proliferation of effector T cells[16]. These characteristics of Treg are related with occurring of cancer. Modulation of Treg can be effective in methods for the prophylaxis and treatment of autoimmune disease[17] and cancer[18], and facilitate organ transplantation[19].

Several mechanisms in which targeting Treg functions remain unclear, however it is suspected to occur by a cell-cell contact dependent mechanism, which possibly involves enhanced secretion of interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and other cytokines. To date, TGF-β has been shown to directly act on naïve T cells to induce Foxp3 expression, converting the naïve T cells to a Treg phenotype[20-22]. TGF-βs are multifunctional

growth factors that play particularly complex roles in the growth, progression, and metastatic potential of cancers[23,24]. Of special interest, transforming growth factor-β1 (TGF-β1) is highly expressed on a various cancer such as triple-negative breast cancer (TNBC), prostate cancer, and lung cancer where it leads to tumor promotion and metastasis[25,26]. In late-stage human tumors, TGF-β1 is often up-regulated where its aberrant expression is associated with development of aggressive tumors and poor prognosis[25,27]. In malignant melanoma, TGF-β1 promotes tumor progression via its autocrine effects including the modulation of tumor stroma and extracellular matrix, the enhancement of angiogenesis, and the inhibition of immune surveillance[28-30], as well as the promotion of tumor invasiveness and metastasis[31,32]. Additionally, melanomas with high intensities of immunostaining for TGF-β1 are significantly linked with enhanced mortality rates in patients[33]. For these reasons, TGF-β1 has attracted much attention as a potential therapeutic target of melanoma, since a monoclonal antibody against TGFβ (GC1008;Fresolimumab) has been successfully employed for such therapies[34].

Although the impact of the TGF-β1 signaling pathway on melanoma progression has been well defined, the molecular mechanisms that underlie the enhanced TGF-β1 production in melanoma cells are still poorly understood. To clarify such mechanisms, melanocortin-1 receptor (MC1R) may help to identify new suitable therapeutic strategies, because it is overexpressed in melanoma cells and involved in some roles in melanoma growth, angiogenesis and metastasis[35-37]. MC1R is a membrane protein with seven membrane-spanning domains that belongs to a five-member subfamily of G-protein-coupled receptors (GPCRs), and exerts the physiological actions as melanocortins (MCs) through Gs-protein-dependent activation of the cyclic AMP (cAMP) signaling pathway. MC1R possesses 317 amino acids in human and 315 in mouse with the structural hallmarks of the GFCRs, including an extracellular N-terminus, seven transmembranous domains and

intraocular C-terminal extension (Figure 1)[31].

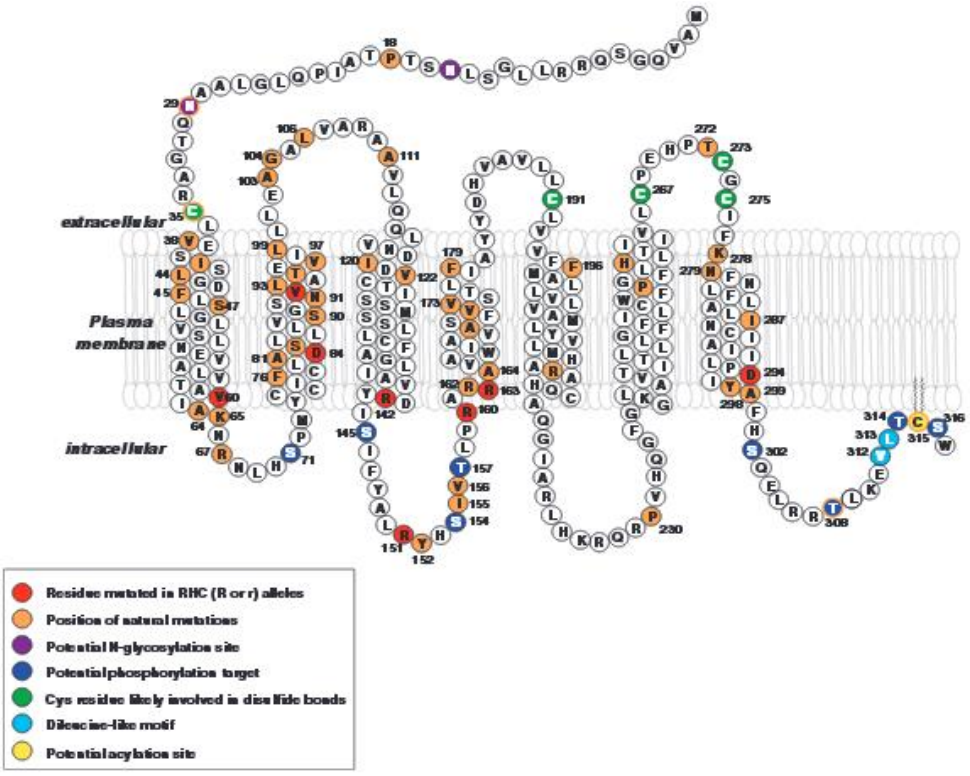


Figure 1 Structure of the human melanocortin-1 receptor

(Reference from García-Borrón, J. C. et al., *Pigment Cell Res* (2005))

The activation of MC1R signaling induces proliferation of melanoma cells, whereas both the activation of MC1R and elevated production of α-melanocyte stimulating hormone (α-MSH) lead to the protection of melanocytes and other cells from ultraviolet (UV) radiation. In the latter cases, the synthesis of melanin, which acts as an antioxidant, is augmented to lower UV damages[38]. MC1R promotes cell migration of melanoma cells as well[39]. Thus, MC1R is considered as a key factor that plays important roles in the melanocyte transformation process. In pathological condition, MC1R expresses on the cell surface of the majority of human melanomas, and correlated with melanoma initiation, progression and metastasis[40].

α-MSH, a specific ligand for MC1R, has been well examined as one of Melanocortins that

includes α-, β- and γ- melanocyte stimulating hormone (MSH) and adrenocorticotrophin (ACTH)[41,42]. These melanocortins peptides have been characterized as pituitary gland-derived peptides, which are structurally derived from a common precursor, proopiomelanocortin (POMC). Originally identified for its effects on pigmentation, α-MSH is a 13-amino-acid peptide with an amino acid sequence (Human:Ac-Ser-Try-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-N) that has been conserved in mammals, and has been recognized as anti-inflammatory and immune-modulatory activities[43,44].

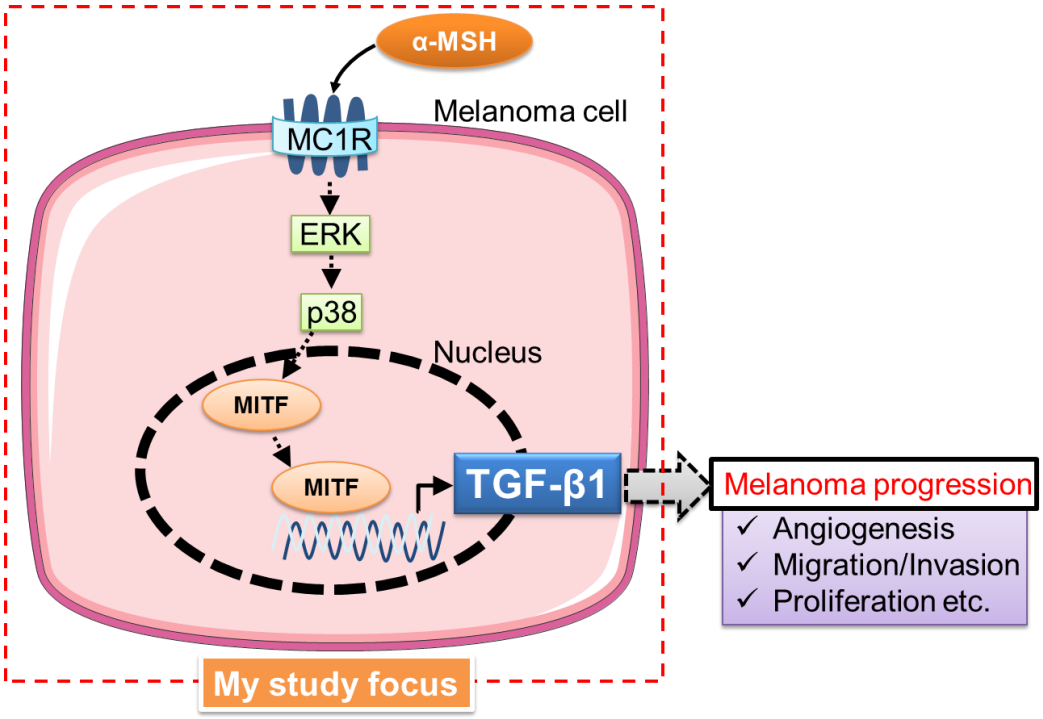


Figure 2 Hypothesis of study

Furthermore, there is a microphothalmia-associated transcription factor (MITF) known as an essential factor in melanoma blast cell differentiation and coat color pigmentation[45,46]. MITF was initially identified as a mastermelanocyte implicated in the coordination of

melanocyte development[36-38], and also supports cell survival in melanocytes through its direct regulation of Bcl2 and cMet[47-49]. MITF can be phosphorylated by ERK1/2 (at Ser^{73}), $p90^{RSK}$ (at Ser^{409})[50], GSK3β (at Ser^{298})[51,52] and/or p38 (at Ser^{307})[53], events most of which lead to enhance the transcriptional activity of MITF[52-54]. Therefore, MITF appears to be involved in the up-regulation of the TGF-β1 pathway.

From the aforementioned background, I hypothesized that since MC1R plays an important role in melanoma progression, it may also play a role in the regulation of TGF-β1 production in melanoma cells. In this study, I evaluated this hypothesis as depicted in Figure 2, and found that α-MSH-mediated activation of the MC1R pathway resulted in enhanced production of TGF-β1 in B16 melanoma cells (B16 cells). Importantly, this effect was mediated by microphthalmia-associated transcription factor (MITF), the major regulator of melanoma cells, and targeting of the α-MSH-MC1R-MITF pathway (designated as the α-MSH-MC1R-MITF axis), which resulted in decreased production of TGF-β1 in vitro, and delayed tumor growth in vivo. Together, my results provide new insight into the molecular mechanisms of TGF-β1 production in melanoma cells.

Abbreviations

ACTH	adenocorticotropic hormone
α-MSH	α-melanocyte stimulating hormone
BMDCs	bone marrow-derived dendritic cells
cDNA	complementary DNA
CREB	cAMP response element binding protein
ERK	extracellular-related kinase
GFP	green fluorescent protein
GPCRs	G-protein-coupled receptors
IL-10	Interleukin-10
MAPK	mitogen-activated protein kinase
MCs	melanocortins
MC1R	melanocortin receptor 1
MITF	microphthalmia-associated transcription factor
mRNA	messenger RNA
NF-κB	nuclear factor-kappa B
PCR	polymerase chain reaction
PI3K	PI3 kinase
PKA	protein kinase A
shRNA	short-hairpin RNA
TNBC	triple-negative breast cancer
TGF-β	transforming growth factor-β
Treg	regulatory T cells
UV	ultraviolet

Note: As a matter of convenience, the format of gene and protein names has been unified between mouse and human in this manuscript.

Methods

1. Cell culture

Mouse B16 cells and NBL-7 (Mink lung epithelium) cell lines were obtained from RIKEN, Japan. Lenti-X293T cells were purchased from Takara Bio Inc., Japan. Human melanoma cell A375M, C8161, and AK1 cell lines were obtained from the Department of Plastic and Reconstructive Surgery, Hokkaido University. B16, NBL-7, A375M, C8161, AK1 and Lenti-X293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS) (Nichirei Biosciences Inc., Japan), 0.6 % L-glutamine, 0.1 mM Non-Essential Amino Acid, 100 U/mL Penicillin and 100 μg/mL streptomycin (Gibco). Finally, cells were incubated in a humid environment with 5 % CO_2 and a temperature of 37 ℃.

2. Reagents

For MC1R stimulation, α-MSH (Peptide Institute. Inc., 4057-V) was used at concentration of 3 ng/ml. Where indicated, an inhibitor of MEK1/2 (U0126), p38 (SB203580), PI3K (LY294002), PKA (H-89) or IKKi (PS-1145) (Sigma-Aldrich) was added to the culture at concentration of 3 μM for 1 hour before α-MSH stimulation.

3. RT-PCR, real time qPCR and genomic PCR

Total RNA was purified from cells using an RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instruction, and used for cDNA synthesis (SuperScript III, Invitrogen). Genomic DNA was prepared using a Purelink genomic DNA mini kit (Life Technologies). cDNA and genomic DNA products were then used to amplify the target sequences using AmpliTaq Gold® 360 Master Mix. The obtained PCR products were separated by electrophoresis in an agarose gel and visualized by GelRed (Biotium) nucleic acid gel staining

and UV illumination. Quantitative RT-PCR analysis was performed to measure levels of TGF-β1 or MC1R mRNA as normalized to the internal control β-actin using POWER SYBR® Green (Invitrogen). Primers sequences are listed in Table 1.

Table 1 Primer sets for PCR reaction

Target		Sequence (5' → 3')	Product size
MC1R	Forward	AGAGCCTTGGTGCCTGTATG	176 bp
MC1R	Reverse	TGACACTTACCATCAGGTCAGAC	
MC2R	Forward	AGCCTCGTGGCAGTTTTGAAA	150 bp
MC2R	Reverse	TCACAATGCTATGGTATTGCAGG	
MC3R	Forward	CAGTCTGATGGAAAACATCCTGG	168 bp
MC3R	Reverse	GTCAGGGAGTCGCTGTTGA	
MC4R	Forward	CCCGGACGGAGGATGCTAT	101 bp
MC4R	Reverse	TCGCCACGATCACTAGAATGT	
MC5R	Forward	AGCCCGGTAAACAGAAGATTCA	183 bp
MC5R	Reverse	CTCTGAGGCGTTCAGGGTAAG	
TGFB1	Forward	CCGCAACAACGCCATCTATG	80 bp
TGFB1	Reverse	CCCGAATGTCTGACGTATTGAAG	
HPRT	Forward	AGTCCCAGCGTCGTGATTAG	127 bp
HPRT	Reverse	TCAGTCCTGTCCATAATCAGTC	
MITF	Forward	CAAATGGCAAATACGTTACCCG	126 bp
MITF	Reverse	CTCCCTTTTTATGTTGGGAAGGT	
hMC1R	Forward	ATCTCTGACGGGCTCTTCCT	109 bp
hMC1R	Reverse	AGCAGTACATGGGTGAGTGC	
hTGF-β1	Forward	CCCAGCATCTGCAAAGCTC	101 bp
hTGF-β1	Reverse	GTCAATGTACAGCTGCCGCA	
β-actin	Forward	TCACCCACACTGTGCCCATCTACG	295 bp
β-actin	Reverse	CAGCGGAACCGCTCATTGCCAATG	

4. NBL-7 proliferation assay

To determine the latency and bioactivity of TGF-β1, $3x10^3$ B16 cells were seeded into a 24 well culture plate and stimulated the next day with 3 ng/ml of α-MSH or PBS. After 44 hours, supernatants of the B16 cell cultures were collected and added to NBL-7 cells (5×10^5

cells per well seeded into a 96-well culture plate). In some experiments, the supernatants of B16 cell cultures were pretreated with a neutralizing anti-TGFβ antibody (R&D Systems, 10 μg/ ml) or a matching control IgG (Biolegend) and incubated at 37 ℃ for 1 hour before adding to a NBL-7 culture. After 48 hours, ^{3}H-thymidine (1 μCi / well) was added to the culture for 4 hours, and incorporation of ^{3}H-thymidine into DNA was measured by MicroBeta2 (PerkinElmer).

5. Lentiviral shRNA-mediated knockdown of *MC1R* and *MITF*

Knockdown of MC1R and MITF was performed using the short-hairpin RNAi Consortium (TCR) sequence database, as listed in Table 2. To generate entry vectors, the oligonucleotides were annealed and ligated into a pENTR4-H1 plasmid vector (provided by Dr. H. Miyoshi) using Bgl-II and Xba-I restriction sites (Toyobo). The cloned insets were confirmed by sequencing with M13 primers (Forward: GTAAAACGACGGCCAG, Reverse: CAGGAAACAGCTATGAC) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Entry vectors and a CS-RfA-EG lentiviral vector (provide by Dr. H. Miyoshi) were employed in a recombination reaction using the Gateway system (Invitrogen) according to the manufacturer's protocol. The generated lentiviral shRNA vectors were then co-transfected along with a packaging set of pCAG-HIVgp, VSV-G and pCMV-VSV-G-RSV plasmids into Lenti-X 293T cells using Polyethyleneimine "MAX" (PEl-max, Polysciences, Inc.). Twelve hours after transfection, the medium was changed to fresh DMEM containing 1 μM Forskolin, and cells were incubated for an additional 24 hours. Subsequently, the supernatants containing the viral particles were collected and filtered through 0.45 μm filters. B16 melanoma cells were infected by incubation with the virus suspension for 3 hours. GFP^{+} B16 cells were isolated by flow sorting (FACS ARIA II, BD), and knockdown efficiency was confirmed by RT-PCR or western blotting.

Table 2 shRNA used for knock down experiments

Target		Sequence (5' → 3')
MC1R	Sense	GATCCCCATCTTCTGTGCGTTGCGTTGTGCTTCCTGT CACATAACGCAGCGCATAGAAGATTTTT
MC1R	Anti-sense	CTAGAAAAATCTTCTATGCGCTGCGTTATGTGACAG GAAGCACAACGCAACGCACAGAAGATGGG
MITF #1	Sense	GATCCCCGCTAGACTTGTGTATTCTGTTGCTTCCTGT CACAATAGAATATACAAGTCTGGCTTTT
MITF #1	Anti-sense	CTAGAAAAGCCAGACTTGTATATTCTATTGTGACAG GAAGCAACAGAATACACAAGTCTAGCGGG
MITF #2	Sense	GATCCCCGTAAATATGTTACCTGTCTCTGCTTCCTGT CACAGAGACGGGTAACGTATTTGCTTTT
MITF #2	Anti-sense	CTAGAAAAGCAAATACGTTACCCGTCTCTGTGACAG GAAGCAGAGACAGGTAACATATTTACGGG

6. Western blotting

Cell lysates of $1x10^6$ B16 cells were prepared with Mammalian Protein Extraction Reagent (M-PER, Thermo Fisher Scientific) and supplemented with Protein Inhibitor Cocktail (Thermo, Fisher Scientific) were used to extract the nuclear fraction. Lysates were then separated in 10 % SDS-PAGE gel, and western blot transfer was done on PVDF membranes (Millipore) using a wet western blot system (Bio-Rad). Primary monoclonal antibodies were as follows: anti-MITF (SantaCruze Biotechnology), anti-ERK phosphor or total (Cell Signaling), anti-p38 (Cell Signaling) and anti-TGFβ (R&D Systems). As a secondary antibody, horseradish peroxidase (HRP) conjugated Abs (GE Healthcare Life Sciences) were used for detection by Chemiluminescence (Super signal WestFemto, Thermo Fisher) or ECL prime (GE heath care). Signals were detected using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences) and quantified using Image J.

7. Reporter gene assay

The coding region of MITF-M was amplified by PCR from cDNA template using a

KOD-Plus-Neo Kit (TOYOBO CO., LTD) and the following primers: Forward: 5'-CACCATGCTGGAAATGCTAGAATACAGTC-3', Reverse: 5'-ACACGCATGCTCCGTTTCT-3'. The coding region was then cloned into a pENTER^{TM}/SD/D-TOPO vector. The entry vector and pEF-DEST51 vector were employed in a recombination reaction using the Gateway system (Invitrogen) according to the manufacturer's protocol. Mouse TGF-β1 promoter fragments, generated by BamII (+55) in combination with SmaI (-1079), were cloned into a pGL2 Basic luciferase reporter vector, kindly provided by Dr. Nakano Naoko (Research Institute for Biological Sciences, Tokyo University of Science). For reporter gene assays, Lenti-X293T cells (4 x 10^5) were cultured in a 6 well tissue culture plate for 24 hours prior to transfection, and then co-transfected with 1μg of the TGF-β1 promoter reporter plasmids and the indicated dose of MITF expression vector using PEI-max. 24 hours after transfection, lysates were prepared from cells using Reporter Lysis Buffer (RLB) (Promega, USA), and used to measure the luciferase activity of the transfected reporters according to the manufacture's protocol (Promega). Luciferase activity was normalized to the β-Gel expression (SV-40 β-galactosidase expression vector, Promega, Madison, WI).

8. Animal experimentation

Six- to eight-week old C57BL/6 female mice were purchased from SLC Japan, Inc. (Tokyo, Japan). Mice (N=5) were inoculated subcutaneously into the back with 3 x 10^5 B16 cells stably transfected with control shRNA or MC1R shRNA. Tumor size was measured every other day for two weeks. Mice were maintained in a temperature-controlled, pathogen-free room at the Institute for Genetic Medicine, Hokkaido University, and treated with human care according to animal procedures approved by Animal Care Committee of Hokkaido University.

9. Flow cytometric analysis

To perform flow cytometric analysis, tumors were separated into dissociated cells according to the collagenase D protocol (Roche). Briefly, tumors were removed from mice on day 14 after transplanting. Collagenase D solution was diluted with RPMI-1640 medium with 2 % FBS to make 10 mg/ml of the working solution. The tumors were incubated with collagenase D at 37 ℃ until dissociation was complete. After passing the resultant cell suspension through a 100 μm filter to remove undigested fragments, cells were centrifuged at 1500 rpm for 5min. The pellets were re-suspended in cell culture medium. The antibodies employed were as follows: APC anti-mouse CD4, PE anti-mouse CD25, FITC anti-mouse CD45, PE anti-mouse Foxp3, and approximate isotype controls. All these antibodies were purchased from Biolegend Pharmingen (San Diego, CA, USA). Intranuclear staining for Foxp3 was carried out using a Mouse Foxp3 Buffer Set according to the instruction manual (BD Pharmingen^{TM}). All samples were measured using FACS Canto II Flow Cytometer (BD Biosciences).

10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance and post-hoc analysis was done using the unpaired t test. Values are shown as means ±S.D. Statistical significant was defined as $p<0.05$.

11. Ethics statement

Animal experiment was performed after obtaining permission from the Institute Animal Care and Use Committee of Hokkaido University. Experiments involving infectious materials were performed in a BSL-2 facility.

Results

The following results were obtained:

1. Enhancement of TGF-β1 production in B16 melanoma cell upon α-MSH stimulation
2. Decrease in TGF-β1 production in α-MSH-stimulated B16 melanoma cells following MC1R Knockdown
3. Discovery of MITF as the factor responsible for enhancing TGF-β1 production in α-MSH-stimulated B16 cells
4. Effect of MC1R Knockdown on B16 melanoma growth in vivo
5. α-MSH stimulation enhances TGF-β1 expression in human melanoma cells

1. Enhancement of TGF-β1 production in B16 cells upon α-MSH stimulation.

To investigate the possible role of MC1R in TGF-β1 production, I first examined the expression of the MC receptors family in B16 cells, compared to bone marrow-derived dendritic cells (BMDCs) as a positive control. While MC1R expression was detected in B16 cells (Figure 3), other MC receptors were not.

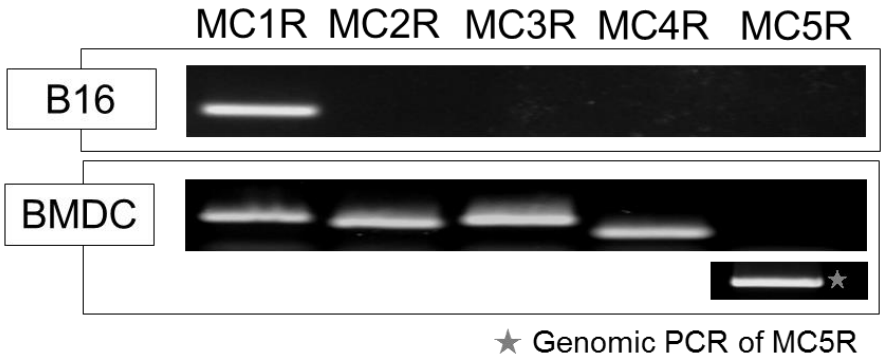


Figure 3 Expression of MC1R in B16 cells.
PCR analysis of MCR family expression was performed using both B16 cells and bone-marrow dendritic cells (BMDCs). MC5R was detected at the genomic level but not in the mRNA of BMDCs, thus genomic PCR of MC5R is shown in a separate panel.

Next, I examined whether MC1R-mediated signaling contributes to TGF-β1 production. To do so, I evaluated the expression level of TGF-β1 mRNA in B16 cells when stimulated with α-MSH. α-MSH is a naturally occurring endogenous peptide hormone of the melanocortin family and serves as a nonselective agonist of the melanocortin receptors[41]. Upon stimulation with α-MSH, MC1R triggers the activation of the ERK1/2 pathway[40], which was also confirmed in B16 cells as shown in Figure 4.

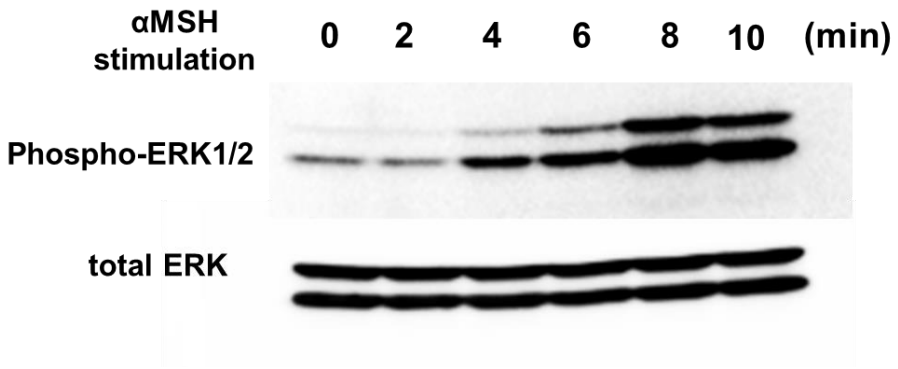


Figure 4 Enhancement of ERK1/2 phosphorylation in B16 cells after α-MSH stimulation.
B16 cells were stimulated with α-MSH stimulation for 0, 2, 4, 6, 8, and 10 minutes. Western blot analysis was performed to compare phosphorylation levels of intracellular kinases.

Importantly, I found that stimulation of B16 cells with α-MSH resulted in up-regulation of TGF-β1 at the mRNA and protein levels (Figure 5A and B). To further confirm the existence of active TGF-β1 in the supernatants of B16 cells, I performed a proliferation inhibition assay using mink lung epithelial cells (NBL-7). As the proliferation of NBL-7 cells was inhibited in response to TGF-β[55-57], I next compared the proliferation of NBL-7 cells when cultured with supernatants collected from non-stimulated or α-MSH-stimulated B16 cell cultures (Figure 5B).

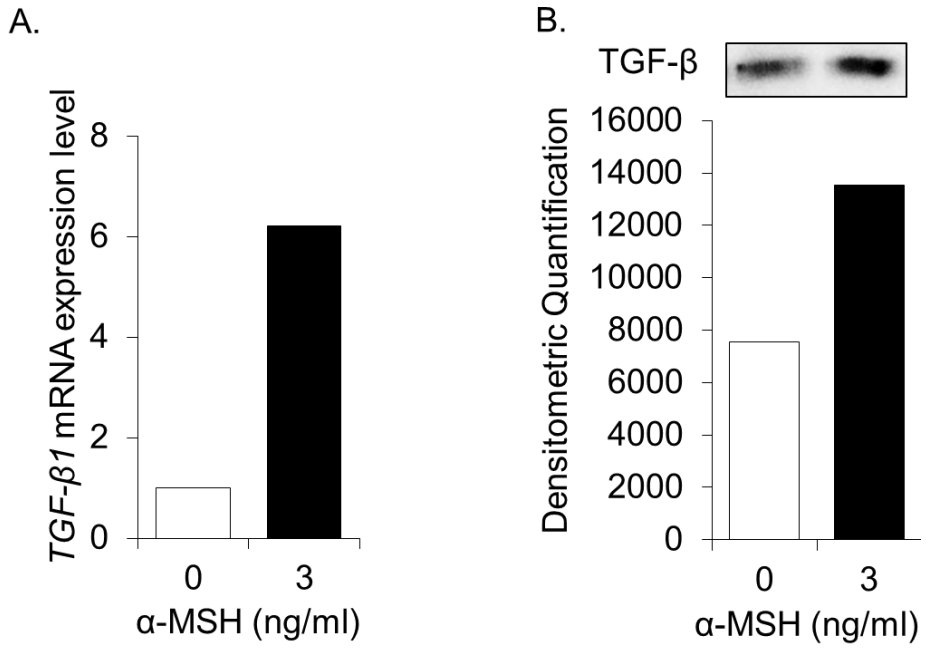


Figure 5 α-MSH-stimulated enhancement of TGF-β1 production in B16 cells. After B16 cells were incubated for 48 hours with or without α-MSH (3 ng/ml), both RT-PCR analysis (A) and Western blot analysis (B) were performed to densitometrically quantify TGF-β in the cultured media. Similar results were obtained in 2 independent experiments.

As expected, I found that proliferation of NBL-7 cells was inhibited when cultured in the presence of α-MSH-stimulated-B16 cell supernatant, which indicates the enrichment of B16 cells cultured with TGF-β1 following α-MSH stimulation (Figure 6).

Furthermore, I found that a neutralizing antibody for TGF-β was effective in abolishing the effects of the supernatants derived from B16 cells as indicated by recovery in NBL-7 proliferation (Figure 6B and C). These data were suggesting that TGF-β1 is the responsible factor for suppressing NBL-7 proliferation in this assay. Together, these results strongly suggest that α-MSH-mediated signaling contributes to TGF-β1 production in B16 cells.

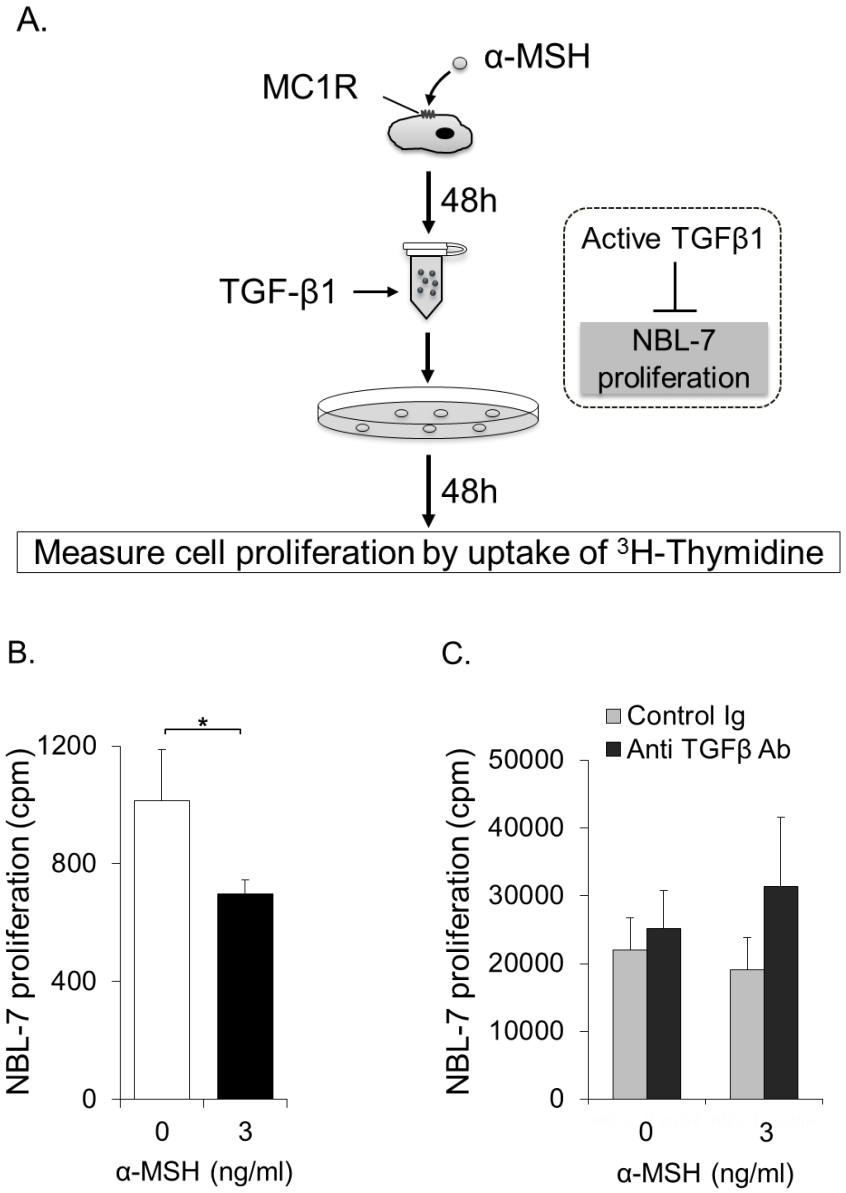


Figure 6 Suppressed proliferation of NBL-7 cells by α-MSH-induced TGF-β1.

A: This schematic describes the NBL-7 proliferation inhibition assay used to evaluate levels of TGF-β1 in the supernatants of B16 cells.

B: Proliferation inhibition assays of NBL-7 cells were performed after B16 cells were incubated in the absence or presence of α-MSH (3ng/ml) for 48 h.

C: Proliferation inhibition assays of NBL-7 cells were performed after B16 cells were incubated as shown in B and the conditioned media were pretreated with either a neutralizing antibody for TGF-β or control Ig.

Data shown are representative of 2 independent experiments. *****P<0.05. ******P<0.01.

2. Decrease in TGF-β1 production in α-MSH-stimulated B16 cells following MC1R Knockdown

As suggested by the aforementioned data, α-MSH stimulation enhances TGF-β1 production in B16 cells. To further confirm the involvement of MC1R in this effect, I utilized short-hairpin RNAs (shRNAs) encoding lentiviral system to knockdown MC1R in B16 cells. Following transfection, GFP^+ B16 cells were isolated by flow sorting (Figure 7A), and knockdown efficiency of MC1R was confirmed by RT-PCR (Figure 7B). In addition to confirmation by mRNA levels, I also confirmed that knockdown of MC1R affected downstream signaling cascades, such as the phosphorylation of p38, which was decreased in B16 cells deficient for MC1R as compared to the control (Figure 7C). Proliferation of B16 cells was not affected following knockdown of MC1R (Figure 7D).

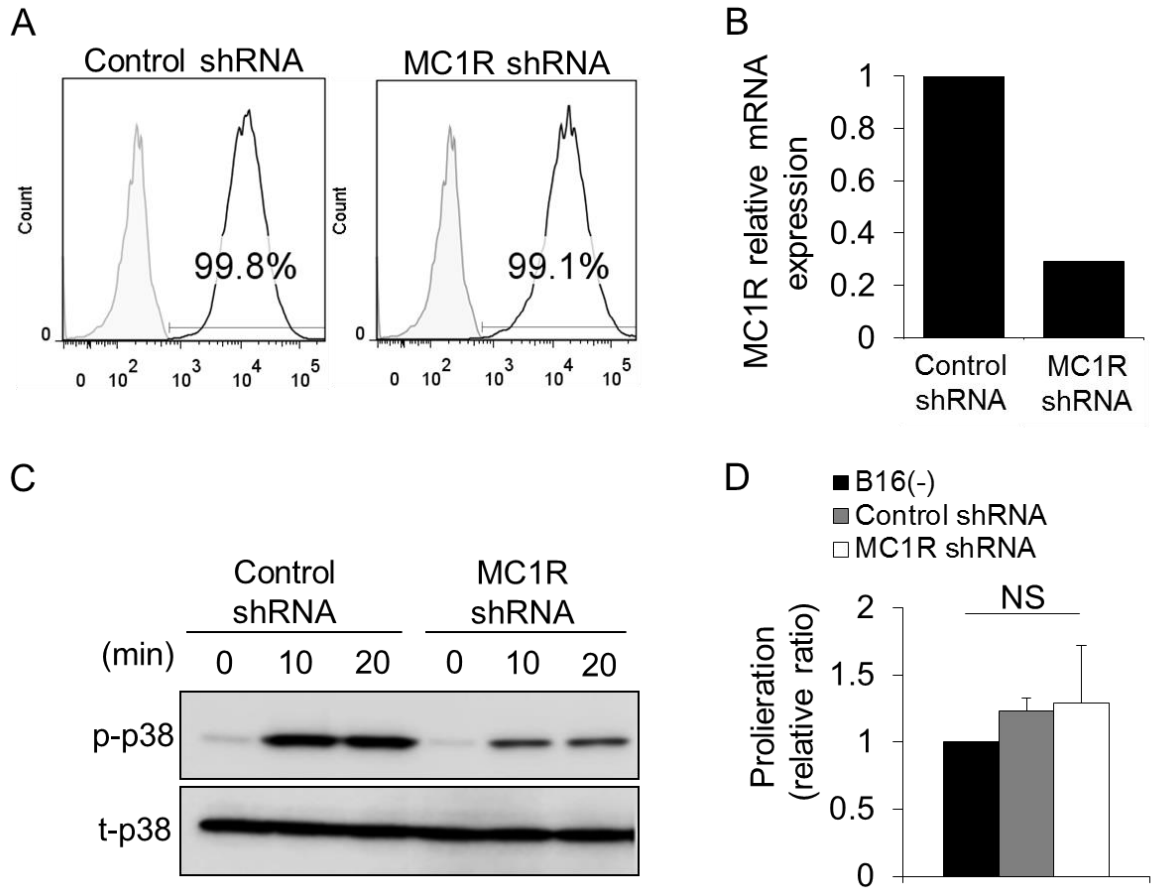


Figure 7 Establishment of MC1R-knockdown B16 cells.

A: Establishment of B16 cells with knockdown for MC1R using a specific shRNA coding lentiviral system. GFP^+ B16 cells were isolated by flow cytometry. Gray histogram: isotype control, White histogram: GFP^+ fraction.

B: Evaluation of MC1R mRNA knockdown efficiency by RT-PCR.

C: Western blot analysis of phosphorylated p38 (upper panel) and total p38 (lower panel). Control and B16 cells knocked down for MC1R were stimulated with α-MSH for various times.

D: Proliferation assay of B16 cells with or without knockdown for MC1R.

I next asked if TGF-β1 production is affected by knockdown of MC1R in B16 cells when stimulated with α-MSH. As expected, stimulation with α-MSH was ineffective in enhancing TGF-β1 production in B16 cells expressing MC1R shRNA compared to the control shRNA (Figure 8A). Additionally, in the NBL-7 cells proliferation inhibition assay, I also found that the proliferation of NBL-7 cells was inhibited when stimulated with the supernatant of α-MSH-stimulated B16 cells with expression of MC1R (Figure 8A). However, this effect was completely abolished when NBL-7 cells were stimulated with the supernatant of α-MSH-stimulated MC1R-knocked down B16 cells (Figure 8B), which reflects decreased TGF-β1 production in α-MSH-stimulated B16 cells after knockdown of MC1R. Thus, these results provide evidence for a functional α-MSH-MC1R signaling axis to regulate TGF-β1 production in B16 cells.

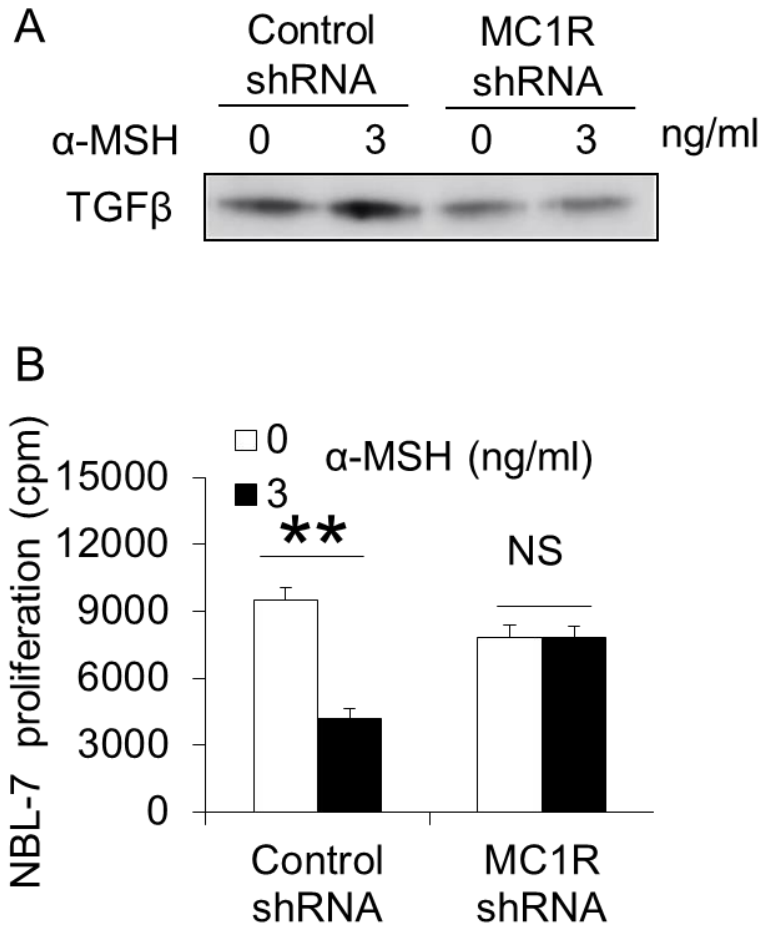


Figure 8 Attenuation of α-MSH-induced effects in B16 cells with MC1R knockdown.
A: Western blot analysis of TGF-β in non-stimulated or α-MSH (3 ng/ml)-stimulated B16 melanoma cells knocked down for MC1R (or control) 72 hours after stimulation.
B: Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3 ng/ml)-stimulated B16 cells knocked down for MC1R (or control). Data shown are representative of 2 independent experiments. NS: non-stimulated. **$P<0.01.

3. Discovery of MITF as the factor responsible for enhancing TGF-β1 production in α-MSH-stimulated B16 cells

To clarify signaling pathways that act downstream of MC1R to regulate TGF-β1 production in α-MSH-stimulated B16 cells, I focused on examining kinase activities, because MC1R has been reported to be activated in several signaling cascades, notably the Protein Kinase A (PKA) - cAMP response element binding protein (CREB) and Mitogen-activated Protein kinase (MAPK) pathways[54,58]. Additionally, recent reports have also identified the role of PI3 kinase (PI3K) and nuclear factor kB (NF-kB) in signaling downstream of MC1R[59]. Therefore, I utilized inhibitors of MEK1/2 (U0126), p38 (SB203580), PI3K (LY294002), PKA (H-89) or IKK (PS-1145) on B16 cultures prior to α-MSH stimulation, and supernatants inhibition assays. In these experiments, I found that the proliferation of NBL-7 cells was inhibited when cultured in the presence of α-MSH-stimulated B16 cells pretreated with inhibitors of PI3K, PKA, and IKK (Figure 9).

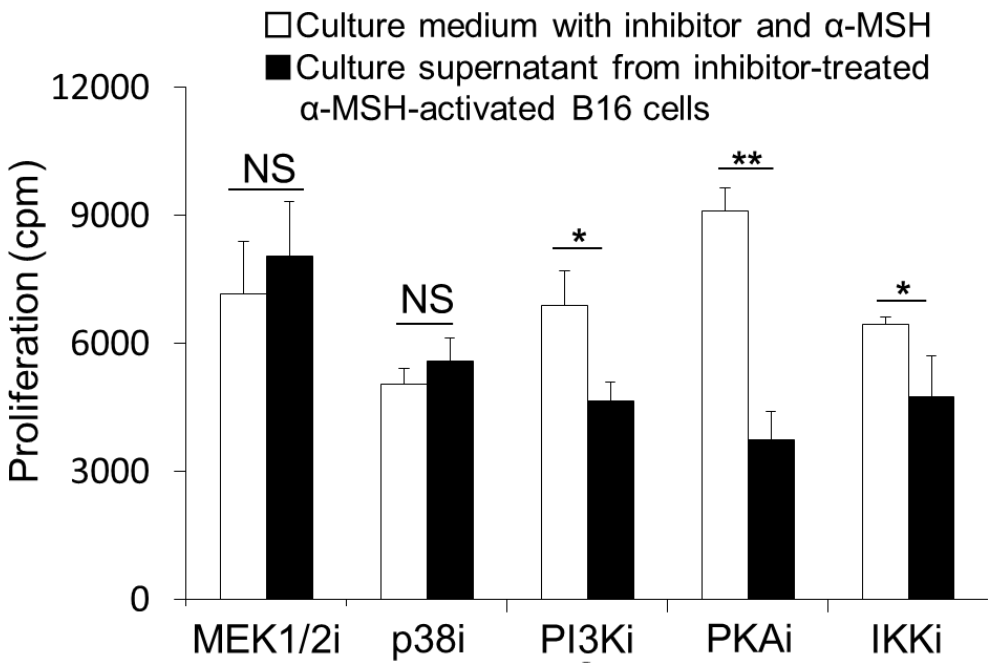


Figure 9 Involvement of ERK and p38 pathways in MC1R-mediated downstream signaling after α-MSH stimulation

Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3 ng/ml)-stimulated B16 cells. An inhibitor of MEK1/2 (U0126), p38 (SB203580), PI3K (LY294002), PKA (H-89) or IKKi (PS-1145) was added to B16 culture prior to α-MSH stimulation to identify the signaling pathway responsible for the expression of TGF-β1.

On the other hand, inhibition of NBL-7 cells proliferation was not observed when B16 cells were pretreated with inhibitors of MEK or p38 pathways, strongly suggesting that these pathways are important for MC1R-meditated signaling, since inhibitors of these two molecules have resulted in decreased TGF-β1 production following α-MSH stimulation.

To further elucidate the molecular mechanisms related to MC1R signaling, I next tried to identify transcription factors that regulate TGF-β1 production in α-MSH-stimulated B16 cells. MITF-is a basic helix-loop-helix zipper transcription factor which acts as a master regulator of several biological activities including development, and the function and survival of melanocytes[54]. Several studies have also reported critical roles for MITF in numerous aspects of melanoma development including proliferation, progression, migration, invasion and metastasis[60]. Importantly, MITF acts downstream of ERK1/2 and p38 MAPK in melanoma cells[50,53].Thus, I focused on MITF as a possible candidate that acts downstream of MC1R to regulate TGF-β1 production. First, I utilized a luciferase reporter assay to evaluate the role of MITF in the regulation of the TGF-β1 promoter. In this assay, I found that luciferase activity of the TGF-β1 promoter reporter was significantly enhanced when co-transfected with an MITF expression plasmid and, more specifically, in a dose-dependent manner (Figure 10), indicating the involvement of MITF in the up-regulation of TGF-β1.

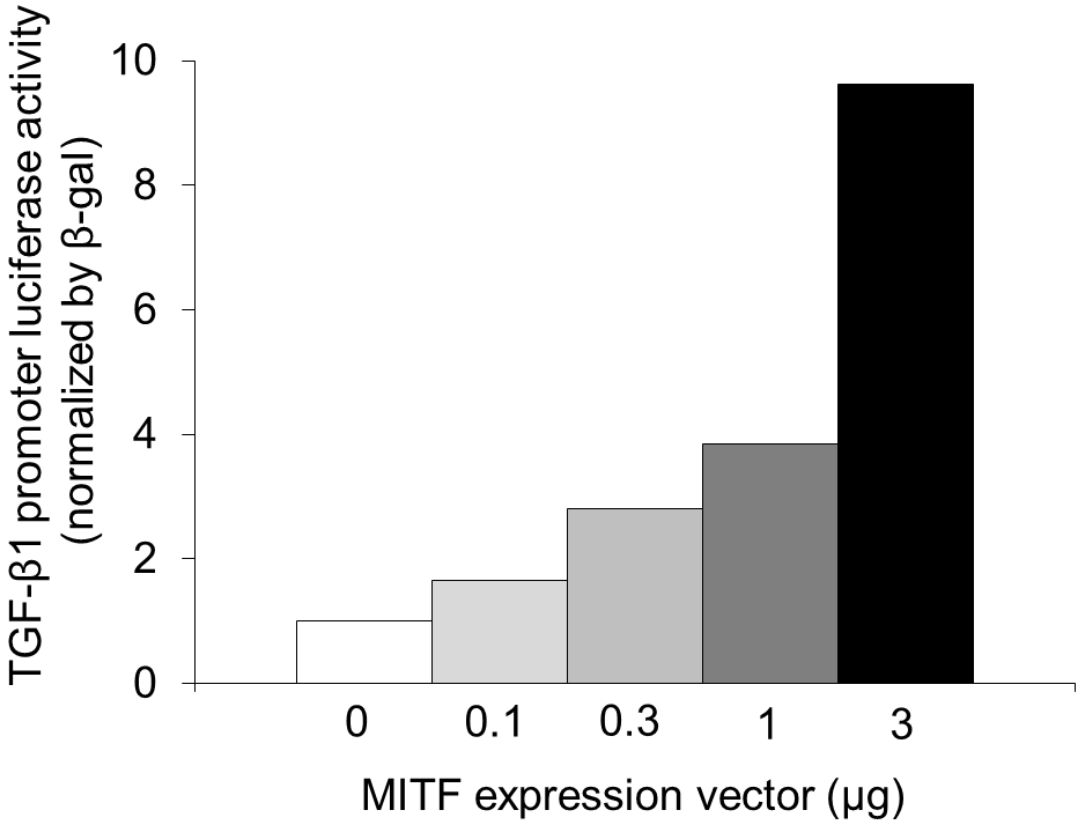


Figure 10 TGF-β1 promoter activities as a function of MITF.
Lenti-X-293T cells were co-transfected with plasmid vectors expressing the TGF-β1 promoter reporter and MITF cDNA. Twenty-four hours after transfection, luciferase reporter assays of TGF-β1 promoter were carried out. Similar results were obtained in 3 independent experiments.

To examine if MITF is involved in the enhancement of TGF-β1 production in α-MSH-stimulated B16 cells, I utilized a short-hairpin RNA (shRNA)-encoding lentiviral system to suppress MITF expression in B16 cells. Two shRNAs, referred to as MITF shRNA#1 and MITF shRNA#2, were developed to knock MITF down. Transfected B16 cells were selected by flow sorting according to their GFP fluorescence, and knockdown efficiency of MITF was confirmed by RT-PCR. Using these two shRNAs, I found shRNA#1 was more effective in knocking down MITF than shRNA#2 (Figure 11A). Proliferation of B16 cells, however, was not affected at all after knockdown of MTIF (Figure 11B). I further examined if TGF-β1 production was affected in these two cell lines by knockdown of MITF in α-MSH-stimulated B16 cells. When MITF was efficiently knocked down in B16 cells using MITF shRNA#1, the proliferation of NBL-7 cells was not inhibited when stimulated with the supernatants of these cells. This was not observed in the case of non-effective MITF shRNA#2 (Figure 11C). These data, taken together, provide evidence that MITF acts downstream of MC1R to enhance TGF-β1 production in B16 cells following α-MSH-stimulation.

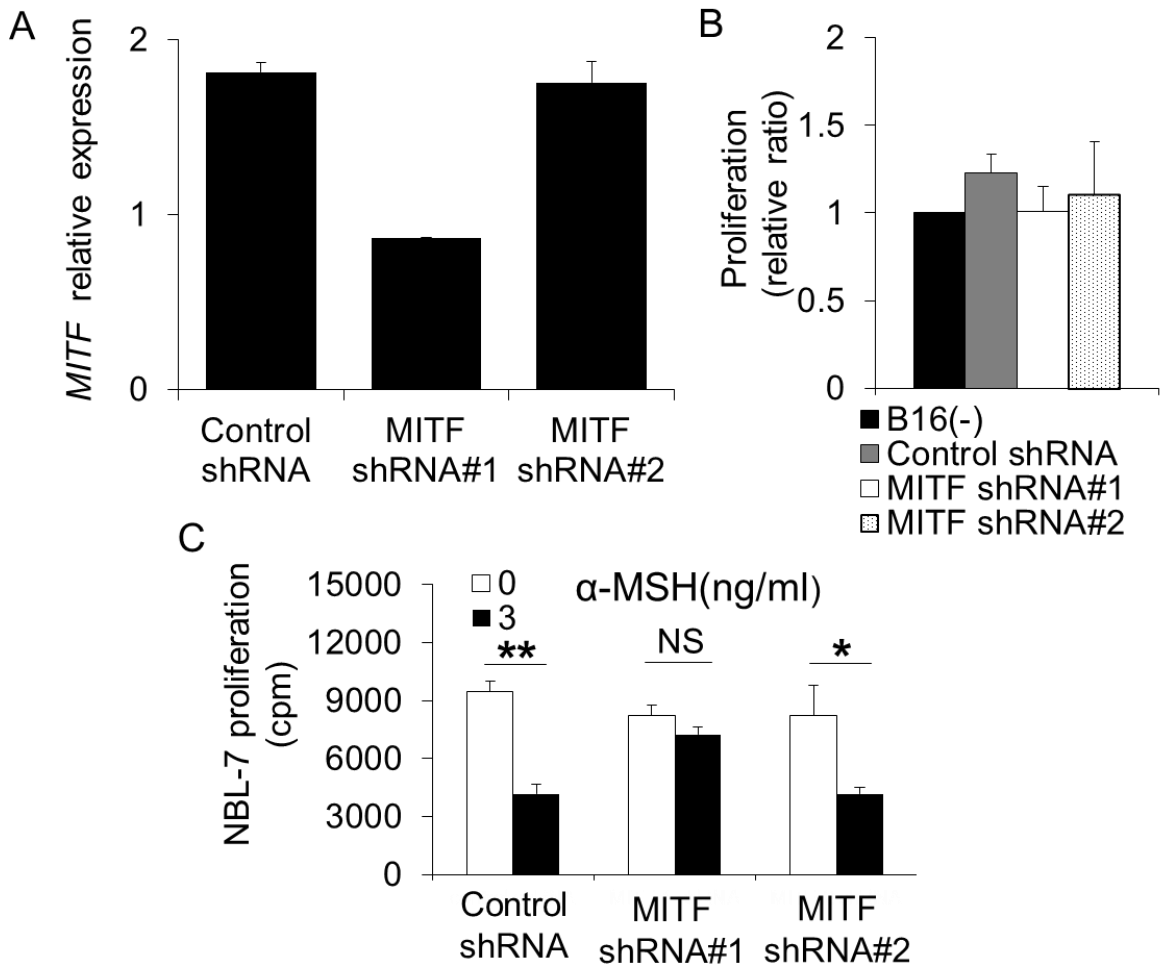


Figure 11 Inhibitory effect of MITF knockdown on α-MSH-stimulated TGF-β1 production in B16 cells.

A: RT-PCR analysis to confirm knockdown efficiency of MITF in B16 cells.

B: Proliferation assay of B16 cells knocked down for MITF (or control) compared to non-treated B16 cells.

C: Proliferation inhibition assays of NBL-7 cells cultured in the supernatants of non-stimulated or α-MSH (3ng/ml)-stimulated B16 cells knocked down for MITF (or control).

Data shown are representative of 2 independent experiments. *****P<0.05. ******P<0.01.

4. Effect of MC1R knockdown on B16 melanoma growth in vivo

Accumulating evidences has unveiled the fundamental roles of TGF-β1 in multiple aspects of cancer progression, including melanoma[61,62]. In this study, I have identified a role for the MC1R signaling pathway in the regulation of TGF-β1 production in B16 cells, which MITF mediated in the downstream. Thus, finally, I investigated whether the targeting of this axis may affect growth of melanoma in vivo. To do so, I simply inoculated B16 cells stably transfected with control shRNA or MC1R shRNA into C57BL/6 mice, and compared tumor growth between the two groups. As expected, tumor growth was delayed in mice inoculated with B16 cells knocked down for MC1R as compared with control cells (Figure 12A and B). Collectively, these data indicate the essential role of the α-MSH-MC1R-MITF axis in regards to the regulation of TGF-β1 production in B16 cells, which consequently has an impact on tumor progression in vivo.

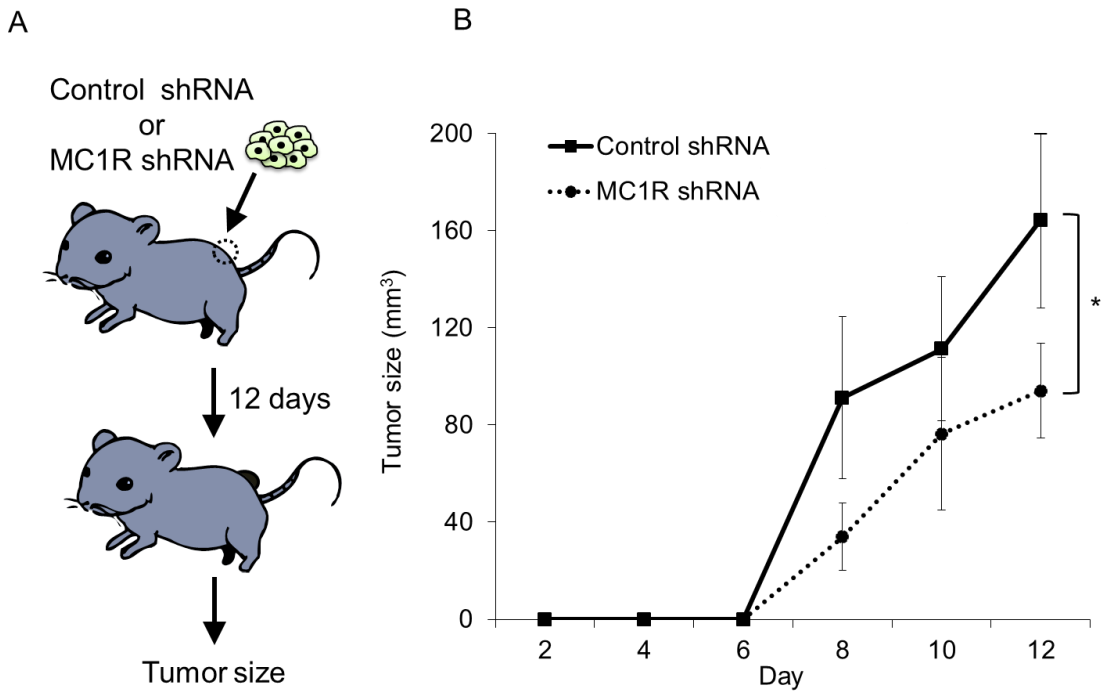
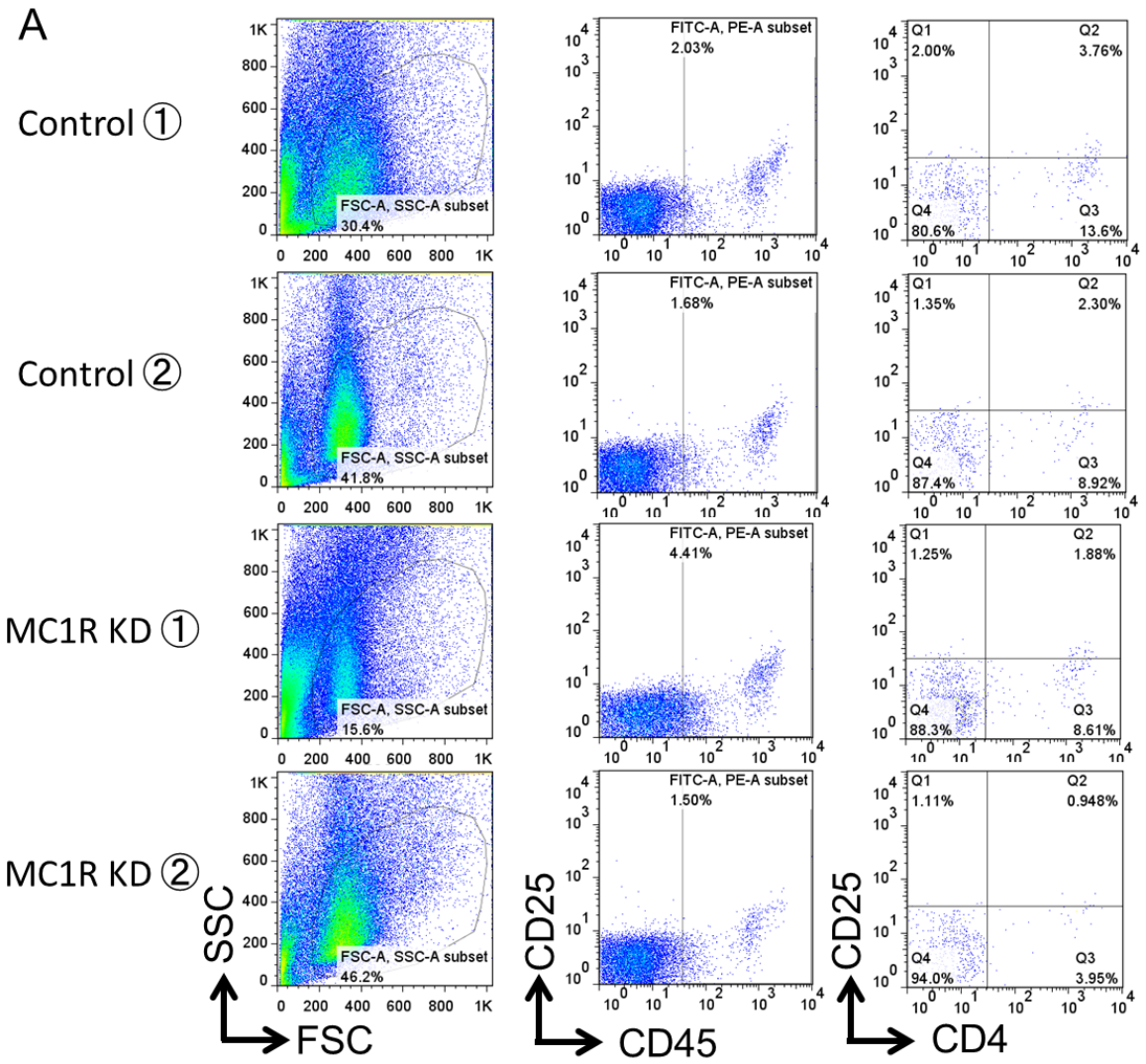


Figure 12 Inhibitory Effect of MC1R knock down on growth of melanoma *in vivo*.
A and B: C57BL/6 mice (n=5 per group) were inoculated subcutaneously (into the back) with B16 cells stably transfected with control shRNA or MC1R shRNA. Tumor growth was measured on the indicated days. *****P<0.05.

I found that there was a significant difference in tumor growth between the control and MC1R knocked down B16 cells (Figure 12B). I predicted that the differences in tumor growth might be affected not only by knockdown for MC1R, but also by induction with Treg in the tumor. Therefore, employing flow cytometry (FACS), I measured the percent of $CD4^{+}CD25^{+}$ and $CD4^{+}Foxp3^{+}$ cells in tumor tissues formed in mice after either control B16 cells or MC1R knockdown cells were injected subcutaneously. Unexpectedly, there was not remarkable difference in the tumor between the control and MC1R knock down (Figure 13A and B). This experiment did not show a relationship between tumor growth and Treg.


A
Control ①
Control ②
MC1R KD ①
MC1R KD ②
FSC-A, SSC-A subset
30.4%
41.8%
15.6%
46.2%
FITC-A, PE-A subset
2.03%
1.68%
4.41%
1.50%
Q1
2.00%
Q2
3.76%
Q4
80.6%
Q3
13.6%
Q1
1.35%
Q2
2.30%
Q4
87.4%
Q3
8.92%
Q1
1.25%
Q2
1.88%
Q4
88.3%
Q3
8.61%
Q1
1.11%
Q2
0.948%
Q4
94.0%
Q3
3.95%
SSC
FSC
CD25
CD45
CD25
CD4

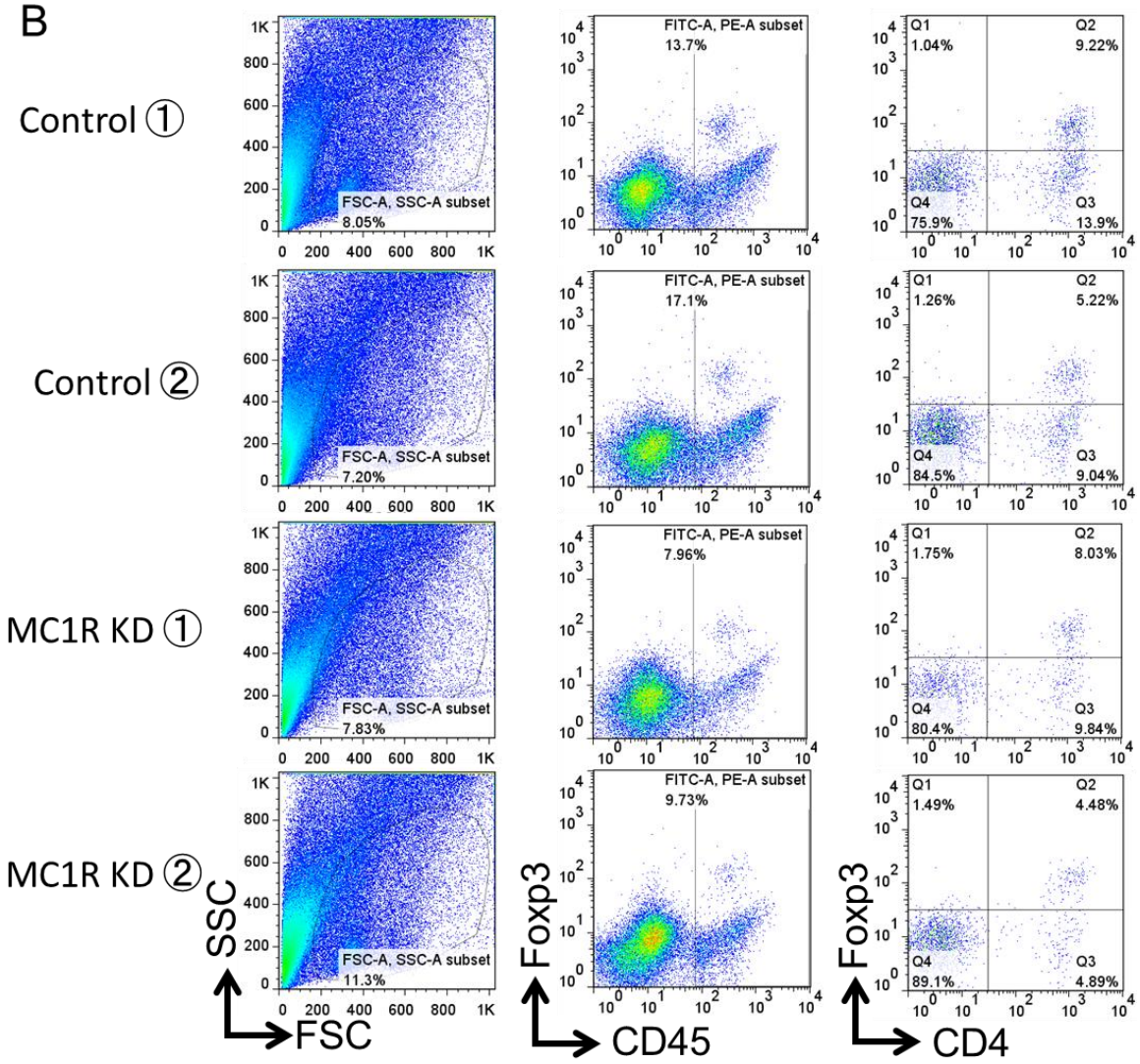


Figure 13 Flow cytometric analysis of Treg phenotype derived from tumors.
A and B: After subcutaneous transplantation, tumors were removed from C57BL/6 mice (n=2 per group) on day 14. Tumors were dissociated into single cells for immunostaining with anti-CD4 antibody, anti-CD25 antibody (A), and anti-Foxp3 antibody (B). Then, dissociated cells were analyzed by flow cytometry. FACS in A and B are depicted with MC1R-knocked down cells (MC1R KD) and control cells (control), respectively.

5. α-MSH stimulation enhances TGF-β1 expression in human melanoma cells

Finally, I asked whether TGF-β1 expression is enhanced in human melanoma cells following α-MSH stimulation similar to B16 cells. To do so, I evaluated MC1R expression in human AK1, C8161 and A375M melanoma cell lines. As shown in Figure 14A, MC1R was expressed in these cell lines with some variations. Importantly, α-MSH stimulation was effective in enhancing TGF-β1 expression (Figure 14B), and more interestingly this enhancement was correlated to MC1R expression, since melanoma cells with higher expression of MC1R such as A375M showed remarkable enhancement in TGF-β1 expression following α-MSH stimulation (Figure 14C). Therefore, these data suggest that the α-MSH-MC1R axis can also contribute to TGF-β1 production in human melanoma cells.

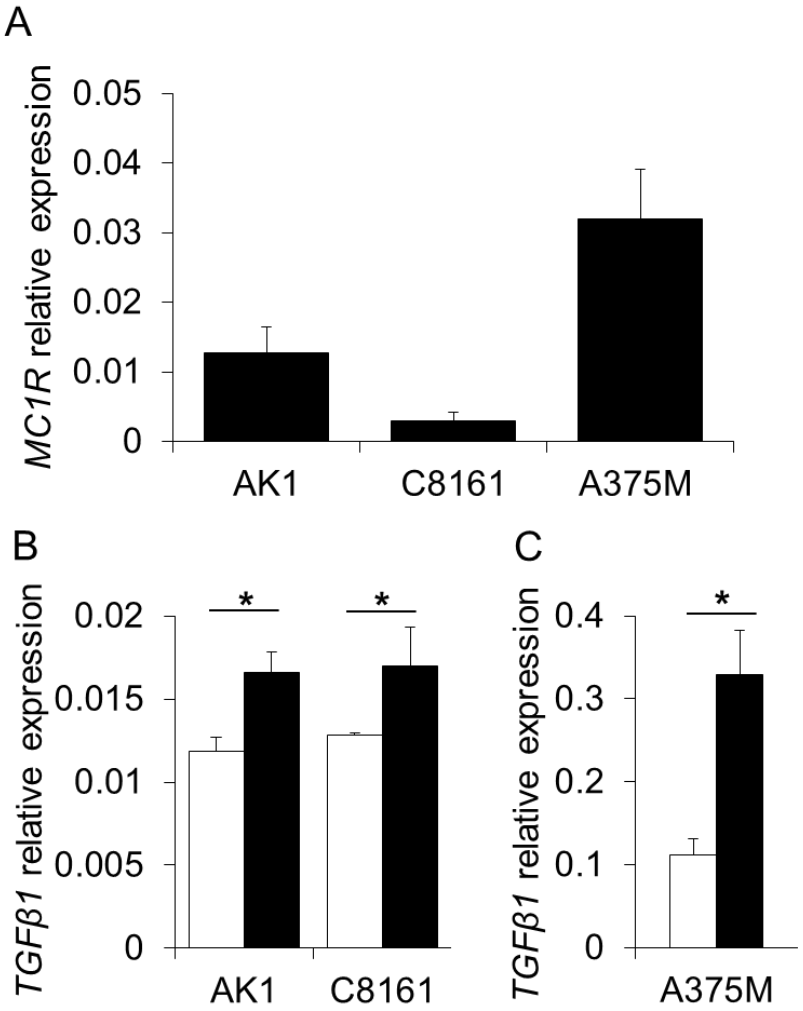


Figure 14 Enhanced expression of TGF-β1 in human melanoma cells by activation of the α-MSH-MC1R axis.

A: PCR analysis of MC1R expression in human melanoma cells; Each value for AK1, C8161 and A375M was normalized to *β-Actin*.

B and C: RT-PCR analysis of TGF-β1 mRNA in non-stimulated (white) or α-MSH (3 ng/ml)-stimulated (black) human melanoma cells 24 hour following stimulation. Levels were normalized to *β-Actin*. *****P<0.05.

Discussion

In the present study, I have demonstrated a novel role for the MC1R-mediated signaling pathway in the regulation of TGF-β1 production in B16 cells as summarized in Figure 15. α-MSH, an agonist of MC1R, activates both p38 and/or ERK1/2, which is then followed by enhanced production of TGF-β1 in B16 cells. Knocking down MC1R or MITF abolishes the α-MSH-stimulated TGF-β1 production. ERK1/2 kinase is reported to up-regulate TGF-β1 via MITF[28]. Therefore, the α-MSH-MC1R-MITF axis appears to be the central signal transduction pathway involved in TGF-β1 production in B16 cells.

What is the physiological or pathological meaning of this axis? TGF-β1, a multifunctional cytokine that plays important roles in different aspects of cellular function, is normally expressed in various human tissues, and is highly expressed in various cancer cell lines[32,63]. More importantly, clinical data reveal that the serum levels of TGF-β1 in melanoma patients are higher when compared with healthy donors[64], and TGF-β1 staining intensities in melanoma cells of the invasion front correlate with poor survival[33]. MC1R, a cell surface endocytic receptor expressed on melanocytes, is highly expressed in transformed melanocytes[3-5]. From my data, MC1R turned to be functional in regard to producing TGF-β1. Thus, the MC1R-mediated up-regulation of TGF-β1 plays indispensable roles in exacerbating the malignancy of melanoma cells.

Quite complicated reactions are taking place in the process to enhance TGF-β1 production. TGF-β1 impacts melanoma progression through activation of various processes such as angiogenesis, metastasis, inflammation, and proliferation[28]. α-MSH stimulation leads to activation of the CREB pathway via cAMP[50,65], and the cAMP pathway mediates the regulation of melanogenesis[54]. MITF acts downstream in the cAMP pathway, suggesting that several signals are produced and transduced to stimulate melanoma development and progression[60]. My data, however, show that the activities of the TGF-β1 promoter are

correlated with MITF expression levels and plausible with some others, which I did not address in the present research. MITF is the target of the MAPK pathway at various levels including its transcription and its protein turnover and function[40,54,58], which is essential for melanocyte differentiation[61], and importantly related to malignant progression[65]. This evidence implies that between the initial step of the a-MSH-MC1R system and the latter one of CREB-MITF in terms of TGF-β1 up-regulation are occurring across several signaling pathways, one of which is MAPK, a second p38-ERK1/2, and others containing PI3K and c-kinase. In addition to these, the secreted the TGF-β1 exerts its autocrine signaling through TGF- β1 receptor in B16 cells[66,67]. TGF-β1 activates a couple of kinases such as p38, PI3K and others as well[68,69], indicating that complicated crosstalk in kinase cascades may develop in process of tumorigenesis in melanomas. To unveil the entry of events triggered by the α-MSH-MC1R-MITF axis, this system will provide a useful model for analyzing how TGF-β1-induced malignant processes during melanoma development and progression.

Does Treg account for α-MSH-induced tumorigenesis in the present system? Treg plays a key role in sustaining immune homeostasis by the suppression of physiological and pathological immune responses[70], and also produce TGF-β and Interleukin-10 (IL-10)[71], blunting the antitumor effect of other immune cells including $CD4^+$ T cells, $CD8^+$ T cells[72-75] and NK cells[76]. Because Treg produces TGF-β1 and secreted TGF-β1 affects melanoma development[66,77], it is intriguing to speculate that the α-MSH-MC1R-MITF axis may stimulate induction of Treg from their precursor cells. However, I found that the frequencies of Treg in tumors isolated from C57BL/6 mice injected subcutaneously with B16 melanoma cells deficient for MC1R were similar to control B16 melanoma cells. These data suggest that the α-MSH-MC1R-MITF axis does not stimulate induction of Treg from their precursor cells in the experimental system as shown in Figure12 and Figure 13.

A couple of strategies might be necessary to down-regulate TGF-β1 synthesis with regard

to development of melanoma therapeutics. Ligands for MC1R are melanocortins such as MSHs and ACTH, each of which is reported to be up-regulated in distinct types of malignant melanoma[44,78], and most of melanomas shows up-regulation of MC1R[31,79]. As observed in murine B16 cells, the activation of the α-MSH-MC1R pathway in a couple of human melanoma cell lines results in enhanced expression of TGF-β1 as well. Increased TGF-β1 definitely acts on melanoma cells though an autocrine mechanism[77], which may cause a synergistic effect between TGF-β1 and MC1R to accelerate tumorigenesis in vivo. Taken together, my results suggest that as a key regulator of melanogenesis and melanocyte functions, MC1R can important contribute to melanoma progression, and therefore its targeting should be a new universal strategy for melanoma treatment. To completely depress TGF-β1 synthesis and its later effects as well, several molecules in the α-MSH-MC1R-MITF-axis as well as in the cascade triggered by the TGF-β1 and TGF-β1 receptor system are available for this line of strategy.

If there is a high positive correlation between MC1R and melanoma progression[44], the development of tumors was expected not so obvious as shown in MC1R-KD mice. However, at any time after inoculation of melanoma cells, the development of melanoma was detected substantially, while delayed in some degree（Figure12）. The most plausible possibility for this is that the shRNA employed did not knock down MC1R gene so completely as to inhibit melanoma progression as well as production of TGF-β1. To analyze this correlation, a complete knockout system should be used such as either KO mice with MC1R gene or genome targeting systems including TALEN or Crisper Cas9[80]. Another possibility is that the subcutaneous inoculation of tumor cells is not appropriate, even if melanoma cells are utilized. It is therefore interesting to examine effect of the α-MSH-MC1R-MITF axis with either the central nervous system or the immune system, because brain and peripheral tissues are known as α-MSH positive tissues[42], and spleen and thymus as immune tissues where Treg are

activated routinely[81,82].

A novel interesting paper was published very recently[83]. This says that the family of Treg subsets, $CD4^{+}CD69^{+}Foxp3^{-}$Treg suppressed T cell response via membrane-bound TGF-β1 and as its result hepatocellular carcinoma progressed actively. Although I did not analyze the population of the subset of Treg in my experiment, there is the possibility that this Treg can be inducible even in my experimental system. At 12 days after injection of B16 cells into mice, there was observed statistical differences of tumor sizes between B16 cells and ones with MC1R knockdown, suggesting that around this timing the interaction between tumor cells and the surrounding tissue cells may begin to trigger the induction of various type of Tregs including $CD4^{+}CD69^{+}Foxp3^{-}$Treg. To address precisely to this possibility on Tregs' induction, further experiments over a long period should be carried out.

Gathering attention centers on new developments in the management of advanced melanoma, role of pembrolizumab (a potent and highly selective humanized monoclonal antibody of IgG4-kappa isotype)[11]. One of such strategies is directed to both PD-1 and PD-1 receptor (PD-L1) and clinical trials have been successfully progressed in the US since 2014. This line of experiments is attempting to shut down the interaction between tumor cells and T-cells to augment antitumor immune response. Whereas this is quite far from my concerns of lowering TGF-β1 synthesis, both of which are resulting in tumor retardation. Furthermore, hampering PD-l action and TGF-β1 production leads to inhibit migration of T-cells and tumor cells, respectively[84], which reduces physical contact of these cells as well. Therefore, additive and/or synergistic strategies are promising in terms of immunotherapy of cancer cells expressing PD-1, PD-L1, TGF-β1, and TGF-β1R as well as melanoma.

In conclusion, my data show that the α-MSH-MC1R-MITF axis exerts its function in mice and possibly in human. It is therefore conceivable that the targeting this axis may provide a novel therapeutic method against melanomas and related tumors in both mouse and human.

However, approximately 20% of genetic ways of working of mouse is different from human, indicating not all the biochemical process is common[85,86]. Therefore, it is absolutely necessary to identify the reactions that are effective for human melanoma treatment by comparing between mouse and human reactions triggered by the α-MSH-MC1R-MITF axis.

Conclusions

1. α-MSH stimulation augments TGF-β1 production in mouse B16 melanoma cells.
2. Knockdown of either MC1R or MITF lowered the α-MSH-induced TGF-β1 production in B16 melanoma cells.
3. The α-MSH-MC1R signaling pathway contributes to TGF-β1 production via ERK, p38, and MITF-mediated mechanisms.
4. Targeting of MC1R by shRNA decreased tumor growth in vivo, but not in vitro.
5. MC1R was also expressed in a few of human melanoma cell lines.
6. The α-MSH-MC1R-MITF axis is regarded as a major signal transduction pathway to up-regulate TGF-β1 in melanoma.

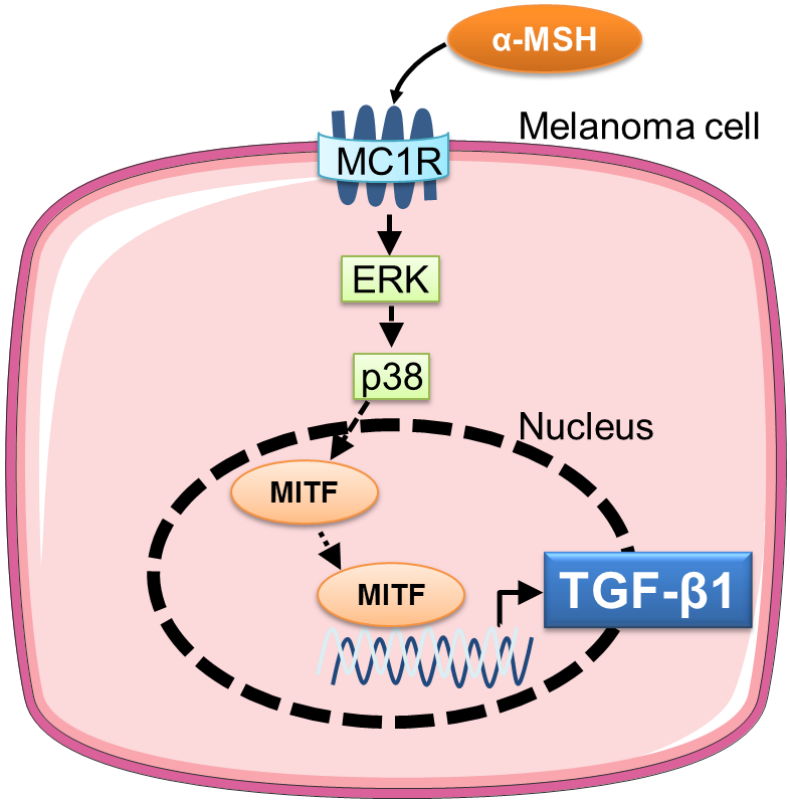


Figure 15 Summary of this study

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