Original Article

α-MSH stimulation contributes to TGF-β1 production via MC1R-MITF signaling pathway in melanoma cell

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Transforming growth factor-β (TGF-β) is a multifunctional cytokine that play critical roles in melanoma progression. Although the impact of TGF-β signaling on melanoma progression has been well characterized, little is known about the molecular mechanisms that control TGF-β production in melanoma cells. In this study, we describe a novel role for Melanocortin Receptor 1 (MC1R) in the regulation of TGF-β production. MC1R is a cell surface endocytic receptor expressed in melanoma cells and serves as a receptor for α-Melanocyte Stimulating Hormone (α-MSH). The activation of MC1R with α-MSH resulted in increased levels of TGF-β, which was mediated by ERK1/2 and p38 signaling pathways. Furthermore, Microphthalmia Transcription Factor (MITF), the master regulator of melanocytes, was found to act downstream of MC1R to regulate TGF-β production. Targeting of MC1R-MITF axis was effective to decrease TGF-β production, and resulted in delayed tumor growth of B16 melanoma *in vivo*. Collectively, these results give new insight into the molecular mechanisms that control TGF-β production in melanoma cells.

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Introduction

Malignant melanoma is considered as one of the most aggressive types of cancer, which show high resistance to conventional chemotherapeutic and immunotherapeutic treatment[1-4]. Melanomas arise as a result of malignant transformation of melanocytes, and are associated with increased production of several growth factors and cytokines that play critical roles in tumor progression. In particular, melanomas produce increasing amounts of the transforming growth factor-β1 (TGF-β1), which is correlated with

disease progression. TGF-β1 is a multifunctional cytokine involved in the regulation of many biological functions of the cell including proliferation, differentiation and survival[5]. In melanoma, TGF-β1 promotes tumor progression via autocrine effects on melanoma cell motility and invasiveness, in addition to paracrine effects including modulation of tumor stroma and extracellular matrix, enhancing angiogenesis and inhibition of immune surveillance[6-9]. For these reasons, TGF-β1 has attracted much attention as a potent therapeutic target of melanoma[9]. Although the impact of TGF-β1 signaling pathway on melanoma progression has been well defined, the molecular mechanisms that underlie the enhanced TGF-β1 production in melanoma cells still poorly understood. Thus, a comprehensive analysis to clarify such mechanisms may help to identify new suitable therapeutic strategies.

Melanocortin 1 receptor (MC1R) is a cell surface endocytic receptor expressed on melanocytes in mouse and human[10]. MC1R can bind to adrenocorticotropic hormone (ACTH) and α-Melanocyte Stimulating Hormone (α-MSH)[11-13]. The activation of MC1R signaling in melanocytes induces tyrosinase expression, resulting in enhanced synthesis of melanin[14]. Thus, MC1R is considered as a key factor that plays important roles in the melanocyte transformation process in pathological conditions such as ultraviolet radiation[14]. In melanoma, MC1R is overexpressed on the cell surface of the majority of human melanomas, and correlated with melanoma initiation, progression and metastasis[15].

From these backgrounds, we hypothesized that since MC1R plays important roles in melanoma progression, it may also play a role in the regulation of TGF-β1 production in melanoma cells. In this study, we have evaluated this hypothesis, and found that α-MSH-mediated activation of MC1R pathway resulted in enhanced production of TGF-β1 in B16 melanoma cells. Importantly, this effect was mediated by MITF, the major regulator of melanoma cells, and the targeting of α-MSH-MC1R-MITF pathway resulted in decreased production of TGF-β1 *in vitro*, and delayed tumor growth *in vivo*. Together, our results provide new insight into the molecular mechanisms of TGF-β1 production in melanoma cells.

Materials and methods

1)Cell culture and reagents

B16 Melanoma 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 A375M, C8161 and AK1 cells lines were obtained from Department of Plastic and Reconstructive Surgery, Hokkaido University. B16, NBL-7, A375M, C8161, AK1 and Lenti-X 293T 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, 0.1mM Non-Essential Amino Acid, 100 U/mL Penicillin and 100μg/mL streptomycin (Gibco). Cells were incubated in a humid environment at 5% CO_2 and 37 °C conditions.

For MC1R stimulation, α-MSH (Peptide Institute. Inc., 4057-V) was used at concentration of 3ng/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 3μM for 1 hour before α-MSH stimulation.

2)RT-PCR, real time qRT-PCR and genomic PCR

Total RNA was purified from cells using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instruction, and used for cDNA synthesis (SuperScript III, Invitrogen). Genomic DNA was prepared by 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 *Tgfb1* or *Mc1r* mRNA as normalized to the internal control *Hprt*, and human *MC1R* or *TGFβ1* mRNA as normalized to the internal control *β-actin* using POWER SYBR®Green (Invitrogen). Sequences of primers are listed in Table 1.

3)NBL-7 proliferation inhibition assay

To determine the latency and bioactivity of Tgfb1, $3x10^3$ of B16 melanoma cells were seeded into a 24 well culture plate and stimulated on the next day with 3 ng/ml of α-MSH or PBS. After 44 hours, supernatants of B16 melanoma cell culture were collected and added to NBL-7 cells ($5x10^5$ cells per well seeded into a 96-well culture plate). In some experiments, the supernatants of B16 cell culture were pretreated with a neutralizing Anti-TGFβ antibody (R & D Systems, 10μg/ml) or matching control IgG (Biolegend) and incubated at 37°C for 1 hour before adding to NBL-7 culture. After 48 hours, ^{3}H-thymidine (1μCi / well) was

Table 1 Primers used 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	

Table 2 shRNA used for knock down experiments

Target		Sequence (5' → 3')
Mc1r	Sense	GATCCCCATCTTCTGTGCGTTGCGTTGTGCTTCCTG TCACATAACGCAGCGCATAGAAGATTTTT
Mc1r	Anti-sense	CTAGAAAAATCTTCTATGCGCTGCGTTATGTGACAG GAAGCACAACGCAACGCACAGAAGATGGG
Mitf #1	Sense	GATCCCCGCTAGACTTGTGTATTCTGTTGCTTCCTG TCACAATAGAATATACAAGTCTGGCTTTT
Mitf #1	Anti-sense	CTAGAAAAGCCAGACTTGTATATTCTATTGTGACAG GAAGCAACAGAATACACAAGTCTAGCGGG
Mitf #2	Sense	GATCCCCGTAAATATGTTACCTGTCTCTGCTTCCTG TCACAGAGACGGGTAACGTATTTGCTTTT
Mitf #2	Anti-sense	CTAGAAAAGCAAATACGTTACCCGTCTCTGTGACAG GAAGCAGAGACAGGTAACATATTTACGGG

added to culture for 4 hours, and DNA incorporation of ^{3}H-thymidine was measured by MicroBeta2 (PerkinElmer).

4)Lentiviral shRNA-mediated knockdown of *Mc1r* and *Mitf*

Knockdown of *Mc1r* and *Mitf* was performed using short-hairpin RNAs (shRNAs). The target sequences used for knockdown were identified from the RNAi Consortium (TRC) sequence database, as listed in Table 2. To generate entry vectors, the oligonucleotides were annealed and ligated into pENTR4-H1 plasmid vector (provided by Dr. H. Miyoshi) using Bgl-II and Xba-I restriction sites (Toyobo). The cloned inserts were finally confirmed by sequencing with M13 primers (Forward: GTAAAACGACGGCCAG, Reverse: CAGGAAACAGCTATGAC) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and samples were applied to 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Entry vectors and

CS-RfA-EG lentiviral vector (provided by Dr. H. Miyoshi) were employed in a recombination reaction using 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-HIV-gp, VSV-G and pCMV-VSV-G-RSV-ReV plasmids into Lenti-X 293T cells using Polyethyleneimine "MAX" (PEI-max, Polysciences, Inc.). 12 hours after transfection, medium was changed into fresh DMEM containing 1μM Forskolin, and cells were incubated for additional 24 hours. Subsequently, the supernatants containing the viral particles were collected, 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.

5)Western blotting

Cell lysates of $1x10^6$ B16 cells were prepared by Mammalian Protein Extraction Reagent (M-PER, Thermo Fisher Scientific) supplemented with Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA). For the detection of p-p38 and p-ERK1/2, cell lysates were prepared at the indicated times after α-MSH stimulation in the presence of phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Thermo Fisher Sciences). For the detection of transcription factor MITF, Nuclear and Cytoplasmic Protein Extraction Reagent (NE-PER, Thermo Fisher Scientific) were used to extract the nuclear fraction. Lysates were then separated on 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 phospho 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 Scientific) or ECL prime (GE health care). Signals were detected using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences) and quantified using Image J.

6)Reporter Gene Assay

The coding region of Mitf-M was amplified by PCR from cDNA template using KOD-Plus-Neo Kit (TOYOBO CO., LTD) and the following primers: Forward: 5'-CACCATGCT GGAAATGCTAGAATACAGTC-3', Reverse : 5'-ACACGCA TGCTCCGTTTCT-3'. The coding region was then cloned into pENTR™/SD/D-TOPO vector. The entry vector and pEF-DEST51 vector were employed in a recombination reaction using Gateway system (Invitrogen) according to the manufacturer's protocol. Mouse TGF-β1 promoter cloned into pGL2 Basic luciferase reporter vector was kindly provided by Dr. Naoko Nakano (Research Institute for Biological Sciences, Tokyo University of Science). For reporter gene assay, Lenti-X 293T cells ($4x10^5$) were cultured in a 6 well tissue culture plate for 24 hours prior to transfection, and then co-transfected with 1 μg 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 luciferase activities of transfected reporters according to the manufacturer's protocol (Promega). Luciferase activity was normalized to the β-Gal expression (SV-40 β-galactosidase expression vector, Promega, Madison, WI).

7)Animal experiment

Six- to eight-weeks old C57BL/6 female mice were purchased from SLC Japan, Inc. (Tokyo, Japan). Mice (N=5) were inoculated subcutaneously into the back with $3X10^5$ of B16 melanoma 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.

8)Statistical Analysis

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

Results

1)α-MSH stimulation enhances Tgfb1 production in B16 melanoma cell

To evaluate the possible role of Mc1r in Tgfβ1 production, we first examined the expression of melanocortin receptors family in B16 melanoma cells. Compared to bone marrow-derived dendritic cells (BMDCs) which serve as a positive control, we found that *Mc1r* was exclusively expressed in

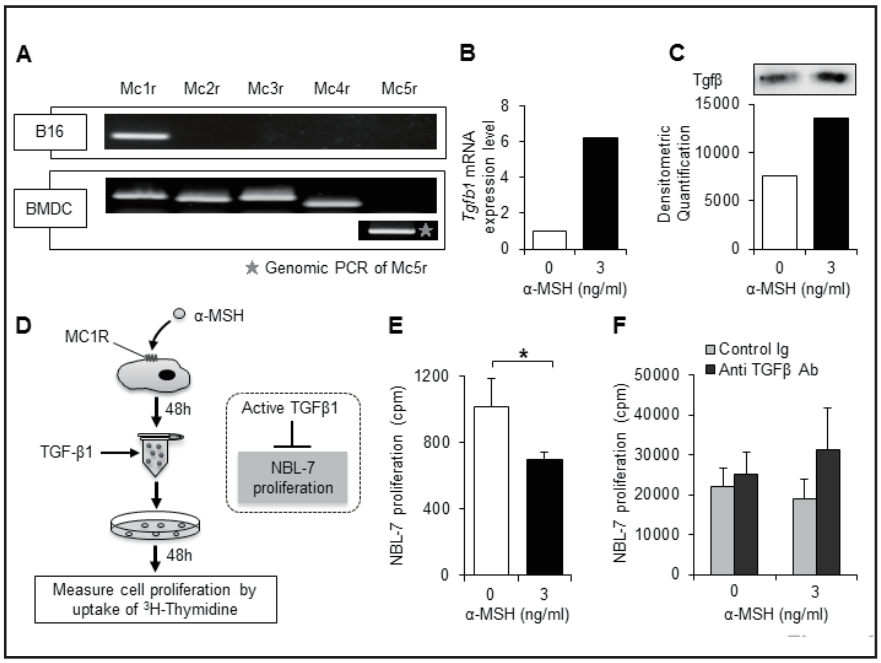

Fig. 1 Tgfb1 production is enhanced in B16 melanoma cells upon α-MSH stimulation

(A)PCR analysis of Mcr family expression in B16 melanoma cells as compared to bone marrow-derived dendritic cells (BMDCs) as a positive control. Mc5r was detected at the genomic level but not in the mRNA of BMDCs, thus genomic PCR of Mc5r was shown in a separate panel.

(B)RT-PCR analysis of *Tgfb1* mRNA in non-stimulated or α-MSH (3ng/ml)-stimulated B16 melanoma cells 48 hours after stimulation.

(C)Western blot analysis and densitometric quantification of Tgfb in the supernatants of non-stimulated or α-MSH (3ng/ml)-stimulated B16 melanoma cells 48 hours after stimulation.

(D)A scheme describes the NBL-7 proliferation inhibition assay used to evaluate levels of Tgfb1 in the supernatants of B16 melanoma cells.

(E)Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3ng/ml)-stimulated B16 melanoma cells.

(F)Proliferation inhibition assay of NBL-7 cells stimulated with the supernatants of B16 cell culture after pretreatment with a neutralizing antibody for Tgfb or control Ig.

Data are shown as representative of 2 independent experiments. *p<0.05.

B16 melanoma cells (Fig. 1A). Next, we examined whether MC1R-mediated signaling contribute to Tgfb1 production. To do so, we evaluated the expression level of *Tgfb1* 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[16]. Upon stimulation with α-MSH, MC1R triggers the activation of ERK1/2 pathway[15], which was also confirmed in B16 cells (data not shown). Importantly, we found that the stimulation of B16 cells with α-MSH resulted in upregulation of Tgf-β1 at mRNA and protein levels (Fig. 1B and 1C). To further confirm the existence of active Tgfb1 in the supernatants of B16 cells, we performed proliferation inhibition assay using mink lung epithelial cells (NBL-7). The proliferation of NBL-7 cells is inhibited in response to TGF-β[17-19], thus we next compared the proliferation of NBL-7 cells when cultured with supernatants collected from non-stimulated or α-MSH-stimulated B16 cell culture (Fig. 1D). As expected, we found that the 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 culture with Tgfb1 following α-MSH stimulation (Fig. 1E). Furthermore, we found that a neutralizing antibody for TGF-β was effective to abolish the effects of B16 supernatants as indicated by recovery in NBL-7 proliferation (Fig. 1F), suggesting that Tgfb1 is the responsible factor for suppressing NBL-7 proliferation in this assay. Together,

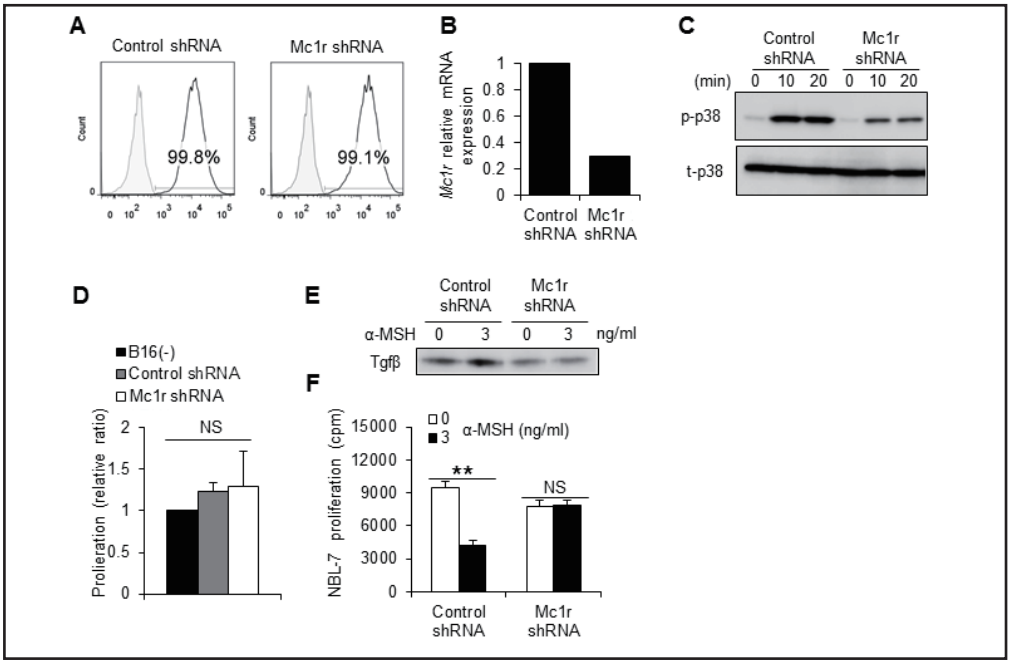

Fig. 2 Tgfb1 production is decreased in α-MSH-stimulated B16 melanoma cells after Knockdown of Mc1r

(A)Establishment of B16 cells knocked down for *Mc1r* using specific shRNA coding lentiviral system. GFP$^+$ B16 cells were isolated by flowcytometry. Gray histogram: isotype control, White histogram: GFP$^+$ fraction.
(B)*Mc1r* mRNA knockdown efficiency was confirmed by RT-PCR.
(C)Western blot analysis of phosphorylated p38 (upper panel) and total p38 (lower panel) compared between α-MSH-stimulated B16 cells knocked down for *Mc1r* or control at the indicated times.
(D)Proliferation assay of B16 cells knocked down for *Mc1r* or control compared to non-treated B16 cells.
(E)Western blot analysis of Tgfβ in non-stimulated or α-MSH (3ng/ml)-stimulated B16 melanoma cell knocked down for *Mc1r* or Control 72 hours after stimulation.
(F)Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3ng/ml)-stimulated B16 cells knocked down for *Mc1r* or control.
Data are shown as representative of 2 independent experiments. NS: non- stimulated. $^{**}p<0.01$.

these results suggest that α-MSH-mediated signaling contribute to Tgf-β1 production in B16 cells.

2)*Mc1r* Knockdown decreases Tgfb1 production in α-MSH-stimulated B16 melanoma cells

As suggested by the above data, α-MSH stimulation enhances Tgfb1 production in B16 cells. To further confirm the involvement of Mc1r in this effect, we utilized short-hairpin RNAs (shRNAs) encoding lentiviral system to knockdown *Mc1r* in B16 cells. Following transfection, GFP$^+$ B16 cells were isolated by flow sorting (Fig. 2A), and knockdown efficiency of *Mc1r* was confirmed by RT-PCR (Fig. 2B). In addition to confirmation by mRNA levels, we also confirmed that knockdown of Mc1r affected downstream signaling cascades, such as the phosphorylation of p38 which was decreased in B16 knock downed for *Mc1r* compared to control (Fig. 2C). Proliferation of B16 cells was not affected following knockdown of *Mc1r* (Fig. 2D). We next asked if Tgfb1 production is affected by knockdown of Mc1r in B16 cells when stimulated with α-MSH. As expected, stimulation with α-MSH was ineffective to enhance Tgfb1 production in B16 cells expressing Mc1r shRNA comparing to control shRNA (Fig. 2E). Additionally, in NBL-7 cells proliferation inhibition assay, we found that the proliferation of NBL-7 cells was efficiently inhibited when stimulated with the supernatant of α-MSH-stimulated B16 cells which express *Mc1r* (Fig. 2E). However, this effect was completely abolished when NBL-7 cells were stimulated with supernatant of α-MSH-stimulated *Mc1r*-knocked down B16 cells (Fig. 2E), which reflects decreased Tgfb1 production in α-MSH-stimulated B16 cells after knockdown of *Mc1r*. Thus, these results provide an evidence for a functional

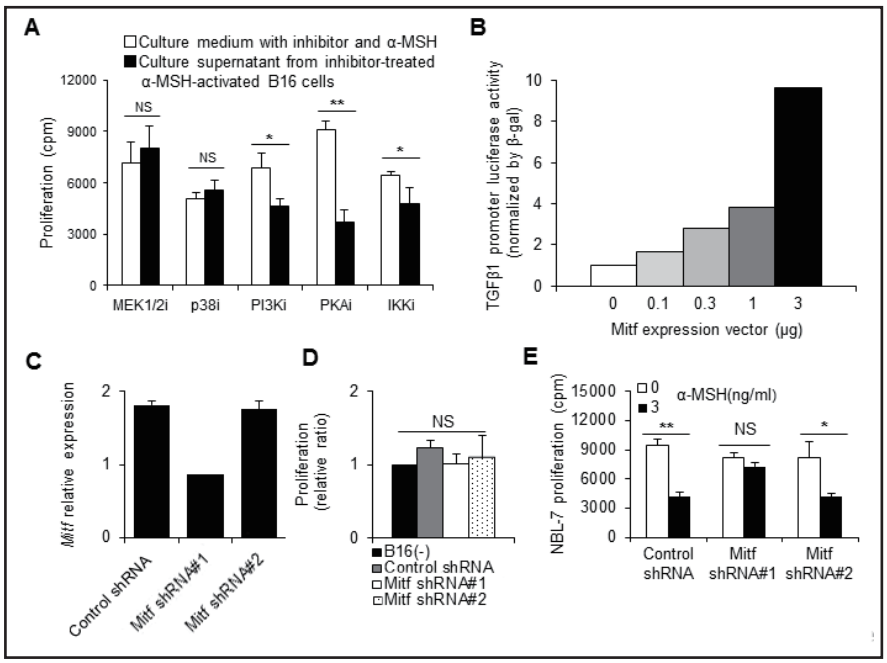

Fig. 3 Mitf acts downstream of Mc1r to enhance Tgfb1 production in B16 melanoma cells

(A)Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3ng/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 responsible signaling pathway for the expression of Tgfb1.

(B)Luciferase reporter assay of TGFβ1 promoter in Lenti-X-293T cells transfected with increased amounts of Mitf expression plasmid 24 hours after stimulation.

(C)RT-PCR analysis to confirm knockdown efficiency of *Mitf* in B16 melanoma cells.

(D)Proliferation assay of B16 cells knocked down for *Mitf* or control compared to non-treated B16 cells.

(E)Proliferation inhibition assay of NBL-7 cells cultured in the presence of supernatants of non-stimulated or α-MSH (3ng/ml)-stimulated B16 cells knocked down for *Mitf* or control.

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

α-MSH-MC1R signaling axis to regulate Tgfb1 production in B16 melanoma cells.

3)Mitf is the responsible factor for enhancing Tgf-β1 production in α-MSH-stimulated B16 cells

Next, we aimed to identify signaling pathways that act downstream of Mc1r to regulate Tgfb1 production in α-MSH-stimulated B16 cells. MC1R has been reported to activate several signaling cascades, notably the Protein Kinase A (PKA) -- cAMP response element binding protein (CREB) and MAP kinase (MAPK) pathways[20-21]. Additionally, recent reports have also identified the role of PI3 kinase (PI3K) and nuclear factor κB (NF-κB) in signaling downstream of MC1R[26]. To identify the responsible pathway, we utilized an inhibitor of MEK1/2 (U0126), p38 (SB203580), PI3K (LY294002), PKA (H-89) or IKK (PS-1145) to B16 culture prior to α-MSH stimulation, and supernatants of culture were used to evaluate Tgfb1 production in a NBL-7 cells proliferation inhibition assay. In this experiment, we found that the proliferation of NBL-7 cells was efficiently inhibited when cultured in the presence of α-MSH-stimulated B16 cells pretreated with inhibitors of PI3K, PKA and IKK (Fig. 3A). On the other hand, the inhibition of NBL-7 cells proliferation was not observed when B16 cells were pretreated with inhibitors of MEK or p38 before α-MSH stimulation (Fig. 3A). Thus, we concluded that Erk1/2 and p38 pathways are important for MC1R-mediated signaling, since inhibitors of these two molecules have resulted in decreased Tgfb1 production following α-MSH stimulation.

To further clarify the molecular mechanisms related to

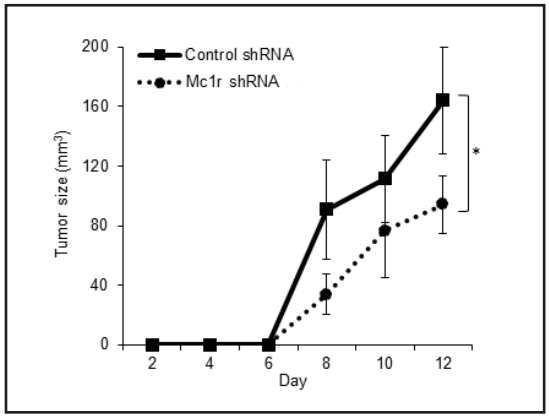

Fig. 4 *Mc1r* knockdown results in delayed growth of B16 melanoma *in vivo*

C57BL/6 mice (n=5 per group) were inoculated subcutaneously into the back with B16 melanoma cells stably transfected with control shRNA or *Mc1r* shRNA, and tumor growth was measured on the indicated days. *$p<0.05$.

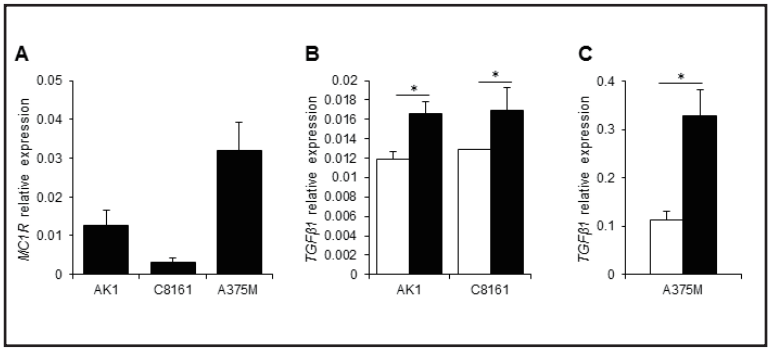

Fig. 5 Activation of α-MSH-MC1R axis enhances *TGF-β1* expression in human melanoma cells

(A)PCR analysis of *MC1R* expression in Ak1, C8161 and A375M human melanoma cells (normalized to *β-Actin*).

(B and C)RT-PCR analysis of *TGF-β1* mRNA in non-stimulated or α-MSH (3ng/ml)-stimulated human melanoma cells 24 hours after stimulation (normalized to *β-Actin*).

MC1R signaling, we next tried to identify transcription factors that regulate Tgfb1 production in α-MSH-stimulated B16 cells. Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor which acts as a master regulator of several biological functions including development, function and survival in melanocytes[20]. Several studies have also reported critical roles of MITF in numerous aspects of melanoma including proliferation, progression, migration, invasion and metastasis[23]. Importantly, MITF acts downstream of ERK1/2 and p38 MAPK in melanoma cells[22, 24]. Thus, we focused on MITF as a possible candidate that acts downstream of Mc1r to regulate Tgfb1 production. First, we utilized luciferase reporter assay to evaluate the role of MITF in the regulation of *TGF-β1* promoter. In this assay, we found that luciferase activity of *TGF-β1* promoter reporter was significantly enhanced when co-transfected with MITF expression plasmid in a dose-dependent manner (Fig. 3B), indicating the involvement of MITF in regulation of *TGF-β1* promoter.

Next, to examine if Mitf is involved in the enhancement of Tgfb1 production in α-MSH-stimulated B16 cells, we utilized short-hairpin RNAs (shRNAs) encoding lentiviral system to suppress Mitf expression in B16 cells. Two shRNAs were developed to knockdown Mitf, and referred to as Mitf shRNA#1 and Mitf shRNA#2. Transfected B16 cells were selected by flow sorting according to GFP fluorescent and knockdown efficiency of Mitf was confirmed by RT-PCR. Using these two shRNAs, we found that shRNA#1 was more effective to knockdown Mitf than shRNA#2 (Fig. 3C). Proliferation of B16 cells was not affected after knockdown of Mitf (Fig. 3D). We next examined if Tgfb1 production was affected in these two cell lines by knockdown of Mitf in α-MSH-stimulated B16 cells in NBL-7 cells proliferation inhibition assay. 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, which was not observed in the case of non-effective Mitf shRNA#2 (Fig. 3E). These data provide evidence that MITF acts downstream of Mc1r to enhance Tgf-β1 production in B16 melanoma cells following α-MSH-stimulation.

4)Effect of Mc1r knockdown on B16 melanoma growth *in vivo*

Accumulating evidence has unveiled the fundamental roles of TGF-β1 in multiple aspects of cancer progression, including melanoma[25-26]. In this study, we have identified a role for Mc1r signaling pathway in the regulation of Tgfb1 production in B16 melanoma cells which was mediated by Mitf. Thus, finally we asked if the targeting of this axis may affect tumor growth of B16 melanoma *in vivo*. To do so, we simply inoculated B16 melanoma cells stably transfected with control shRNA or Mc1r shRNA into B6 mice, and compared tumor growth between the two groups. Comparing to control group, we found that tumor growth

was delayed in mice inoculated with B16 cells knocked down for Mc1r (Fig. 4). Collectively, these data indicate the importance of α-MSH-MC1R-MITF axis in the regulation of Tgfb1 production in B16 cells, which consequently has an impact on tumor progression *in vivo*.

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

Finally, we asked whether *TGF-β1* expression is enhanced in human melanoma cells following α-MSH stimulation similar to B16 cells. To do so, we first evaluated *MC1R* expression in human Ak1, C8161 and A375M melanoma cell lines. We found that MC1R is expressed in these cell lines with some variations (Fig. 5A). Importantly, α-MSH stimulation was effective to enhance *TGF-β1* expression (Fig. 5B and 5C), 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 (Fig. 5C). Thus, these data suggest that α-MSH-MC1R axis can also contribute to TGF-β1 production in human melanoma cells.

Discussion

In this study, we demonstrated a novel role for MC1R-mediated signal pathway in the regulation of TGF-β1 production in melanoma cells. TGF-β1, a multifunctional cytokine that play important roles in different aspects of the cellular function, is normally expressed in various tissues, and highly expressed in various cancer cell lines[27-28]. Importantly, clinical data showed that the serum levels of TGF-β1 in melanoma patients are higher compared with healthy donors[29], suggesting that TGF-β1 is correlated with melanoma progression in these patients. MC1R, a cell surface endocytic receptor expressed on melanocytes, was also found to be highly expressed in transformed melanocytes[3, 4, 30]. From these backgrounds, we hypothesized that MC1R-meidated signaling pathway might be related to TGF-β1 production in melanoma cells. Indeed, we found that stimulating MC1R-expressing melanoma cells with α-MSH, an agonist peptide hormone of the melanocortin family, resulted in enhanced production of TGF-β1. Knock-down of MC1R in melanoma cells has abolished the enhanced production of TGF-β1 after stimulation with α-MSH, suggesting that α-MSH-MC1R-mediated signaling pathway is involved in enhancing TGF-β1production melanoma cells.

Regarding the molecular mechanism, we found that α-MSH-MC1R stimulation resulted in enhanced TGF-β1production via MITF-mediated pathway. It is well known that α-MSH stimulation leads to activation of CREB pathway via cAMP[24, 31]. Additionally, accumulating evidence has indicated the involvement of cAMP pathway in the regulation of melanogenesis[20]. Furthermore, MITF acts downstream of cAMP and transduces several signals related to melanoma development and progression[23]. By unveiling the involvement of this axis in TGF-β1 production, this report provides an additional evidence of the importance of this pathway in melanogenesis.

Our data showed that the activities of TGF-β1 promoter was correlated with MITF expression levels. In melanoma, MITF is the target of MAPK pathway at various levels including its transcription and its protein turnover and function[15, 20, 21], which is essential for melanocyte differentiation[25], and importantly related to malignant progression[31].

Based on our *in vitro* results, we also examined if targeting of α-MSH-MC1R-MITF axis may affect tumor growth *in vivo*. We found that tumor growth was delayed in mice inoculated with melanoma cells knocked-down for MC1R compared to wild type control.

Similar to murine B16 melanoma cells, activation of α-MSH-MC1R pathway in human melanoma cells resulted in enhanced *TGF-β1* expression. Together, our results suggest that MC1R, a key regulator of melanogenesis and melanocyte functions, can importantly contribute to melanoma progression through enhancing melanoma cells to produce more TGF-β, and thus may serve as a new therapeutic target to be considered in future strategies of melanoma treatment.

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Conflict of interests

The authors declare no conflict of interest.

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