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NOTE

Pharmacology

Resveratrol can induce differentiating phenotypes in canine oral mucosal melanoma cells

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ABSTRACT. This report described the differentiation induction of canine oral mucosal melanoma (OMM) cells by resveratrol. Exposure of canine OMM cells to resveratrol (maximum dose: 50 μM and treatment period: 72 hr) induced differentiating features like melanocytes, and enhanced chemosensitivity against cisplatin, but alone had no influence on cell viability. Additionally, resveratrol significantly enhanced mRNA expression of key melanoma differentiation markers such as microphthalmia-associated transcription factor (MITF). Of several inhibitors against mitogen-activated protein kinase subtypes, only the c-Jun N-terminal kinase (JNK) inhibitor, SP600125, induced melanocyte-like morphological change and enhanced MITF mRNA expression. Furthermore, resveratrol also suppressed JNK activation in OMM cells by approximately 33%. Overall, these findings suggest that resveratrol induces differentiation in canine OMM cells, due to the inhibition of JNK signaling.

KEYWORDS: canine oral mucosal melanoma cell, cell viability, c-Jun N-terminal kinase, microphthalmia-associated transcription factor, resveratrol

Mucosal melanoma (MM) is an aggressive subtype of melanoma originating from melanocytes in the mucosal membranes [5, 11, 13]. MM is a rare entity in humans, by contrast MM is common in canines and the majority of malignancies occur most often in the oral cavity, causing significant mortality in domestic dogs [5, 11]. Human and canine OMM share multiple clinical and biological similarities [13], both are reported to have severe resistance to various chemotherapies and poorer prognosis than other melanoma subtypes [13], with median survival times of canines with advanced OMM being less than 5 months following surgery [11, 13] and no significant increase to survival rate even with multimodal therapy including surgery, radiation, and chemotherapy [2, 12].

Melanoma cell phenotype switching has been identified as an important event that drive metastasis and drug resistance [8]. Melanoma cells can reversibly change between two major phenotypes that co-exist in bulk tumor tissues, "proliferative, differentiated, and often therapy-sensitive" and "invasive, dedifferentiated, and often therapy-resistant" [7, 8, 14]. Accordingly, the dedifferentiation of melanoma has been demonstrated as a major mechanism for the acquired therapy resistance [7, 14]. Hence, it is anticipated that either by forced differentiation or inhibition of transitions from the proliferative to invasive phenotype could represent a potential development in future therapeutic approaches against melanoma.

Resveratrol, a naturally occurring polyphenol, has diverse biological activities and capacity to target many signaling pathways involved in cancer malignancy [6]. Moreover, resveratrol has been shown to induce phenotypic differentiation changes in human melanoma, and canine bone marrow mesenchymal stem cells [18, 19], but the effects on canine OMM have not been reported.

In this context, this study was undertaken to investigate if resveratrol could induce differentiation in canine oral melanoma cells, leading to attenuation of chemoresistance in the cells.

All cultures and chemicals were obtained from Nacalai tesque (Kyoto, Japan), unless otherwise indicated. SB 203580 and SP600125 were obtained from ChemScene LLC (Monmouth Junction, NJ, USA). Cisplatin was sourced from Tokyo Chemical Industry (Tokyo, Japan). All antibodies for western blotting were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The TLM-1 canine oral melanoma cell line originating from a canine (Gordon setter) oral melanoma was obtained from Kerafast (Boston, MA,

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USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) and 0.5% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

To determine cell viability, a WST-8 assay was carried out. The cells were seeded in a 96-well plate (5×10^3 cells/well), cultured for 24 hr, and subsequently treated with each reagent (treatment periods, doses and other conditions are indicated in their respective figure legends). After each treatment, 10 µL of WST-8 solution was applied to each well containing 100 µL of cell suspension and incubated for a further 30 min at 37°C in 5% CO_2. Color development was monitored at 450 nm using a multi-well plate reader (SUNRISE Rainbow RC-R, Tecan Japan, Kawasaki, Japan).

To check cell features, crystal violet (CV) staining was carried out. The cells were seeded in a 24-well plate (5×10^4 cells/well), cultured for 24 hr, and subsequently treated with each reagent according to treatment conditions described in their respective figure legends. After the treatment, the culture supernatant was discarded, and 300 µL of 4% paraformaldehyde solution was applied to each well and fixed for 15 min. After the fixed cells were washed three times with phosphate-buffered saline, 30 µL of 4% CV solution was added to each well. CV staining was carried out at 37°C in a humidified atmosphere with 5% CO_2 for 12 hr, and photos were taken of the cells stained with CV at 40X magnification, unless otherwise indicated.

Total RNA was isolated from the cells using the FavorPrep Tissue Total RNA Extraction Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). Total RNA (300 ng for each sample) was used for cDNA synthesis using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). cDNA templates were analyzed by real-time PCR using the Thermal Cycler Dice Real Time System Lite (TAKARA BIO INC., Kusatsu, Japan) and THUNDER-BIRD™ SYBR qPCR Mix (Toyobo, Osaka, Japan), with the following program: 10 sec at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Primer sets (Nihon Gene Research Laboratories, Sendai, Japan) are shown as follows. MITF, 5′-GGGATTGATGGATCCTGCTTTG-3′(forward), 5′-GGCTGGACAGGAGTTGCTGA-3′(reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-AAGGCTGAGAACGGG AAACT-3′ (forward), 5′-GGAGGCATTGCTGACAATCT-3′ (reverse); tyrosinase, 5′- GATGAGTACATGGGAGGGCG-3′(forward), 5′-CTGCTTTCTATTGCGCCGAC-3′(reverse); glycoprotein 100 (GP100), 5′- CCTTCTCCGTGAGTGTGTCT-3′(forward), 5′-GAGATCAGGGTCCCGGTATG-3′(reverse). Gene expression data were normalized to the expression of the reference gene GAPDH.

Each protein level in each sample was determined by western blotting as follow. The cells were harvested and lysed in ice-cold Laemmli sample buffer (Bio-Rad, Berkeley, CA, USA) containing protease inhibitor cocktail and phosphatase inhibitor. The cells were incubated on ice for 20 min following centrifugation at 12,000 rpm at 4°C for 10 min. The samples were electrophoresed through a 12% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were blocked with Blocking One P for 1 hr, incubated with primary antibodies for 1 hr, and then incubated with the secondary antibody for 1 hr. Detection was accomplished using Chemi-Lumi One Super and C-DiGit (LI-COR, Lincoln, NE, USA). A densiometric analysis of each immune band was performed using Image Studio for C-DiGit (LI-COR). Molecular sizing was conducted using Rainbow MW markers (Amersham Japan, Tokyo, Japan). Protein concentrations were assessed using the DC Protein Assay System (Bio-Rad).

Results of the statistical analysis are expressed as mean ± standard deviation of the mean (SD). Differences among groups were analyzed by one-way ANOVA followed by the Tukey–Kramer test, and differences between two groups were analyzed by the Student's *t*-test. All statistical analyses were performed using Ekuseru-Toukei 2015 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences with *P*-values of 0.05 or less were considered statistically significant.

In this study, we investigated if resveratrol could induce differentiation in OMM cells from invasive phenotype to proliferative phenotype. If so, we also checked the contribution of the phenotype change to attenuation of chemoresistance in OMM cells. As shown Fig. 1A, with resveratrol treatment (dose, 50 µM; period, 48 hr and 72 hr) canine OMM underwent differentiation as assessed by changes to melanocyte morphology. Corresponding to this observation, mRNA level of MITF showed a time-dependent increase in OMM cells by 50 µM resveratrol treatment (Fig. 1B). Resveratrol (50 µM, 72 hr) treatment also led to a significant increase in the expression of the MTIF transcriptional targets, tyrosinase and GP100 (Fig. 1C). MITF plays a central role in mediating phenotype switching and down regulation of MITF is a major feature of dedifferentiation of melanoma cells to a mesenchymal-like invasive phenotype [7, 8, 14]. It has been reported that melanoma cells with a more differentiated profile exhibit high expression of MITF [7, 8, 14]. Tyrosinase and GP100 are part of the common upregulated geneset among many known MITF targets directly involved in melanocyte differentiation [17]. Consistent with our findings, increased expression of MITF in addition to the well-characterized differentiation-associated MITF downstream targets, tyrosinase and GP100, support the assumption that resveratrol induces a differentiated phenotype in canine OMM cells. Furthermore, we demonstrated that the treatment under the conditions used in this study, resveratrol (50 µM, 72 hr) had no effect on the viability of OMM cells (Fig. 1D). The effect of resveratrol on cell viability has been shown to be dose dependent and varies amongst melanoma cell types [18, 20]. In mouse melanoma cells, resveratrol ≤50 µM reduced cell viability, however concentrations of 50 µM did not cause significant cell death. It was also shown that grape extract (100 µg/mL) promoted MITF expression in mouse melanoma cells [20]. However, in human metastatic melanoma cells, resveratrol concentrations as high as 100 µM had little effect on cell viability, while resveratrol treatment (75 µM, 72 hr) still induced differentiation and substantially contributed to phenotypic changes [18].

It has been reported that mitogen-activated protein kinase (MAPK) is critical for MITF abundance and function in melanoma cells [14]. Based on these findings, we checked which MAPK subtype(s) could be associated with resveratrol-mediated differentiation in OMM cells, using several inhibitors against each MAPK subtype. As shown in Fig. 2A, only a c-Jun N-terminal kinase (JNK) inhibitor, SP600125, showed a similar morphological change with that caused by resveratrol treatment (50 µM, 48 hr). As well, a significant increase of MITF mRNA level was only observed under JNK inhibition in similar with that in the resveratrol-treated group (Fig. 2B). In addition, resveratrol independently suppressed JNK activation in OMM cells. Resveratrol treatment (50 µM, 24 hr) led

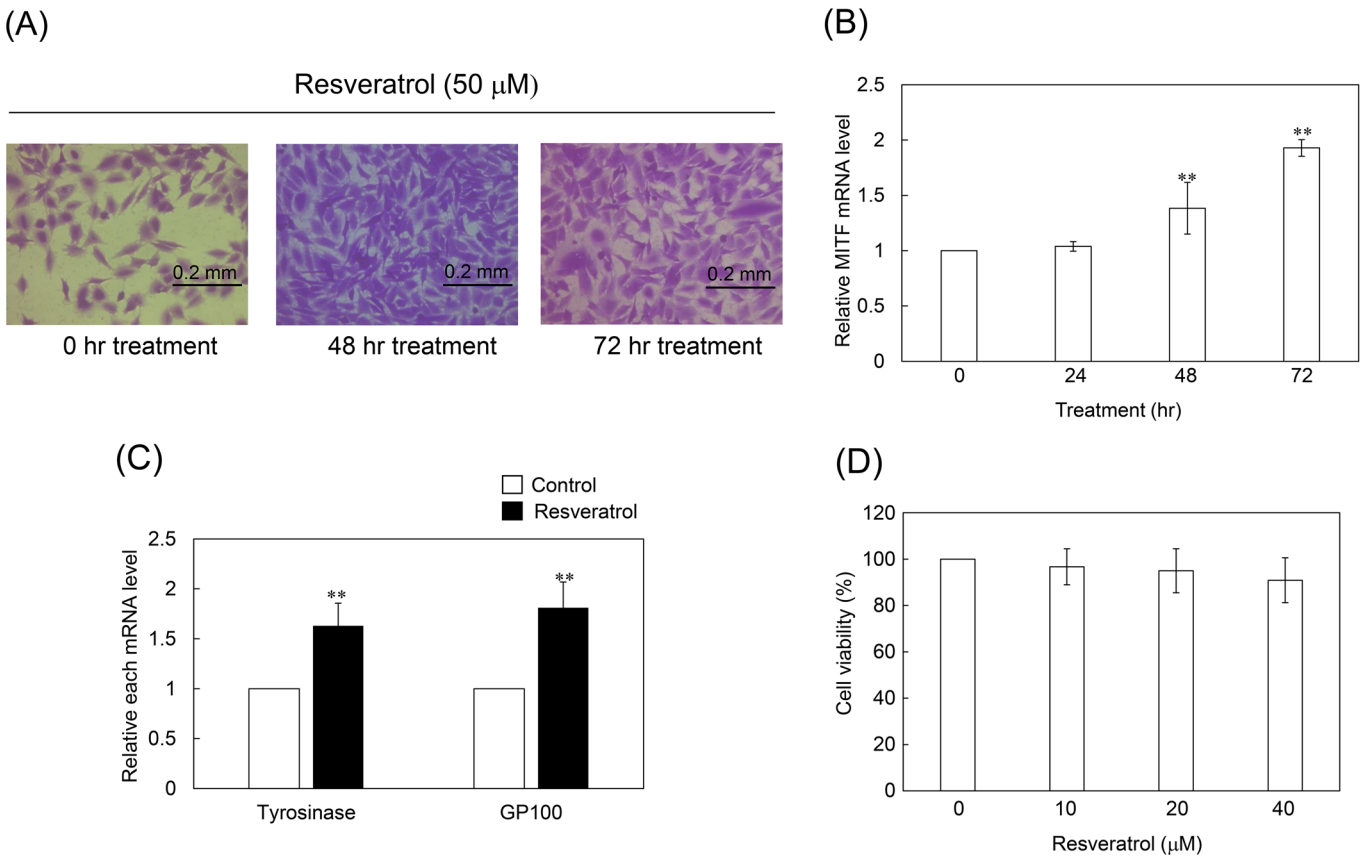

Fig. 1. Resveratrol induces differentiation in oral mucosal melanoma (OMM) cells. (**A**) Morphological changes of OMM cells by resveratrol treatment (50 μM) and period (48 hr and 72 hr). Each sample after the treatment was stained by crystal violet (CV) method, and this image was representative one of three independent experiments. (**B**) Time-dependent enhancement of mRNA level in microphthalmia-associated transcription factor (MITF), a representative marker of differentiation in melanoma, by 50 μM resveratrol treatment. Value in 0 hr group was considered as 1.0, and the number of each group was three. $^{**}P<0.01$ vs. 0 hr group. (**C**) The increases in mRNA levels for tyrosinase and glycoprotein 100 (GP100) by resveratrol treatment (50 μM, 72 hr). Value in control was considered as 1.0, and the number of each group was three. $^{**}P<0.01$ vs. control. (**D**) The effect of resveratrol on cell viability in OMM cells. The cells were treated with resveratrol at indicated doses for 24, 48 and 72 hr. After that, cell viability was determined by WST-8 method. Cell viability in 0 μM group was considered as 100%, and the number of each group was six.

to an approximately 33% decrease in the activated level of JNK verified as the ratio of phosphorylated JNK to total JNK, and the difference showed a statistical significance (Fig. 2C). These results demonstrated that promotion of differentiation in OMM cells by resveratrol lies in the blockade of JNK activation. We further tested OMM cells with anisomycin, a potent stimulator of the p38 MAPK (p38) and JNK pathways, widely used in various cancer types including melanomas [10, 16]. Interestingly, the significant change in MITF mRNA level (Fig. 2D) was not observed upon JNK activation with anisomycin at both concentration levels (25 nM and 50 nM, 48 hr). Based on the reported concentration of anisomycin administered [10, 16], responses to JNK activation in melanomas appeared to be dependent upon its malignancy phenotype. In metastatic melanoma cells, anisomycin in lower concentrations (40 nM) selectively activated only p38 while JNK activation was reported to be achieved only with doses of anisomycin exceeding 500 nM [16]. Moreover, in metastatic melanoma cells, JNK activity has been shown to be upregulated and remained constitutively active, with higher levels of JNK phosphorylation being associated with more aggressive metastatic phenotypes [1]. From these observations, it could be speculated that the constitutively higher JNK activation status in OMM cells contributed to no response towards the JNK activator. In a previous report, it has been shown that JNK overexpression stimulates phosphorylation and cytoplasmic retention of cyclic AMP response element-binding protein (CREB)-regulated transcription coactivator (CRTC) 3, contributing to the inhibition of MITF expression, and on the contrary, that the inhibition of JNK up-regulates MITF level via the dephosphorylation and nuclear localization of CRTC3 [10]. Therefore, we could not exclude the possibility that resveratrol-mediated differentiation of OMM cells depends on CRTC3-stimulated CREB transactivation caused by JNK inhibition.

Finally, we estimated if anti-melanoma activity of cisplatin, which was clinically utilized for canine melanoma treatment [2], could be enhanced in resveratrol exposed OMM cells. Our results, Fig. 3 showed that the viability of canine OMM cells was affected by cisplatin treatment in a concentration-dependent manner. It was observed that resveratrol (50 μM) can potentiate the cells' sensitivity to the cytotoxic effects of 20 μM or even as high as 40 μM cisplatin treatment. Combination therapy of resveratrol and cisplatin

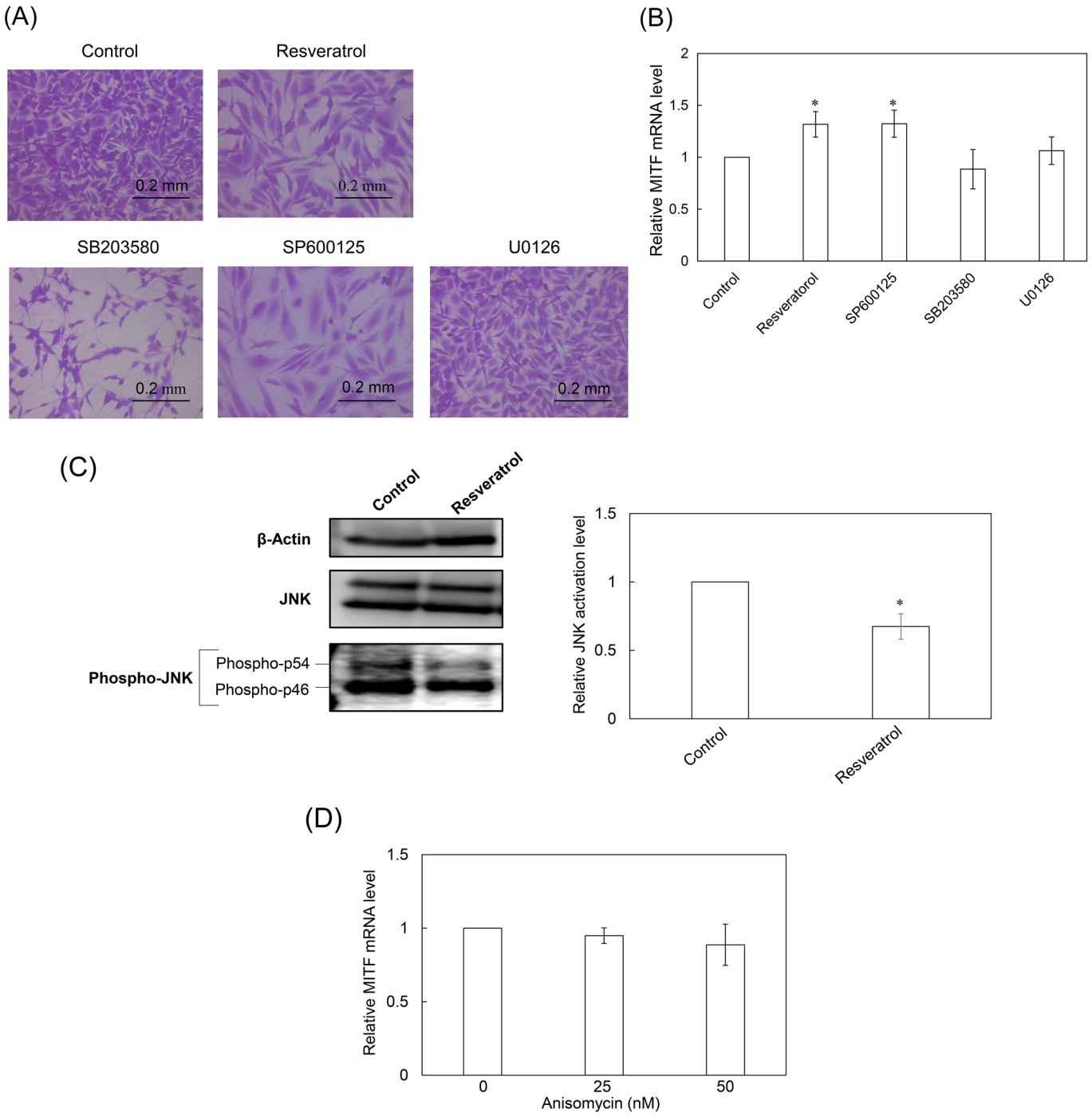

Fig. 2. c-Jun N-terminal kinase (JNK) inhibition contributes to resveratrol-induced differentiation in OMM cells. The cells were treated with resveratrol (50 μM), and each mitogen-activated protein kinase (MAPK) subtype inhibitor, SP600125 (JNK inhibitor, 10 μM), SB203580 (p38 MAPK (p38) inhibitor, 10 μM) and U0126 (extracellular-regulated kinase inhibitor, 10 μM), and anisomycin (JNK activator, 25 nM and 50 nM), respectively for each indicated time. After that, each assay was carried out. (**A**) Morphological changes in OMM cells after each treatment for 48 hr. Each sample was stained with CV methods, and this image was representative one of three independent experiments. (**B**) Changes of MITF mRNA levels in each group after the treatment for 48 hr. Value in control was considered as 1.0, and the number of each group was three. **P*<0.05 vs. control. (**C**) The effect of resveratrol (50 μM) on the activation of JNK in OMM cells after the treatment for 24 hr. After that, the activation of JNK (estimated as the phosphorylation level) was determined by western blotting as described previously. Relative JNK activation level was evaluated as the ratio to control, value in control was considered as 1.0 and the number of each group was three. **P*<0.05 vs. control. (**D**) Changes of MITF mRNA levels in each group after treatment with anisomycin for 48 hr. Value in control (0 nM) was considered as 1.0, and the number of each group was three. **P*<0.05 vs. control (0 nM).

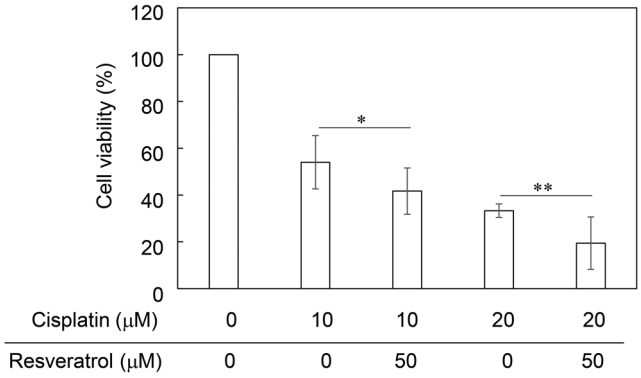

Fig. 3. Resveratrol enhances cisplatin-induced cytotoxicity in OMM cells. The cells were treated with 50 μM resveratrol for 6 hr before the application of cisplatin at indicated doses. After that, the co-treatment of resveratrol and cisplatin for 48 hr was performed, and subsequently, cell viability was determined by WST-8 method. Cell viability of no treatment group was considered as 100%, and the number of each group was six. *P<0.05 and **P<0.01 vs. cisplatin treatment group.

broadly applied in the treatment of various cancer types has been shown to improve efficacy and alleviate toxicity [3, 4]. For example, resveratrol (40 μM) reduces the toxic effect of cisplatin (20 μM) used in high concentration on normal HUVEC cells while contributing to an increase in sensitivity to cisplatin in head and neck cancer cells [3]. Another study has also shown that resveratrol (~17.5 μM) increases the therapeutic efficacy of cisplatin (~66 μM) in a highly resistant K1735 and B16F10 mouse melanoma cells [4]. In canines, a reported safe oral dosage of resveratrol between 600–1,200 mg/kg/day resulted in dose-related increases to plasma resveratrol levels of 20–50 μM [9]. Hence, the effective dose of resveratrol (50 μM) used in our study is compatible with the reported attainable plasma levels in canines [9]. This could indicate that resveratrol has clinical potential for canine or even human melanoma therapy, given that both share biological similarities [13]. Although resveratrol demonstrates an enhancing effect on cisplatin chemosensitivity, the contribution of the effect of resveratrol-induced differentiation on cisplatin chemosensitivity is currently unknown and requires further investigations. Notably, in mouse melanoma models, resveratrol enhances cisplatin's therapeutic effect through the upregulation of connexin 43 (Cx43) expression [4]. Previously, we have reported [15] that Cx43 function is impaired in canine OMM cells. Further research is therefore required to explore the influence of resveratrol on the upregulation of Cx43 in canine OMM cells and consequently an enhancement in cisplatin chemosensitivity.

Overall, our study highlighted the role of resveratrol in promoting differentiation in canine OMM cells via JNK inhibition, which led to a profound increase in MITF expression and drove melanoma differentiation to mitigate chemoresistance.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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REFERENCES

1. Alexaki VI, Javelaud D, Mauviel A. 2008. JNK supports survival in melanoma cells by controlling cell cycle arrest and apoptosis. *Pigment Cell Melanoma Res* **21**: 429–438. [Medline] [CrossRef]
2. Barabas K, Milner R, Lurie D, Adin C. 2008. Cisplatin: a review of toxicities and therapeutic applications. *Vet Comp Oncol* **6**: 1–18. [Medline] [CrossRef]
3. Bostan M, Petrică-Matei GG, Radu N, Hainarosie R, Stefanescu CD, Diaconu CC, Roman V. 2020. The effect of resveratrol or curcumin on head and neck cancer cells sensitivity to the cytotoxic effects of cisplatin. *Nutrients* **12**: 2596. [Medline] [CrossRef]
4. Cheng YJ, Chang MY, Chang WW, Wang WK, Liu CF, Lin ST, Lee CH. 2015. Resveratrol enhances chemosensitivity in mouse melanoma model through connexin 43 upregulation. *Environ Toxicol* **30**: 877–886. [Medline] [CrossRef]
5. Gillard M, Cadieu E, De Brito C, Abadie J, Vergier B, Devauchelle P, Degorce F, Dréano S, Primot A, Dorso L, Lagadic M, Galibert F, Hédan B, Galibert MD, André C. 2014. Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Cell Melanoma Res* **27**: 90–102. [Medline] [CrossRef]
6. Guo K, Feng Y, Zheng X, Sun L, Wasan HS, Ruan S, Shen M. 2021. Resveratrol and its analogs: potent agents to reverse epithelial-to-mesenchymal transition in tumors. *Front Oncol* **11**: 644134. [Medline] [CrossRef]
7. Hoek KS, Goding CR. 2010. Cancer stem cells versus phenotype-switching in melanoma. *Pigment Cell Melanoma Res* **23**: 746–759. [Medline] [CrossRef]
8. Hoek KS, Eichhoff OM, Schlegel NC, Döbbeling U, Kobert N, Schaerer L, Hemmi S, Dummer R. 2008. *In vivo* switching of human melanoma cells between proliferative and invasive states. *Cancer Res* **68**: 650–656. [Medline] [CrossRef]
9. Johnson WD, Morrissey RL, Usborne AL, Kapetanovic I, Crowell JA, Muzzio M, McCormick DL. 2011. Subchronic oral toxicity and cardiovascular safety pharmacology studies of resveratrol, a naturally occurring polyphenol with cancer preventive activity. *Food Chem Toxicol* **49**: 3319–3327. [Medline] [CrossRef]

10. Kim JH, Hong AR, Kim YH, Yoo H, Kang SW, Chang SE, Song Y. 2020. JNK suppresses melanogenesis by interfering with CREB-regulated transcription coactivator 3-dependent MITF expression. *Theranostics* **10**: 4017–4029. [Medline] [CrossRef]
11. Nishiya AT, Massoco CO, Felizzola CR, Perlmann E, Batschinski K, Tedardi MV, Garcia JS, Mendonça PP, Teixeira TF, Zaidan Dagli ML. 2016. Comparative aspects of canine melanoma. *Vet Sci* **3**: 7. [Medline] [CrossRef]
12. Pazzi P, Steenkamp G, Rixon AJ. 2022. Treatment of canine oral melanomas: a critical review of the literature. *Vet Sci* **9**: 196. [Medline] [CrossRef]
13. Prouteau A, André C. 2019. Canine melanomas as models for human melanomas: clinical, histological, and genetic comparison. *Genes (Basel)* **10**: 501. [Medline] [CrossRef]
14. Roesch A, Paschen A, Landsberg J, Helfrich I, Becker JC, Schadendorf D. 2016. Phenotypic tumour cell plasticity as a resistance mechanism and therapeutic target in melanoma. *Eur J Cancer* **59**: 109–112. [Medline] [CrossRef]
15. Sato A, da Fonseca IIM, Nagamine MK, de Toledo GF, Olio R, Hernandez-Blazquez FJ, Yano T, Yeh ES, Dagli MLZ. 2021. Effects of alpha-connexin carboxyl-terminal peptide (aCT1) and bowman-birk protease inhibitor (BBI) on canine oral mucosal melanoma (OMM) cells. *Front Vet Sci* **8**: 670451. [Medline] [CrossRef]
16. Slipicevic A, Øy GF, Rosnes AK, Stakkestad Ø, Emilsen E, Engesæter B, Mælandsmo GM, Flørenes VA. 2013. Low-dose anisomycin sensitizes melanoma cells to TRAIL induced apoptosis. *Cancer Biol Ther* **14**: 146–154. [Medline] [CrossRef]
17. Vachtenheim J, Borovanský J. 2010. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. *Exp Dermatol* **19**: 617–627. [Medline] [CrossRef]
18. Yang S, Meyskens FL Jr. 2005. Alterations in activating protein 1 composition correlate with phenotypic differentiation changes induced by resveratrol in human melanoma. *Mol Pharmacol* **67**: 298–308. [Medline] [CrossRef]
19. Zhao XE, Yang Z, Zhang H, Yao G, Liu J, Wei Q, Ma B. 2018. Resveratrol promotes osteogenic differentiation of canine bone marrow mesenchymal stem cells through wnt/beta-catenin signaling pathway. *Cell Reprogram* **20**: 371–381. [Medline] [CrossRef]
20. Zhou S, Riadh D, Sakamoto K. 2021. Grape extract promoted α-msh-induced melanogenesis in B16F10 melanoma cells, which was inverse to resveratrol. *Molecules* **26**: 5959. [Medline] [CrossRef]