Tyrosinase suppresses vasculogenic mimicry in human melanoma cells

HIROKI KAMO, RYOTA KAWAHARA and SIRO SIMIZU

Department of Applied Chemistry, Faculty of Science and Technology, Keio University,
Yokohama, Kanagawa 223-8522, Japan

Abstract. Melanoma is a type of skin cancer that derives from melanocytes; this tumor is highly metastatic and causes poor clinical outcomes in patients. Vasculogenic mimicry (VM), a vascular-like network that is formed by tumor cells instead of endothelial cells, promotes the growth and metastasis of tumors by providing tumors with oxygen- and nutrient-containing blood. VM correlates with a poor prognosis in patients with melanoma, but the melanoma-specific mechanisms of VM are unknown. The present study revealed that treatment with the melanogenesis stimulators 3-isobutyl 1-methylxanthine (IBMX) and α-melanocyte-stimulating hormone (α-MSH) significantly inhibited VM in MNT-1 human pigmented melanoma cells. Tyrosinase (TYR), an essential enzyme in melanin production, was upregulated on treatment with α-MSH and IBMX, prompting an examination of the association between TYR and VM. A TYR inhibitor, arbutin, promoted VM in melanoma cells. Furthermore, CRISPR/Cas9-mediated knockout (KO) of TYR increased VM by melanoma cells. Notably, even in non-pigmented melanoma cells, TYR attenuated VM. Although re-expression of wild-type TYR suppressed VM in TYR-KO cells, T373K TYR, a frequently detected mutation in individuals with albinism, failed to inhibit VM. Overall, these results demonstrated that TYR negatively regulates VM, providing novel insights into the antioncogenic function of TYR in melanomas.

Correspondence to: Professor Siro Simizu, Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan
E-mail: simizu@applc.keio.ac.jp

Abbreviations: VM, vasculogenic mimicry; α-MSH, α-melanocyte-stimulating hormone; IBMX, 3-Isobutyl 1-methylxanthine; cAMP, cyclic adenosine monophosphate; TYR, tyrosinase

Key words: α-MSH, arbutin, CRISPR/Cas9, IBMX, melanogenesis, melanoma, tyrosinase, vasculogenic mimicry

Introduction

Melanoma is malignant form of skin cancer, due to its high metastatic potential. BRAF mutants are well-known oncogenic drivers in malignant melanoma, and small molecules have been developed to target BRAF, including vemurafenib and dabrafenib (1-3). In recent years, monoclonal antibodies against programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte protein 4 (CTLA-4) have been administered to melanoma patients as immune checkpoint inhibitors (4,5). These drugs suppress the progression of melanoma, but melanoma-specific mechanisms of tumorigenesis are incompletely understood.

Melanocytes, the cells from which melanomas originate, express tyrosinase (TYR) to produce melanin. TYR is a type I membrane glycoprotein that catalyzes the hydroxylation of L-tyrosine and the oxidation of L-3,4-dihydroxyphenylalanine-the rate-limiting reactions in the synthesis of melanin (6,7). The reaction intermediates act as not only substrates for melanin synthesis but also promotors of melanogenesis (8). In melanocytic cells, melanin synthesis is regulated by secreted hormones. α-melanocyte-stimulating hormone (α-MSH), a representative melanogenesis-stimulating hormone, initially binds to melanocortin-1 receptor (MC1R) on the cell surface. MC1R is a seven-transmembrane G protein-coupled receptor and upregulates cAMP synthesis in an adenylyl cyclase-dependent manner (9,10). cAMP functions as a second messenger that activates protein kinase A (PKA), effecting the phosphorylation of cAMP response element-binding protein (CREB) (11). Phosphorylated CREB then upregulates the transcription of microphthalmia-associated transcription factor (MITF), resulting in the expression of several melanogenic genes, including *TYR* (12).

MITF-amplified melanoma is malignant, and melanin deposition is often considered to be a hallmark of malignant melanoma (13). However, melanogenesis is not essential for tumorigenesis in melanocytes because amelanotic melanomas exist. By contrast, Hendrix and colleagues have suggested that the expression of TYR is low in aggressive melanoma (14). Moreover, the loss of TYR correlates with poor survival in melanoma (15). Although these reports suggest that TYR suppresses the progression of melanoma, there is no direct evidence that TYR functions as a tumor suppressor.

Vasculogenic mimicry (VM) is one means by which blood is supplied for tumor growth. During VM, vascular-like networks are formed by tumor cells, instead of by vascular endothelial cells (16). VM was first described in uveal melanoma by Maniotis *et al* in 1999 (17) and has been observed in several aggressive cancers, such as breast, ovarian, prostate, and lung cancer and sarcoma (18-22). VM is associated with an extremely poor prognosis in melanoma patients (23-26) and thus is a crucial factor in aggressive melanoma. In addition, VM is linked to metastasis in tumor cells (27). Given that melanoma cells have high metastatic potential, certain melanoma-specific proteins might regulate the onset of VM and cell motility. Important regulators of VM, including VE-cadherin, have previously been identified (28), but the tissue-specific mediators of VM remain unknown.

In this study, we found that stimulators of melanogenesis inhibit VM in MNT-1 human melanoma cells. Because TYR is central to the melanin synthesis pathway, we focused on TYR and determined its effects on VM. We observed that TYR negatively regulates VM in human pigmented and amelanotic melanoma cell lines. Further, a loss-of-function TYR mutant did not downregulate the development of VM. Our findings constitute evidence that the enzymatic activity of TYR is crucial for the suppression of VM in melanomas.

Materials and methods

Cell culture. The MNT-1 human pigmented melanoma cell line, kindly gifted by Profs. Michael S. Marks (Children's Hospital of Philadelphia and University of Pennsylvania, PA) and Cheah Shiau Chuen (UCSI University, Kuala Lumpur, Malaysia), was cultured with Dulbecco's Modified Eagle Medium (Nissui Pharmaceutical, Tokyo, Japan) that was supplemented with 7% (v/v) FBS, 10% (v/v) AIM-V Medium liquid (Thermo Fisher Scientific, Inc. Waltham, MA), 100 U/ml penicillin G, 100 mg/l kanamycin, 2.25 g/l NaHCO$_3$, and 600 mg/l L-glutamine at 37°C in a humidified incubator with 5% CO$_2$. The SK-MEL-28 human amelanotic melanoma (RIKEN BioResource Center, Tsukuba, Japan), WM266-4 (American Type Culture Collection, Manassas, VA), 293T human embryonic kidney (RIKEN BioResource Research Center), and HT1080 human fibrosarcoma (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) cell lines were cultured in Dulbecco's Modified Eagle Medium that was supplemented with 7% (v/v) FBS, 100 U/ml penicillin G, 100 mg/l kanamycin, 2.25 g/l NaHCO$_3$, and 600 mg/l L-glutamine at 37°C in a humidified incubator with 5% CO$_2$.

Reagents. Arbutin (Merck KGaA, Darmstadt, Germany) and α-melanocyte-stimulating hormone (α-MSH; Peptide Institute, Inc., Osaka, Japan) were dissolved in sterilized water. 3-isobutyl-1-methylxanthine (IBMX; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO).

Melanin content assay. Cell pellets were dissolved with 10% (v/v) DMSO that contained 1 N NaOH at 70°C for 1 h. The melanin content was quantified by measuring the absorbance at 405 nm. Each absorbance value was normalized by the amount of total protein.

TYR activity assay. The enzymatic activity of cellular TYR was quantified using the Tyrosinase Activity Assay Kit (Abcam # ab252899; Cambridge, UK) according to the manufacturer's instructions.

VM assay. The *in vitro* VM assay was conducted as described (29-31). Initially, 96-well plates were coated with 40 µl/well of Matrigel® Growth Factor Reduced (Corning, Corning, NY) and incubated for 30 min at 37°C. Cells were suspended and added to the Matrigel-coated wells at 2.0×10^4 cells/well and cultured at 37°C in a humidified incubator with 5% CO$_2$. In each well, images of five independent, randomly selected fields were captured using phase-contrast microscopy (Leica DMi1, Leica, Wetzlar, Germany), and the number of tubes was counted. A tube was defined as an area that was surrounded by cells.

MTT assay. Cells were seeded at 2.0×10^3 cells/well in the presence of vehicle, IBMX, or α-MSH in 96-well plates and cultured for 48 h. Thiazolyl blue tetrazolium bromide was added to each well, after which the cells were cultured for 4 h at 37°C. The media was removed, and the MTT formazan product was dissolved in 100 µl DMSO. The absorbance at 570 nm was measured to quantify the number of living cells.

Establishment of TYR knockout cells. Knockout (KO) of TYR was performed using the CRISPR/Cas9 system as described (31,32). We used the D10A Cas9 mutant to avoid off-target effects (called the Nickase system). Thus, we designed 2 nearby targets in exon 1 of *TYR*; the sequences of the oligonucleotides for generating the guide RNAs were as follows: target 1, 5'-CACCGGGCTCTAGGGAAATGG CCAG-3' (forward) and 5'-AAACCTGGCCATTTCCCTAGA GCCC-3' (reverse); and target 2, 5'-CACCGTGTCTCCTCT AAGAACCTGA-3' (forward) and 5'-AAACTCAGGTTCTTA GAGGAGACAC-3' (reverse). Each pair of oligonucleotides was annealed and inserted into the *Bbs*I restriction site of pSpCas9n(BB)-2A-Puro (PX462) V2.0 (gifted by Feng Zhang, Addgene, Cambridge, MA). MNT-1 and SK-MEL-28 cells were cotransfected with these plasmids using Lipofectamine 3000™ (Thermo Fisher Scientific, Inc.) and then treated with 1.25 µg/ml puromycin dihydrochloride (Merck KGaA) to select transfectants. Clonal TYR-KO cells were established by limiting dilution method.

Construction of TYR expression vectors. TYR cDNA was amplified from the pcDNA4(TO)-tyrosinase plasmid (33), a kind gift of Prof. Takafumi Hasegawa (Tohoku University, Sendai, Japan), by polymerase chain reaction (PCR) using the following primers: 5'-TTTTCTCGAGATGCTCCTGGCTGT TTTGTACTGC-3' (forward) and 5'-TTTTGCGGCCGCTTA TAAATGGCTCTGATACAAGCTGTGG-3' (reverse). To avoid recognition by Cas9, we constructed Cas9-resistant *TYR* cDNA by overlap extension PCR with the following primers: 5'-GCG TGAGCAGCAAAAATCTCATGGAAAAGGAATGCTGTC CACCGTG-3' (forward) and 5'-AAGCCCGTGGAAAGTGTC CGGCGCTGGTCTGGAAACTCCACAGCAG-3' (reverse). The T373K point mutation was generated by overlap extension PCR with the following primers: 5'-AATGGAAAAATGTCC CAGGTACAGGGATCTG-3' (forward) and 5'-TTTGGATGA
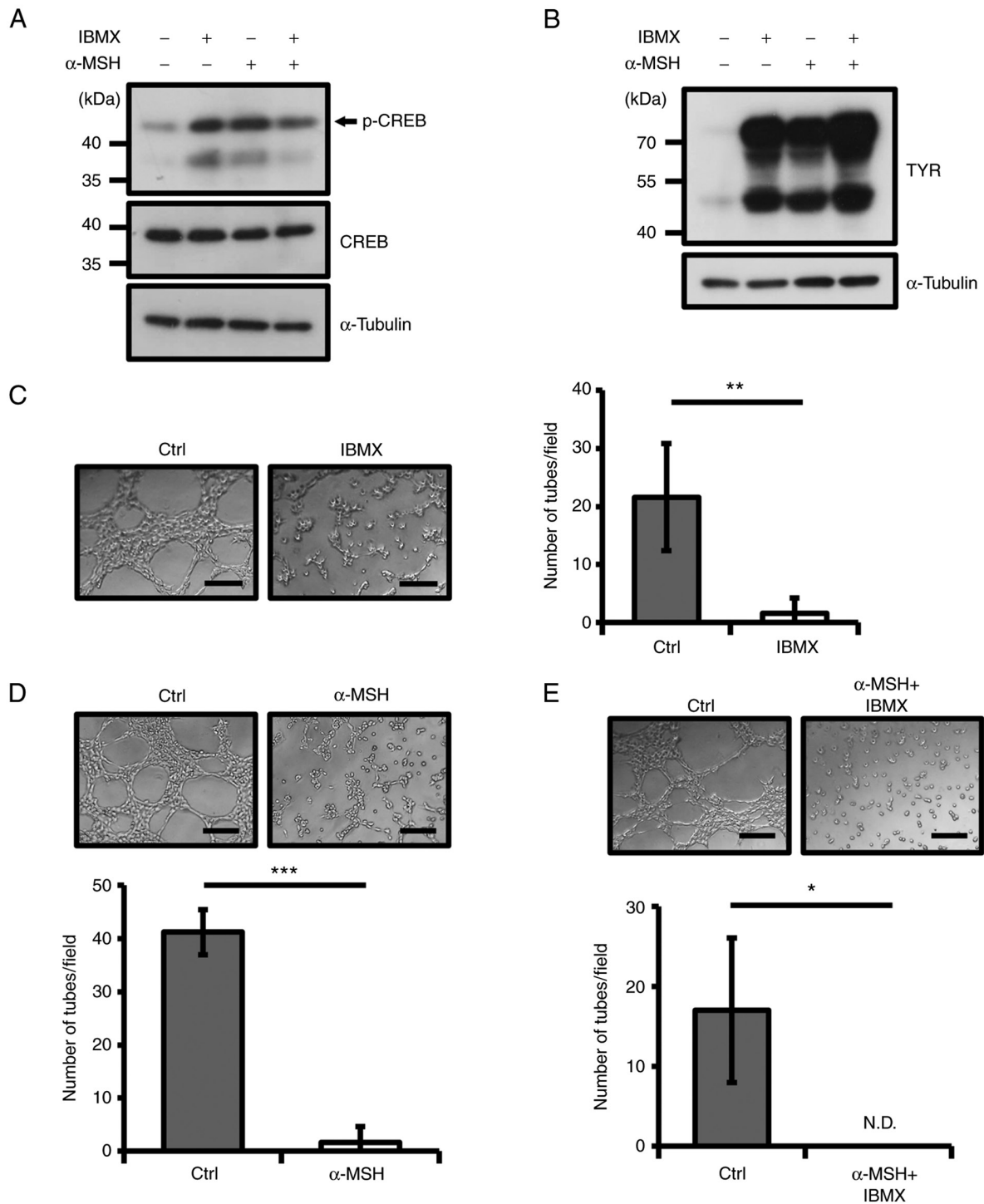
Figure 1. Stimulation with IBMX and α-MSH inhibits VM in MNT-1 cells. (A and B) MNT-1 cells were treated with 1 μM α-MSH, 100 μM IBMX or 1 μM α-MSH + 100 μM IBMX for (A) 30 min or (B) 48 h. The cells were lysed, and western blotting was performed. (C) MNT-1 cells were pretreated with vehicle or 100 μM IBMX for 48 h, and VM assay was performed. (left) Images of VM were captured 5 h after cell seeding; representative images are presented (scale bars, 200 μm). (right) VM was quantified by counting tube numbers. (D and E) MNT-1 cells were pretreated with vehicle, (D) 1 μM α-MSH or (E) 100 μM IBMX + 1 μM α-MSH for 48 h, and VM assay was performed. (upper) Images of VM were captured 5 h after cell seeding; representative images are presented (scale bars, 200 μm). (lower) VM was quantified by counting tube numbers. Data shown are the means ± SD (n=5). *P<0.05, **P<0.01, ***P<0.001. ND, not detected; p-, phosphorylated; Ctrl, control; IBMX, 3-Isobutyl 1-methylxanthine; a-MSH, α-melanocyte-stimulating hormone; VM, vasculogenic mimicry; TYR, tyrosinase.

AATAAAGAAATCACCATTTCTG-3' (reverse). The resulting amplicons were inserted into the *Xho*I/*Not*I restriction site of CSII-CMV-MCS-IRES2-Bsd (RIKEN BioResource Center). The CSII-CMV-MCS-IRES2-Bsd-GFP plasmid (34) was used as a control.

293T cells were transfected with these plasmids using Lentivirus High-Titer Packaging Mix (Takara Bio Inc.) and cultured for 6 h. The cells were washed with phosphate-buffered saline and cultured with fresh medium for 42 h to facilitate the production of virus particles. TYR-KO MNT-1 and WM266-4

cells were then treated with the lentivirus-containing conditioned media. After infection, cells were selected with 12.5 μg/ml blasticidin S (FUJIFILM Wako Pure Chemical Corporation).

Western blot. Western blot was performed as described (31,35). Cells were cultured and lysed in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 1 mM phenylmethyl-sulfonyl fluoride] with PhosSTOP phosphatase inhibitor cocktail (Merck KGaA) on ice with sonication. The lysate was centrifuged at 15,300 x g for 10 min, and the superna-tant was collected. The amount of protein in each cell lysate was measured by Coomassie Brilliant Blue G-250 staining (Bio-Rad Laboratories, Inc., Hercules, CA).

Loading buffer [350 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 0.012% (w/v) bromophenol blue, 6% (w/v) SDS, and 30% (v/v) 2-mercaptoethanol] was added to each lysate and boiled for 3 min. The samples were electrophoresed on 9% SDS-polyacrylamide gels, after which the proteins were transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk at room temperature for 30 min and immunoblotted with monoclonal anti-TYR (Abcam # ab170905), monoclonal anti-α-tubulin (Merck KGaA #T5168), monoclonal anti-CREB (Cell Signaling Technology #9197; Danvers, MA), and anti-phospho-CREB (Cell Signaling Technology #9191) at room temperature for 1 h. HRP-linked anti-rabbit IgG (Cytiva #NA934; Marlborough, MA) and HRP-linked anti-mouse IgG (Cytiva #NA931) were added to the membranes for 1 h at room temperature. Signals were detected by enhanced chemiluminescence using Western Lightning Plus-ECL (PerkinElmer, Inc., Waltham, MA) or Immobilon Western Chemiluminescent HRP substrate (Merck KGaA) and exposed to RX-U films (FUJIFILM, Tokyo, Japan) in a dark room.

Statistical analysis. Differences between 2 groups were analyzed by two-tailed student's t-test (unpaired). Datasets with 3 groups or over were analyzed using one-way ANOVA with Tukey's test using SPSS (version 27; IBM, Armonk, NY). The results were expressed as mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

Stimulation with IBMX and α-MSH inhibits VM in MNT-1 cells. To determine the significance of cAMP/CREB/TYR signaling in VM, we treated MNT-1 cells with the cAMP signaling activator IBMX and confirmed that IBMX induces phos-phorylation of CREB and upregulates TYR (Fig. 1A and B). IBMX also inhibited VM in MNT-1 cells (Fig. 1C). Further, α-MSH, a potent activator of cAMP signaling, increased CREB phosphorylation and TYR levels (Fig. 1A and B). Consistent with this result, α-MSH impeded VM in MNT-1 cells (Fig. 1D).

Cotreatment with IBMX and α-MSH enhanced the expres-sion of TYR and inhibited VM in MNT-1 cells (Fig. 1B and E). IBMX and α-MSH did not affect cell viability individually or in combination (Fig. S1), confirming that their suppressive activities on VM were not attributed to cell death. These data
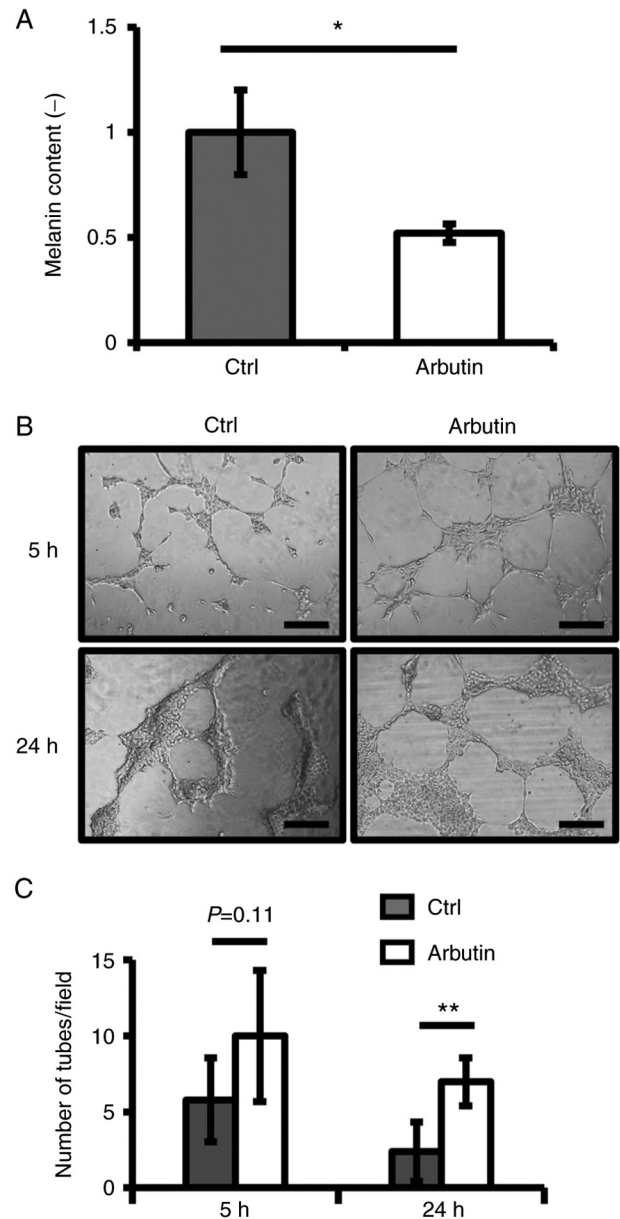

Figure 2. Arbutin promotes VM in MNT-1 cells. (A) MNT-1 cells were pretreated with vehicle or 500 μM arbutin for 48 h and a melanin content assay was performed. (B) MNT-1 cells were pretreated with vehicle or 500 μM arbutin for 48 h, and VM assay was performed. Images of VM were captured at the indicated hours (5 and 24 h) after cell seeding; representa-tive images of MNT-1 cells are presented (scale bar, 200 μm). (C) VM in MNT-1 cells was quantified by counting tube numbers. Data shown are the means ± SD (n=5). *P<0.05, **P<0.01. NS, not significant; Ctrl, control.

suggest that IBMX and α-MSH inhibit VM, consistent with the activation of the cAMP/CREB/TYR axis.

TYR inhibitor promotes VM in MNT-1 cells. Because α-MSH and IBMX upregulated TYR (Fig. 1B), we examined the function of TYR in VM. Arbutin is a well-known TYR inhibitor and has inhibitory effects on melanin synthesis (36), and we confirmed the reduction of melanin content in arbutin-treated MNT-1 cells (Fig. 2A). By contrast, arbutin promoted VM (Fig. 2B and C), prompting us to study the effects of arbutin on VM in non-melanoma cell lines. HT1080 is a TYR-non-expressing tumor cell line (Fig. S2A). By VM
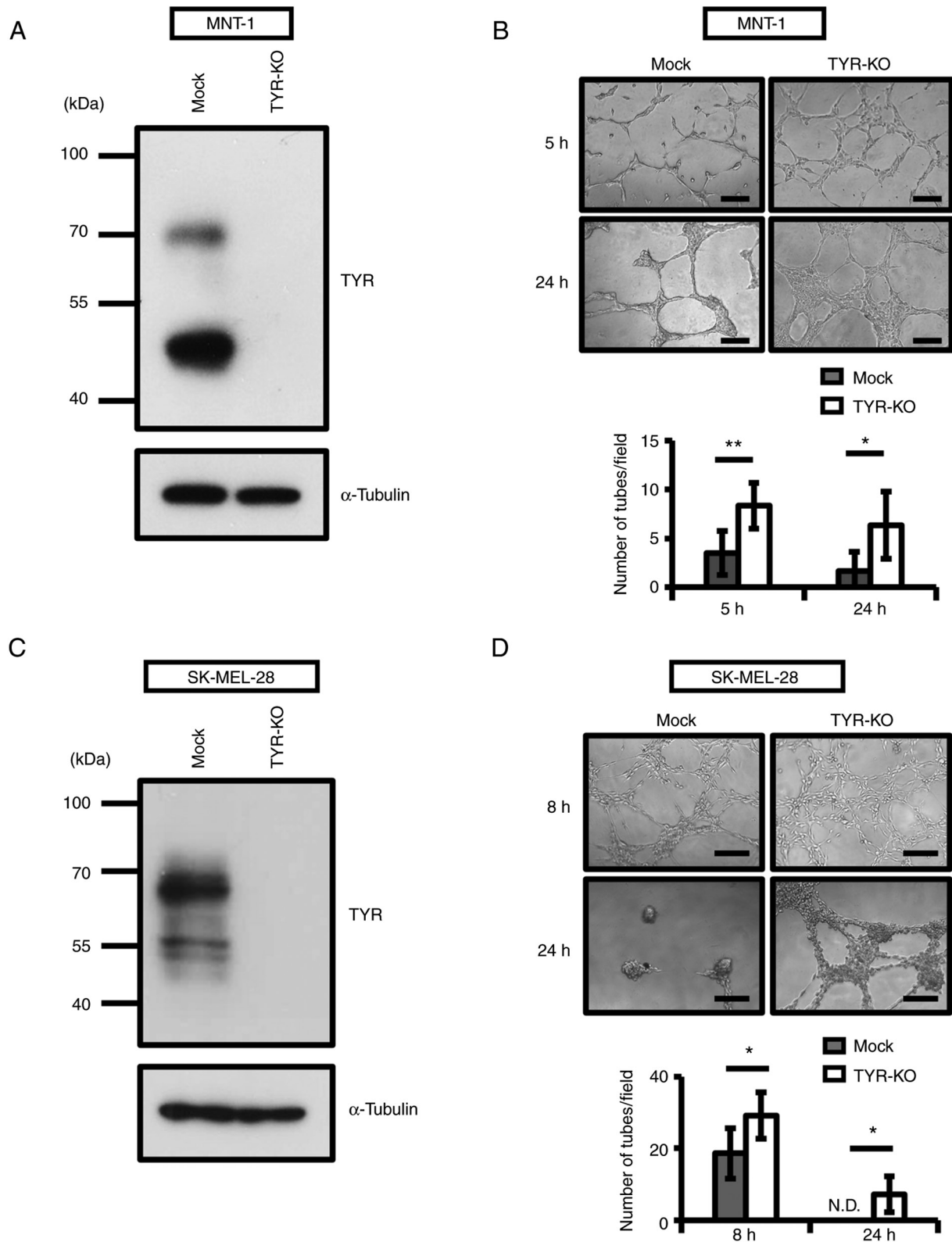
Figure 3. KO of TYR facilitates VM in MNT-1 and SK-MEL-28 cells. (A) TYR-KO MNT-1 cell lines were established using CRISPR/Cas9. (B) VM assay of mock and TYR-KO MNT-1 cells at the indicated times (scale bars, 200 µm). VM in MNT-1 cells was quantified by counting tube numbers. (C) TYR-KO SK-MEL-28 cell lines were established using CRISPR/Cas9. (D) VM assay of mock and TYR-KO SK-MEL-28 cells at the indicated times (scale bars, 200 µm). VM in SK-MEL-28 cells was quantified by counting tube numbers. Data shown are the means ± SD (n=5). *P<0.05, **P<0.01. KO, knockout; TYR, tyrosinase; ND, not detected; VM, vasculogenic mimicry.

assay, arbutin did not increase tube numbers in HT1080 cells (Fig. S2B), indicating that arbutin suppresses VM by inhibiting TYR in tumor cell lines.

TYR suppresses VM in melanoma cell lines. To verify the function of TYR in VM, we established a TYR-KO MNT-1 cell line using the CRISPR/Cas9 system (Fig. 3A). As expected, the
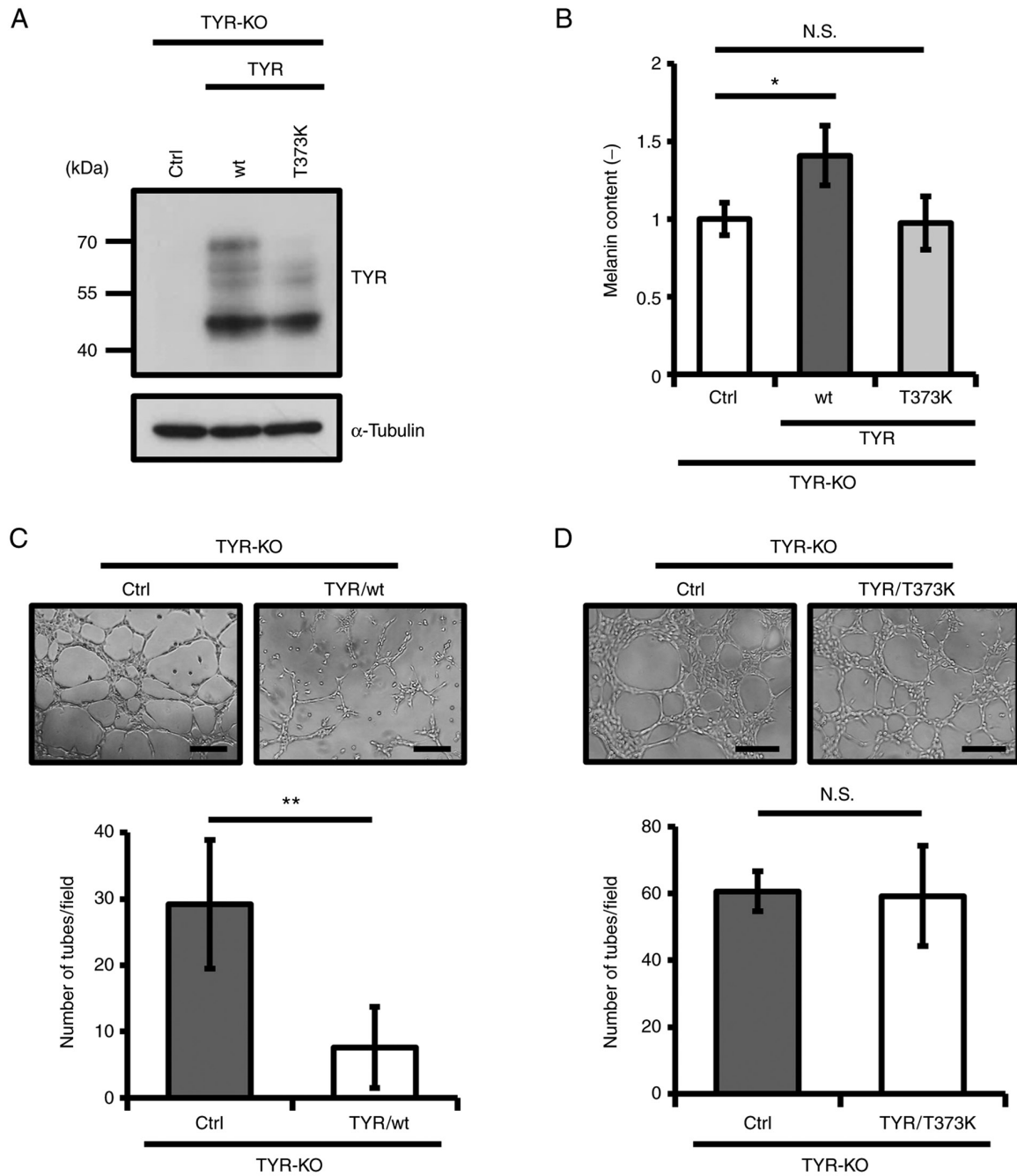
Figure 4. TYR regulates VM through its enzymatic activity. (A) Wt or T373K TYR was re-expressed in TYR-KO MNT-1 cells to establish stably TYR-re-expressing cell lines. Rescue of TYR was confirmed by western blotting. CSII-CMV-MCS-IRES2-Bsd-GFP vector was used as Ctrl. (B) Melanin content assay was performed in the indicated cells. VM assay in TYR-rescued MNT-1 cells; (upper) representative images of (C) TYR/wt-rescued and (D) TYR/T373K-rescued cells (scale bars, 200 μm). (lower) VM in (C) TYR/wt-rescued and (D) TYR/T373K-rescued cells were quantified by counting tube numbers. Data shown are the means ± SD (n=5). *P<0.05, **P<0.01. Wt, wild-type; TYR, tyrosinase; KO, knockout; NS, not significant.

enzymatic activity of TYR decreased significantly, and thus, melanin content was diminished in TYR-KO MNT-1 cells (Fig. S3A and SB). Consistent with the results after treatment with arbutin, VM was promoted in TYR-KO MNT-1 cells (Fig. 3B). Given that amelanotic melanomas also express TYR endogenously, we examined whether TYR has suppressive activity against VM even in amelanotic melanoma cells. To test this, we deleted *TYR* in SK-MEL-28 human amelanotic melanoma cells by CRISPR/Cas9 and confirmed its enzymatic activity (Figs. 3C and S4A). As shown in Fig. 3D, depletion of TYR promoted VM in SK-MEL-28, as well as pigmented

MNT-1 cells. We also confirmed that overexpression of TYR increases its enzymatic activity and attenuates VM in WM266-4 human amelanotic melanoma cells (Fig. S4B-SD). In addition, KO of TYR attenuated inhibitory effect of α-MSH on VM in MNT-1 cells (Figs. 1D and S5). These results demonstrate that TYR suppresses VM in pigmented and amelanotic melanoma cells.

Enzymatic activity of TYR is critical for TYR-mediated inhibition of VM. Human *TYR* often carries mutations, some of which cause albinism (37). The T373K mutation is frequently
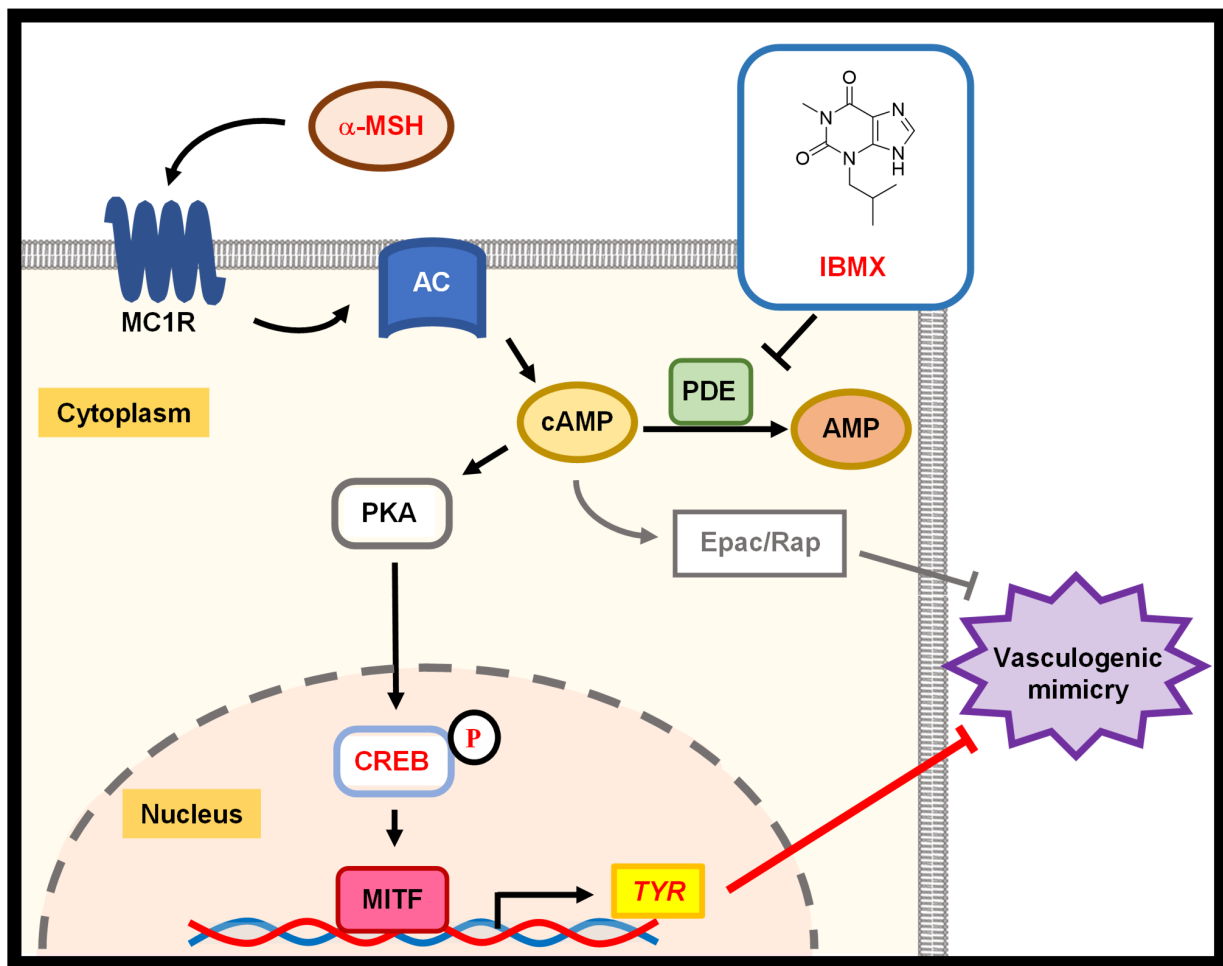
Figure 5. Schematic representation of cAMP/PKA/CREB signaling in VM formation. TYR is upregulated by CREB and suppresses VM formation in melanoma cells. α-MSH, α-melanocyte-stimulating hormone; AC, adenylyl cyclase; MC1R, melanocortin-1 receptor; PDE, phosphodiesterase; IBMX, 3-Isobutyl 1-methylxanthine; MITF, microphthalmia-associated transcription factor; TYR, tyrosinase; VM, vasculogenic mimicry.

observed in albinos, attenuating the enzymatic activity of TYR (38). Thus, we re-expressed wild-type (wt) or T373K TYR in TYR-KO MNT-1 cells to establish TYR-rescued MNT-1 cell lines (Fig. 4A). Whereas re-expression of wt TYR rescued its enzymatic activity and melanin production, re-expression of T373K TYR did not, as expected (Figs. 4B and S6). Notably, rescue with wt TYR decreased tube numbers, but T373K TYR did not affect VM in TYR-KO MNT-1 cells (Fig. 4C and D). These results suggest that the enzymatic activity of TYR is required for regulating VM.

Discussion

Advanced cancer is difficult to prevent using surgical and pharmaceutical approaches, necessitating the identification of clear hallmarks of aggressiveness in tumors to treat patients. In the past 2 decades, VM has garnered interest as an indicator of tumor malignancy (27,28), but the mechanisms by which it develops are poorly understood. Melanoma is an aggressive and metastatic tumor, and numerous reports have demonstrated that VM causes a poor prognosis in melanoma patients (23-26). In this study, we aimed to determine the melanoma-specific molecular mechanisms of VM.

Pigmentation is a unique property of melanomas. cAMP facilitates melanin synthesis through downstream signaling; thus, we treated MNT-1 human pigmented melanoma cells with IBMX and α-MSH, which enhance the activity of the cAMP/PKA axis (39). As a result, these compounds significantly inhibited VM, and the inhibition of TYR promoted it, indicating that the activation of TYR and the consequent synthesis of melanin correlate negatively with the potential for VM. However, TYR regulated VM even in SK-MEL-28 and WM266-4 human amelanotic melanoma cells. Thus, TYR itself might be a negative regulator of VM without melanin synthesis. It has been suggested that the enzymatic activity of TYR regulates some biological events (40,41). Our data reinforce this concept, because enzymatically inactive TYR did not affect VM. On the other hand, numerous reports have indicated that melanin production affects various cellular behaviors in normal and malignant melanocytes (42-45). Therefore, future work is warranted to determine whether the presence of melanin affects VM.

cAMP activates several signaling pathways and suppresses VM in melanoma cells through cAMP/Epac/Rap1 signaling (46). However, whether other pathways that are stimulated by cAMP affect VM is unknown (46,47). In the current study, we focused on the cAMP/PKA/CREB/TYR axis, because this pathway is an important cascade in melanogenesis.

Our results demonstrated that IBMX and α-MSH suppress VM with the upregulation of phosphorylated CREB and TYR in MNT-1 cells. α-MSH decreased tube numbers in TYR-KO MNT-1 cells, albeit to a lesser extent than in parental MNT-1 cells. Thus, TYR is critical for CREB-mediated regulation of VM (Fig. 5).

Epidemiological data suggest that melanoma-associated hypopigmentation after immunological therapy for metastatic melanoma correlates with an improved prognosis (48-50). Furthermore, inhibition of melanogenesis leads to favorable results in the treatment of melanoma (51-53). However, several reports indicate that depigmentation of melanoma constitutes a sign of tumor progression that accompanies greater metastasis (54-57). Because VM is closely related to the high metastatic potential of tumors, TYR expression might be a salient marker of the low potential for metastasis and VM in melanomas. Because antigen-specific T cells recognize TYR and are involved in tumor rejection (48,58), a loss of TYR might affect immune escape from CD8+ T cells (49). Further, TYR per se downregulates cell migration, cell survival, epithelial mesenchymal transition, and tumorigenesis in melanoma (54,59). Thus, the loss of TYR might allow melanoma cells to escape the immune system and tumor-suppressive activity, accelerating tumor progression.

We have unveiled a novel function for TYR-suppression of VM in human melanoma cells, independent of its melanogenic activity. Our findings provide new insights into melanoma-specific mechanisms of tumorigenesis, guiding the development of therapeutic approaches for melanoma patients in whom VM arises.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HK, RK and SS designed the study. HK performed all experiments and analyzed the data. HK and RK confirmed the authenticity of all the raw data. HK, RK and SS wrote the original draft. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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