Original Article

Effects of Prostamide/Prostaglandin F Synthase on Melanogenesis and Tumor Metastatic Potential of B16 Melanoma

Anzu Yamagishi[1], Kensuke Iwasa[2], Shinji Yamamoto[3], Chikara Haruta[4], Kei Maruyama[5], Keisuke Yoshikawa[6*]

[1-6]Department of Pharmacology, Faculty of Medicine, Saitama Medical University, 38 Moro-hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan.

ABSTRACT

Introduction: Prostaglandin (PG) $F_{2\alpha}$ and prostaglandin ethanolamide (prostamide, PM) $F_{2\alpha}$ are cyclooxygenase-catalyzed metabolites of arachidonic acid and arachidonyl etanolamide (anandamide), respectively. PM/Prostaglandin F synthase (PM/PGFS) catalyzes the conversion of PGH_2 to $PGF_{2\alpha}$ and PMH_2 to $PMF_{2\alpha}$. In this study, we established *PM/PGFS*-knock out (KO) B16 melanoma cells to analyze the role of PM/PGFS in melanogenesis and tumor metastatic potential.

Methods: *PM/PGFS*-KO B16 melanoma cells were generated by genome editing using CRISPR/Cas9 and cultured as single cells by limited dilution. We measured $PGF_{2\alpha}$ and melanin contents and assessed cell growth, *matrix metalloproteinase-2 (*MMP-2, type IV collagenase) levels, and intracellular signaling molecules such as CREB, AKT, JNK, and p38. Tumor metastasis in the lungs was evaluated by intravenous injection of B16 melanoma cells into mice.

Results: *PM/PGFS* deletion decreased $PGF_{2\alpha}$ production; however, it did not alter melanogenesis, cell growth, MMP-2 levels, intracellular signaling, and tumor metastasis.

Conclusion: PM/PGFS enzyme has no effects on melanogenesis, cell growth, and tumor metastasis in the B16 melanoma cells.

Keywords: CRISPR/Cas9, *matrix metalloproteinase-2,* melanin, PM/PGFS

Introduction

Prostaglandin (PG) $F_{2\alpha}$ is a product of cyclooxygenase-catalyzed metabolism of arachidonic acid. $PGF_{2\alpha}$ induces luteolysis during the estrous cycle, plays essential roles in parturition with its strong uterine contractile actions, and has vasoconstriction and bronchoconstriction activities[1]. Prostaglandin ethanolamide (prostamide, PM) $F_{2\alpha}$ is a cyclooxygenase-2-catalyzed metabolite of arachidonyl etanolamide (anandamide) that exerts biological activities, such as pain transmission[2] and adipogenesis[3]. The enzyme prostamide/prostaglandin F synthase (PM/PGFS) catalyzes the conversion of PGH_2 to $PGF_{2\alpha}$ and PMH_2 to $PMF_{2\alpha}$, and is mainly expressed in the central nervous system[4]. We have previously reported that PM/PGFS is constitutively expressed in the myelin sheaths and cultured oligodendrocytes[5]. PM/PGFS has been reported to regulate intraocular pressure[6].

B16 melanoma cells are frequently used as tumor models to investigate the mechanism of metastasis. A murine model of experimental metastasis in lung can be obtained by

Access this article online

Quick Response Code:

Website: www.pnrjournal.com

DOI: 10.47750/pnr.2022.13.03.012

Address for correspondence: Keisuke Yoshikawa, Department of Pharmacology, Faculty of Medicine, Saitama Medical University, 38 Moro-hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan.

Phone: +81-49-276-1157

E-mail address: keisukey@saitama-med.ac.jp

Received: 14 June, 2022 **Accepted:** 23 July, 2022

Published: 17 August 2022

How to cite this article: Yamagishi A, Iwasa K, Yamamoto S, Haruta C, Maruyama K, Yoshikawa K. Effects of Prostamide/Prostaglandin F Synthase on Melanogenesis and Tumor Metastatic Potential of B16 Melanoma. J Pharm Negative Results 2022;13(3):79-84

intravenous injection of B16 melanoma cells[7]. These cells produce melanin and are used to study melanogenesis[8].

$PGF_{2\alpha}$ is a key regulator of matrix metalloproteinase (MMP)-2 expression[9,10]. MMP-2 is a type IV collagenase, which plays major role in degrading the extracellular matrix during cancer invasion and metastasis[11,12]. $PGF_{2\alpha}$ enhances migration and invasion of B16 melanoma cells *in vitro* Matrigel assay[13]. Therefore, $PGF_{2\alpha}$ might increase tumor metastasis through the enhancement of MMP-2 expression. In this study, we aimed to analyze the role of PM/PGFS in tumor metastasis and melanogenesis using *PM/PGFS*-knock out (KO) B16 melanoma cells.

Materials and Methods

Cell Culture

B16 mouse melanoma cells (JCRB0202) were obtained from the RIKEN Cell Bank (Osaka, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10 % (v/v) fetal bovine serum and 1 % penicillin–streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere with 5 % CO_2 at 37 °C.

Generation of *PM/PGFS-KO B16* Melanoma Cells

Experimental protocols were approved by DNA experiment Safety Committee of Saitama Medical University. *PM/PGFS*-KO B16 melanoma cells were generated using a Guide-it CRISPR/Cas9 system (Takara Bio Inc., Shiga, Japan). *PM/PGFS*-specific gRNAs (No. 1 Forward: 5'-CCGGGTGGTGGACCTTGGTCGCGT-3' and Reverse: 5'-AAACACGCGACCAAGGTCCACCAC-3'; PAM sequence; AGG, No. 2 Forward: 5'-CCGGCACACGCTC CTACGCGACCA-3'andReverse:5'-AAACTGGTCGCGTA GGAGCGTGTG-3'; PAM sequence; AGG) were designed using CRISPR direct[14] and synthetic oligos were ligated into Guide-it-ZsGreen1 vector. The vectors were transfected into B16 melanoma cells with Lipofectamine (Invitrogen). B16 melanoma cells expressing ZsGreen were selected and cultured as single cells by limited dilution. A Guide-it Genotype confirmation kit (Takara Bio Inc.) was used to determine the homozygous mutants. In-del detection and cloning of targeting site were performed using a Guide-it Indel Identification kit (Takara Bio Inc.). The colonies for KO cells were identified by the changes in DNA sequences.

RNA Extraction and Reverse transcription (RT)-PCR

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Total RNA was reverse-transcribed using a PrimeScript RT reagent kit (Takara Bio Inc.). The following primer sequences were used for RT-PCR: *PM/PGFS* (Forward: 5'- ATGAATGTGGTGGACCTTGG -3' and Reverse: 5'- AACCACCATCCAGAAACTCCT -3').

Enzyme-linked Immunosorbent Assay (ELISA)

B16 melanoma cells were plated (5×10^5 cells/well) in 6-well plates and incubated for 24 h. The cells were then incubated with 10 μM arachidonic acid (MP Biom, #150384, Solon, OH). After 24 h, the medium was replaced with phosphate-buffered saline (PBS) containing 3 μM of a Ca^{2+} ionophore, calcimycin (ab120287, Abcam, Cambridge, MA). After 3 h, the culture supernatant was collected by centrifugation, snap-frozen, and stored at -80 °C. The $PGF_{2\alpha}$ concentration was measured using an EIA kit (Cayman Chemicals, Ann Arbor, MI).

Liquid Chromatography-tandem Mass Spectrometry Analysis of Prostamides

Prostamides were purified by solid phase extraction with Oasis HLB columns (Waters Corporation, Milford, MA). The methods for purification and liquid chromatography were same as previously described[15]. Briefly, prostamides were extracted from cells or supernatants with 1 ng of internal standards ($PGF_{2\alpha}$-EA-d_4, PGE_2-d_4; Cayman chemicals). Prostamides were separated on a Waters X-Bridge C18 (3.5 μm, 150 mm × 1.0 mm i.d., Waters Corporation) using with a high-performance liquid chromatography system (Nexera LC-30AD, Shimadzu Corporation, Kyoto, Japan) and analyzed using a triple quadrupole mass spectrometer (LCMS-8040; Shimadzu Corporation). Prostamides were identified by multiple reaction monitoring in negative ion mode. Data were analyzed using LabSolutions software (Shimadzu Corporation).

Cell Growth Assay

Cell growth was determined using the microculture tetrazolium technique (MTT). MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolically active cells. Cells (1×10^5 cells/well) in 24-well plates were treated and 50 μl of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazolium bromide] (FUJIFILM, Osaka, Japan) (5 mg/ml in PBS) was added to each well. The plates were incubated in a humidified atmosphere of 5 % of CO_2 at 37 °C for 4 h. After removing the medium, formazan crystals were dissolved in 200 μl isopropanol /HCl (100 : 0.34), and the absorbance was measured using a micro plate reader (Bio-Tek, Redmond, WA) at 570 nm relative to 630 nm.

Determination of Melanin Content

B16 melanoma cells (1 × 105 cells/well) were seeded in 24-well plates and incubated for 24 h. The medium was then replaced with 499 ml of fresh medium and 1 ml of DMSO containing a-Melanocyte Stimulating Hormone (MSH, LKT Laboratories, St Paul, MN), forskolin (20 mM, Cayman Chemicals), and phorbol-12-myristate-13-acetate (PMA, 100 mM, Sigma-Aldrich, Deisen-hofen, Germany) was added. The control cells were treated with DMSO only. Cells were incubated for 48 h and the medium was replaced with

fresh medium containing a-MSH, forskolin, and PMA. After 48 h, the remaining adherent cells were assayed for melanin content. Therefore, the cells were continuously exposed to the reagents for four days. After removing the medium and washing cells with PBS, cell pellet was dissolved in 1 ml of 1 N NaOH. The crude cell extracts were assayed using a microplate reader (Bio-Tek) at 415 nm to determine melanin contents. Results were calculated as percentage with respect to the melanin content of the control cells.

Western Blot Analysis

Cells were homogenized on ice using radioimmunoprecipitation assay RIPA buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 0.1 % SDS, 0.5 % DOC] containing a protease inhibitor cocktail (Calbiochem, San Diego, CA) (1 : 1000 dilution) with a tissue homogenizer (Brinkmann Instruments, Westbury, NY). Protein concentrations were determined using a BCA protein assay kit (Nacalai Tesque, Tokyo, Japan). Proteins (10 μg /lane) in lysates were separated by 12 % SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Redmond, WA). After blocking with 5 % skim milk (MEGMILK SNOW BRAND Co Ltd, Tokyo, Japan) in PBS containing 0.05 % Tween 20 (polyoxyethylene sorbitan monolaurate, Nacalai Tesque) (PBS-T), the membranes were incubated with primary antibodies overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) and then washing thrice with PBS-T. The membranes were then incubated with chemiluminescence reagent (Chemi-Lumi One Super, Nacalai Tesque; ImmunoStar LD, FUJIFILM). Images of the membranes were captured using a C-DiGit blot scanner (LI-COR, Lincoln, NE) and subjected to ImageJ analysis. Each membrane was probed with anti-GAPDH antibody (1 : 1000, ABS16, Millipore, Billerica, MA), and the bands were used as loading controls. A pre-stained molecular weight marker was used to confirm expected sizes of the target proteins. The primary antibodies used were anti-cAMP response element binding protein (CREB, 1 : 1000, ab32515) and anti-p-CREB (1 : 1000, ab9674) purchased from Abcam; and anti-AKT (1 : 1000, #587F11), anti-p-AKT (1 : 1000, #9271S), anti-Jun amino terminal kinase (JNK, 1 : 1000, #9252) dilution, anti-p-JNK (1 : 2000, #9255S), anti-p38 (1 : 1000, #9212), and anti-p-p38 (1 : 2000, #28B10) purchased from Cell Signaling Technology.

In vivo Tumor Study

The mice were housed in appropriate animal care facilities at Saitama Medical University, and were handled according to established international guidelines. Experimental protocols were approved by the Animal Research Committee of Saitama Medical University. B16 melanoma and KO cells (1×10^6) suspended in PBS were intravenously injected into the tail veins of 10-week-old male C57BL/6J mice (Tokyo Laboratory Animal Science, Tokyo, Japan). Two weeks after the intravenous injection, the mice were euthanized, and the lungs were removed. After formalin fixation, photographs of the lungs were captured and the spherical colonies of black melanoma were counted. The lungs were weighed and the proportion of lung tumors in the lung tissues were calculated.

Statistics

Multiple comparisons were performed by one-way ANOVA followed by Newman-Keuls post-hoc test or two-way ANOVA followed by post-hoc Tukey test. All data were analyzed using Graph Pad Prism Ver. 5.01 (Graph Pad Software, Inc., San Diego, CA) and expressed as mean ± SEM. p values < 0.05 were considered statistically significant.

Results

PM/PGFS-KO B16 melanoma cells were generated using CRISPER/Cas9 system with two gRNAs, and single cells were cloned. Cloning of targeting site suggests that KO cells were homozygous mutants. Sequencing showed the presence of a 10-base deficiency accompanying a frame shift in two strains (Figure 1). The amino acid changes from position 8 occurred and premature termination, i.e. a stop codon was contained in the middle of the amino acid sequence in KO1 and KO2 (Figure 1). We tested the mRNA expression of *PM/PGFS* using RT-PCR with primers specific to the deleted region. Amplification product of *PM/PGFS* was observed in wild-type (WT), but not in KO1 and KO2 cells (Figure 1). Additionally, western blot detected PM/PGFS expression in WT, but not in KO1 and KO2 cells (Figure 1). These data confirmed that KO cells were successfully generated.

We evaluated $PGF_{2\alpha}$ production from these cells to investigate the effect of *PM/PGFS* deletion. $PGF_{2\alpha}$ production was decreased in KO1 and KO2 cells compared to that in WT cells (Figure 2). $PMF_{2\alpha}$ production was under the detection limit of mass spectrometry analysis (data not shown). $PMF_{2\alpha}$ production was less than 4 pg per well (6-well plates). We then measured the melanin contents produced by these cells. *PM/PGFS* deletion did not significantly change the melanogenic activity of B16 melanoma cells unstimulated

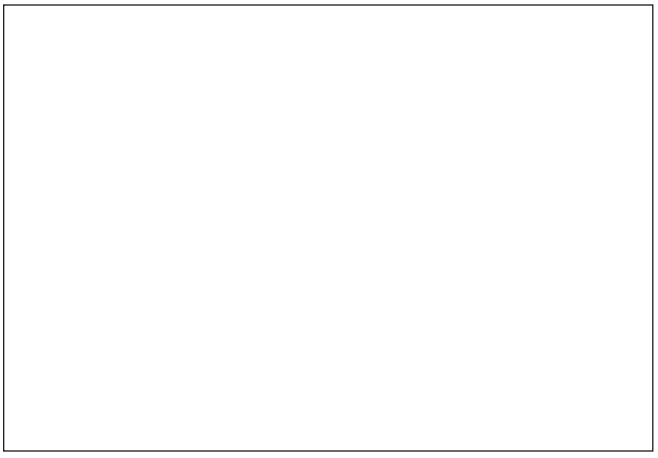

Fig. 1: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

or stimulated with α-MSH, forskolin, and PMA (Figure 3). We investigated the effect of *PM/PGFS* deletion on cell growth by assessing the number of viable cells using MTT assay. *PM/PGFS* deletion did not alter the cell growth of B16 melanoma cells (Figure 4). Moreover, *PM/PGFS* deletion did not change MMP-2 expression (Figure 5). Intracellular signaling molecules, such as CREB, AKT, JNK, and p38, regulate cell proliferation. The expression of CREB, AKT, JNK, and p38, and their phosphorylated forms were unaltered by *PM/PGFS* deletion (Figure 5).

The tumor-bearing mouse model was obtained by artificial metastasis in the lung through intravenous injection of B16 melanoma cells[7]. Pulmonary metastasis was established by injecting WT and KO cells into the tail veins of mice (Figure 6). The weights of the lungs did not change in the injected mice (Figure 6). Moreover, treatment with the KO cells did not change the extent of lung metastasis and melanotic area (Figure 6).

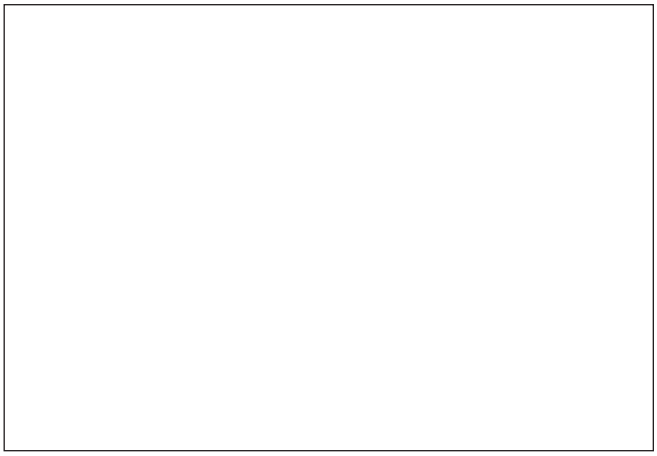

Fig. 2: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Discussion

We generated *PM/PGFS*-KO B16 melanoma cells using CRISPR/Cas9 system to investigate the role of *PM/PGFS* in melanogenesis and tumor metastasis. $PGF_{2\alpha}$ production was decreased in the KO cells. However, *PM/PGFS* deletion did not alter melanogenic activity, cell growth, MMP-2 expression, and intracellular signaling in B16 melanoma cells, and tumor metastasis in the tumor-bearing mice injected with the KO cells.

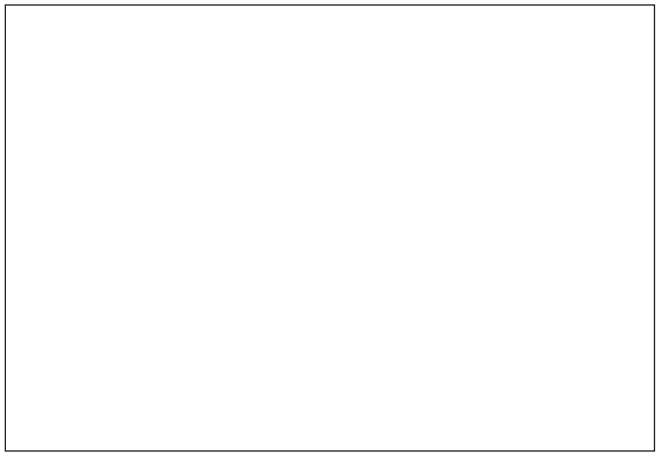

Fig. 3: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

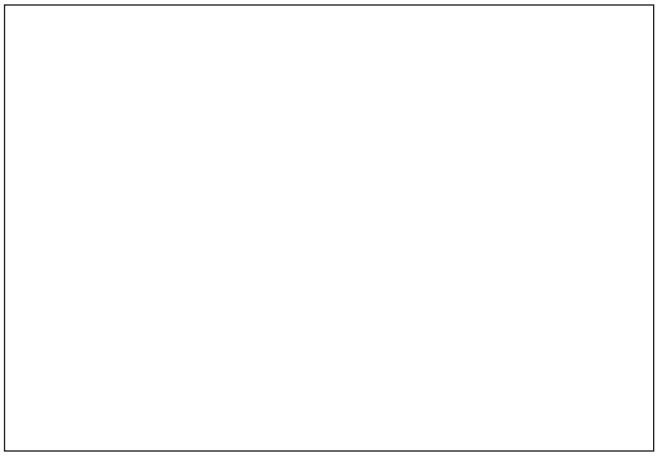

Fig. 4: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Fig. 5: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Fig. 6: xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

PM/PGFS deletion caused premature termination of its protein synthesis and undetectable expression of PM/PGFS at the mRNA and protein levels in KO B16 melanoma cells. Moreover, *PM/PGFS* deletion caused low $PGF_{2\alpha}$ production from B16 melanoma cells. These results suggested that *PM/PGFS*-KO B16 melanoma cells were successfully generated. On the other hand, $PMF_{2\alpha}$ production was not observed in WT and *PM/PGFS*-KO B16 melanoma cells. B16 melanoma cells probably had low potential to produce series of PMs, because not only $PMF_{2\alpha}$, PME_2 and PMD_2 also were not detected by mass spectrometry (data not shown). Therefore, B16 melanoma cells may contain small amount of anandamide, a substrate for PM/PGFS.

α-MSH, forskolin, and PMA are potent activators of melanogenesis[16,17]. *PM/PGFS* deletion did not affect melanogenesis in the presence or absence of these activators, suggesting that PM/PGFS does not affect melanogenesis in B16 melanoma cells.

PM/PGFS deletion had no effect on the growth and tumor metastatic potential of B16 melanoma cells. Therefore, $PGF_{2\alpha}$ produced by PM/PGFS did not affect cell growth and tumor metastasis. This is contrasting to previous evidences that $PGF_{2\alpha}$ upregulates MMP-2 activity and enhances invasion of B16 melanoma cells[9,10,13]. A comparatively higher dose of $PGF_{2\alpha}$ (1 μg/ml) was exogenously used in previous studies[10,13], which might be responsible for MMP-2 activation. In contrast, approximately 100 pg/ml of $PGF_{2\alpha}$ produced by the cells in our study was probably insufficient to activate MMP-2. Intracellular signaling molecules such as CREB, AKT, JNK, and p38 are important regulators of activities in B16 melanoma cells[18-20]. *PM/PGFS* deletion did not change cellular events in these cells probably owing to unaffected intracellular signaling pathways. Taken together, our results suggest that PM/PGFS has no effects on melanogenesis, cell growth, and tumor metastatic potential of B16 melanoma cells.

Acknowledgements

We greatly appreciate the Mass spectrometry analysis of prostamide $F_{2\alpha}$ from Drs. H Nakanishi and T Ohto (LIPIDOME LAB Co., Ltd, Akita, Japan). The authors thank the Division of Laboratory Animal Medicine, Biomedical Research Center, Saitama Medical University, for maintaining the mice.

We greatly appreciate the valuable comments and suggestions from Drs. K Watanabe and H Moriuchi. We thank Messrs. T Ogimi, K Tosaki, and H Higashi (Saitama Medical University Faculty of Health and Medical) for research support.

References

1. Pate JL, Johnson-Larson CJ, Ottobre JS. Life or death decisions in the corpus luteum. *Reproduction in Domestic Animal*. 2012;47 Suppl 4:297-303
2. Gatta L, Piscitelli F, Giordano C, Boccella S, Lichtman A, Maione S, et al. Discovery of prostamide $F_{2\alpha}$ and its role in inflammatory pain and dorsal horn nociceptive neuron hyperexcitability. *PloS one*. 2012;7:e31111
3. Silvestri C, Martella A, Poloso NJ, Piscitelli F, Capasso R, Izzo A, et al. Anandamide-derived prostamide $F_{2\alpha}$ negatively regulates adipogenesis. *The Journal of Biological Chemistry*. 2013;288:23307-23321
4. Moriuchi H, Koda N, Okuda-Ashitaka E, Daiyasu H, Ogasawara K, Toh H, et al. Molecular characterization of a novel type of prostamide/prostaglandin F synthase, belonging to the thioredoxin-like superfamily. *The Journal of Biological Chemistry*. 2008;283:792-801
5. Yoshikawa K, Takei S, Hasegawa-Ishii S, Chiba Y, Furukawa A, Kawamura N, et al. Preferential localization of prostamide/prostaglandin F synthase in myelin sheaths of the central nervous system. *Brain Research*. 2011;1367:22-32
6. Bertrand JA, Woodward DF, Sherwood JM, Spenlehauer A, Silvestri C, Piscitelli F, et al. Deletion of the gene encoding prostamide/prostaglandin F synthase reveals an important role in regulating intraocular pressure. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*. 2021;165:102235
7. Giavazzi R, Decio A. Syngeneic murine metastasis models: B16 melanoma. *Methods in Molecular Biology*. 2014;1070:131-140
8. Solano F, Briganti S, Picardo M, Ghanem G. Hypopigmenting agents: An updated review on biological, chemical and clinical aspects. *Pigment Cell Research*. 2006;19:550-571
9. Weinreb RN, Kashiwagi K, Kashiwagi F, Tsukahara S, Lindsey JD. Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells. *Investigative Ophthalmology & Visual Science*. 1997;38:2772-2780
10. Reich R, Martin GR. Identification of arachidonic acid pathways required for the invasive and metastatic activity of malignant tumor cells. *Prostaglandins*. 1996;51:1-17
11. Jezierska A, Motyl T. Matrix metalloproteinase-2 involvement in breast cancer progression: a mini-review. *Medical Science Monitor.* 2009;15:Ra32-40
12. Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Experimental Lung Research*. 2005;31:599-621
13. Tsai CS, Luo SF, Ning CC, Lin CL, Jiang MC, Liao CF. Acetylsalicylic acid regulates MMP-2 activity and inhibits colorectal invasion of murine B16F0 melanoma cells in C57BL/6J mice: effects of prostaglandin F(2)alpha. *Biomedicine & Pharmacotherapy*. 2009;63:522-527
14. Naito Y, Hino K, Bono H, Ui-Tei K. CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics (Oxford, England)*. 2015;31:1120-1123
15. Hijioka M, Futokoro R, Ohto-Nakanishi T, Nakanishi H, Katsuki H, Kitamura Y. Microglia-released leukotriene B(4) promotes neutrophil infiltration and microglial activation following intracerebral hemorrhage. *International Immunopharmacology*. 2020;85:106678
16. Flori E, Rosati E, Cardinali G, Kovacs D, Bellei B, Picardo M, et al. The α-melanocyte stimulating hormone/peroxisome proliferator activated receptor-γ pathway down-regulates proliferation in melanoma cell lines. *Journal of Experimental & Clinical Cancer Research : CR*. 2017;36:142

17. Zhao X, Geltinger C, Kishikawa S, Ohshima K, Murata T, Nomura N, et al. Treatment of mouse melanoma cells with phorbol 12-myristate 13-acetate counteracts mannosylerythritol lipid-induced growth arrest and apoptosis. *Cytotechnology*. 2000;33:123-130

18. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiological Reviews*. 2004;84:1155-1228

19. Sabbah M, Krayem M, Najem A, Sales F. Dasatinib Stimulates Its Own Mechanism of Resistance by Activating a CRTC3/MITF/Bcl-2 Pathway in Melanoma with Mutant or Amplified c-Kit. *Molecular Cancer Research*. 2021;19:1221-1233

20. Wang D, Xu W, Chen X, Han J, Yu L, Gao C, et al. Icariin induces cell differentiation and cell cycle arrest in mouse melanoma B16 cells via Erk1/2-p38-JNK-dependent pathway. *Oncotarget*. 2017;8:99504-99513