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RESEARCH ARTICLE

Anti-tumor effects of paeonol in melanoma by up-regulating miR-143 and suppressing its target WNT5B

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Abstract

Previous studies have shown the potent anti-tumor effect of paeonol, a natural compound extracted from traditional Chinese herb medicine. However, the therapeutic effect and underlying mechanisms of paeonol in melanoma are largely unknown. Two melanoma cell lines (M14 and A375) were cultured in the presence of paeonol. Cell proliferation was determined by colony formation and cell-counting kit 8 assays. Real-time quantitative reverse transcription polymerase chain reaction and Western blot were performed to detect mRNA and protein expression, respectively. Paeonol treatment significantly inhibited the colony formation and proliferation of melanoma cells by enhancing the expression of the well-characterized tumor suppressor microRNA (miR)-143. Block of miR-143 diminished the inhibitory effects of paeonol on cell growth. miR-143 directly bound to Wnt family member 5B (WNT5B) gene. Paeonol therefore down-regulated WNT5B expression through miR-143. Moreover, paeonol suppressed melanoma growth *in vivo*. Our study reveals that paeonol effectively suppresses melanoma cell growth both *in vitro* and *in vivo* through miR-143/WNT5B axis, which shows potential for the treatment of melanoma.

Keywords: *melanoma*; *paeonol*; *miR-143*; *WNT5B*

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Melanoma is progressively transformed from melanocytes, and becomes metastatic at late stage. Although metastatic melanoma could be treated with radiotherapy, surgery, and chemotherapy, the survival rate of patients is low (1). Single-agent chemotherapy is widely used in the treatment of melanoma patients; however, the objective response rate (ORR) is only 5 to 20% (2). Combination chemotherapy gives out better ORR, but is associated with greater toxicity (3, 4). Thus, medicines with lower toxicity are needed for melanoma treatment.

Paeonol is a traditional Chinese herb medicine. As a component of traditional Chinese medicine, it is widely used in anti-inflammation, anti-oxidation, hypnosis, and sedation, etc. Recent studies demonstrated the anti-tumor activities of paeonol in hepatocellular carcinoma, breast cancer, gastric cancer, colon cancer, breast carcinoma and ovarian cancer (5–10). However, the underlying mechanism of paeonol against tumor cells is still unclear. A previous study suggests that paeonol is able to inhibit the metastasis of a melanoma cell line B16F10 (11). But how paeonol inhibits melanoma cell proliferation is still an open question.

Recently, studies have identified a group of microRNAs (miRNAs) that are involved in the development of melanoma. For example, miR-143 was found to be down-regulated in melanoma tissues. miR-143 overexpression could inhibit cell proliferation and induce apoptosis in melanoma, suggesting that miR-143 functions as a tumor suppressor (12). Likewise, miR-143 inhibited the growth of several other cancer cells, including bladder cancer, colorectal cancer and non-small-cell lung cancer (13–15).

Morphogen pathways, such as bone morphogenetic protein (BMP), Wnt, and Notch are heavily involved in most cancers, including melanoma. For instance, Wnt signaling plays important roles in the differentiation of

melanoblasts during development (16). At the same time, Wnt5A-mediated Wnt signaling is able to dedifferentiate melanocytes into a more stem cell-like state (17). And Wnt5A expression positively correlates with tumor grade of melanoma and affects the cell motility of metastatic melanoma (18).

In this study, we explored the effects of paeonol on melanoma cell proliferation and its underlying mechanisms.

Materials and methods

Tissues and cells

Human melanoma cell lines, including M14 and A375, were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle Medium in the presence of fetal bovine serum (10%) at 37°C. miR-143 mimics or inhibitors (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Waltham, MA) were employed for cell transfection, which lasted for 24–48 h.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen) was used to extract the total RNA. miR-143 expression was examined using a Hairpin-it TM miRNAs qPCR kit (Genepharma). WNT5B expression was detected by SYBR green qPCR assay (Takara, Dalian, China). $2^{-\Delta\Delta CT}$ method was used for data analysis.

Cell-counting kit 8

Cell proliferation was detected using cell-counting kit 8 (CCK-8) (Beyotime, Haimen, China). Cells (0.5×10^4) were seeded in the 96-well plate in the presence of dimethyl sulfoxide (DMSO) or paeonol for 24–96 h. Then, CCK-8 (10 μL) reagent was added and incubated the cells for 1 h. The optical density at 450 nm was recorded by a microplate reader.

Colony formation assay

Cells (600 cells per well) were plated into six-well plates, and cultured for 10–15 days. Cells were fixed with methanol, then stained with 0.1% crystal violet to visualize the colonies.

Luciferase reporter assay

Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI) and normalized to Renilla activity following traditional procedures.

Western blot

Protein was extracted using radioimmunoprecipitation lysis buffer (Beyotime), and the concentration was determined using the bicinchoninic acid protein assay kit (Beyotime). Protein was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking, the membranes were incubated with corresponding primary antibodies overnight at 4°C. The blots were further incubated with horse radish peroxidase-conjugated secondary antibodies for 1 h. Membranes were visualized using enhanced chemiluminescence (Millipore).

Xenograft tumor model

M14 cells were suspended in serum-free 1640 (1×10^6), and were injected in the posterior flank of the nude mice. After 8 days, the mice were treated with 100 mg/kg paeonol or DMSO by tail intravenous injection. The tumors were measured every 2 days until day 20, and were excised, photographed and weighed. Animal studies were approved by Wuxi No. 2 People's Hospital.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and analyzed using SPSS (version 13.0, SPSS, Chicago, IL). Student's *t*-test or one-way analysis of variance (ANOVA) was employed for the statistical comparison.

Results

Paeonol inhibited the proliferation and colony formation

Upon paeonol treatment, the proliferation of M14 cells was significantly reduced after 24 h (Fig. 1a). After 96 h of paeonol treatment, only half of the cells were viable compared to the cells treated with vehicle (Fig. 1a). Similarly, the proliferation rate of A375 cells was also decreased by around 50% with paeonol treatment (Fig. 1b). We further examined the colony formation ability of the cells when they were treated with paeonol. Our data indicated that when either M14 cells or A375 cells were treated with paeonol, the number of colonies was reduced by ~85% compared to control cells (Fig. 1c).

Paeonol up-regulated tumor suppressor miR-143

miR-143 functions as a tumor suppressor in various cancers including melanoma (12–15, 19, 20). Since paeonol inhibits melanoma cell proliferation, we asked whether paeonol performed the anti-tumor function by regulating expression of miR-143. The result showed that the expression of miR-143 was induced more than 4-fold in M14 cells incubated with paeonol (Fig. 2a). Similar level of induction was also observed in A375 cells upon paeonol treatment (Fig. 2b). To verify the specificity of the probe of miR-143 used in the real-time qPCR assays, we also designed anti-miR-143 oligonucleotide. It was indicated that the anti-miR-143 successfully blocked paeonol-mediated up-regulation of miR-143 in M14 and A375 cells

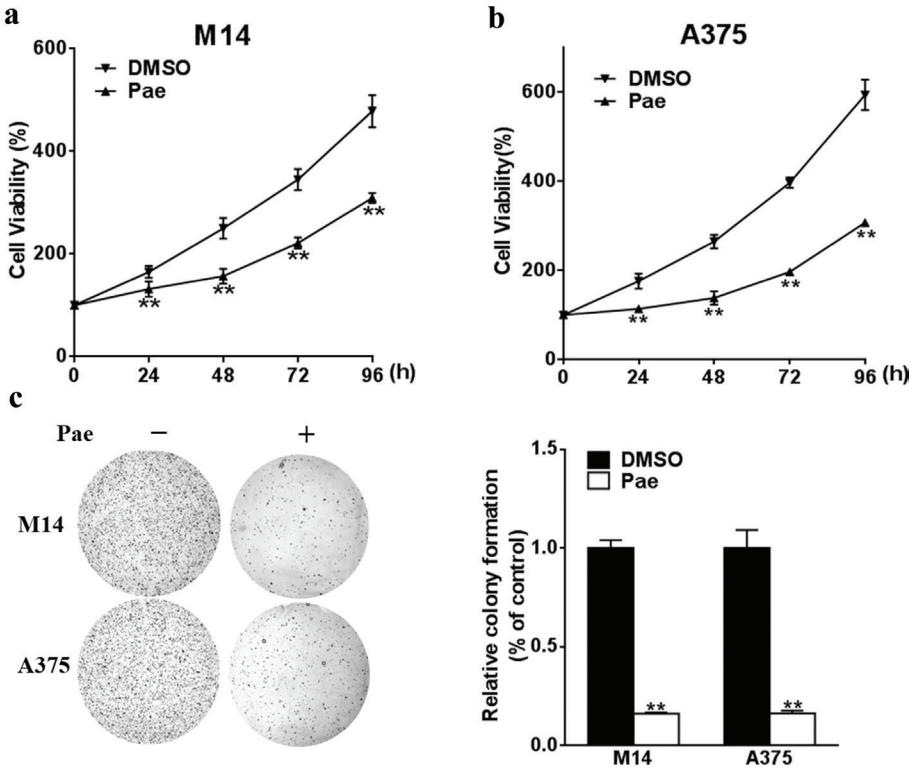

Fig. 1. Paeonol (Pae) inhibits cell proliferation and growth of melanoma. M14 and A375 melanoma cells were treated with 500 μM Pae or DMSO. (a, b) CCK-8 assay was used to test cell proliferation. (c) Colony formation assay was employed to analyze the cell growth ability. ** $P < 0.01$ versus control.

(Fig. 2c, d), suggesting that the genuine miR-143 was controlled by paeonol.

Silence of miR-143 reversed the adverse effect of paeonol on melanoma

Next, we asked whether miR-143 plays a critical role in the anti-tumor effect of paeonol on melanoma. To address this question, we transfected anti-miRNA-143 to interfere the induction of miRNA-143 in M14 and A375 cells treated with paeonol (Fig. 2c, d). Strikingly, silence of miRNA-143 significantly diminished the function of paeonol on inhibiting the proliferation of M14 and A375 cells (Fig. 3a, b). In parallel, colony formation assays demonstrated that the cells transfected with anti-miRNA-143 did not respond to paeonol treatment any more (Fig. 3c, d).

WNT5B is the target of miR-143 and was suppressed by paeonol

Since miR-143 functions as a tumor suppressor and is essential for the adverse effect of paeonol on melanoma, we extended our study to identify the target gene of miR-143 in melanoma. Through comprehensive sequence analysis, we found that the 3′ UTR of *WNT5B* is complementary to the sequence of miR-143 (Fig. 4a), which makes it a candidate target of miR-143. Therefore, we cloned the 3′ UTR of *WNT5B*, as well as a corresponding mutant one into reporter plasmid and co-transfected them with either miR-143 mimics or negative control. Comparing to negative control, miRNA-143 mimics dramatically inhibited the activity of 3′ UTR of *WNT5B* but not the corresponsive mutant one, indicating that this element is the authentic target of miR-143 (Fig. 4b). Meanwhile, we treat the cells transfected with 3′ UTR reporter with paeonol. Consistently, the reporter activity of 3′ UTR is repressed by paeonol, in contrast to the mutant one (Fig. 4c). Next, we examined whether miR-143 regulates WNT5B via its 3′ UTR. Western blot was employed to detect the protein level of WNT5B in M14 and A375 cells. As expected, WNT5B protein is decreased with paeonol treatment, while the reduction is attenuated by anti-miR-143 (Fig. 4d and e). Taken together, our data revealed that WNT5B is a direct target of miR-143, and is controlled by miR-143 via its 3′ UTR in melanoma cells.

Paeonol suppresses tumor formation of melanoma in vivo

The finding of paeonol inhibiting melanoma cell growth led us to further test its therapeutic potential *in vivo*. We injected M14 cells into nude mice to form tumor xenografts for 8 days and then treated the mice with paeonol or vehicle for another 12 days. The tumor xenografts from paeonol treatment group were significantly smaller than

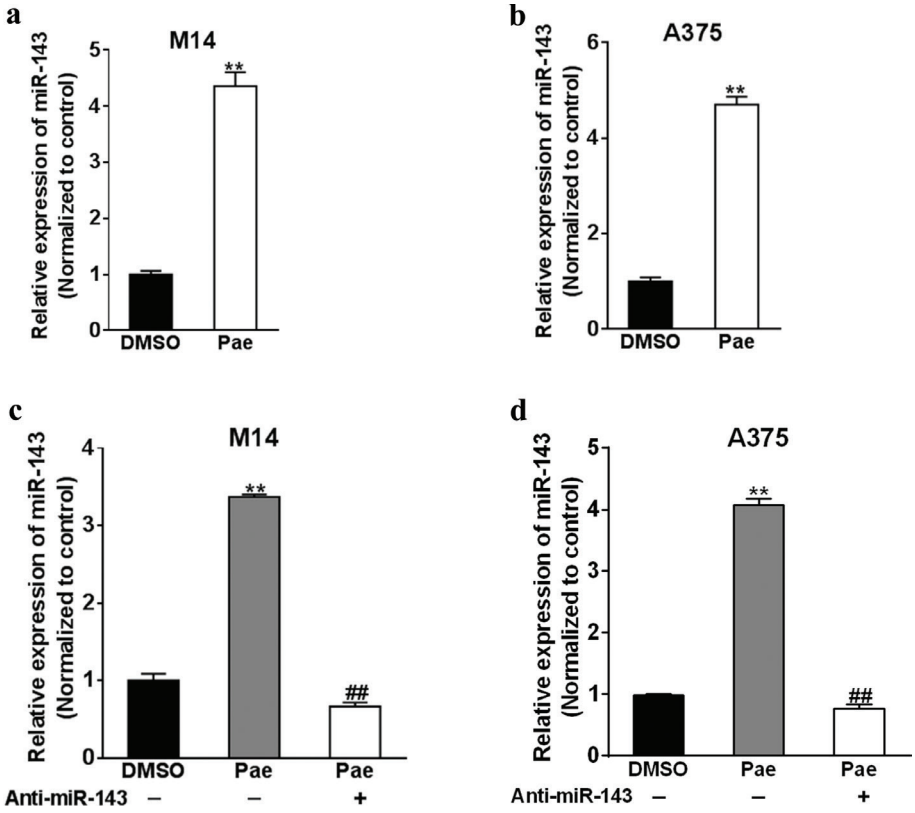

Fig. 2. Paeonol (Pae) induces the expression levels of miR-143. (a, b) M14 and A375 melanoma cells were treated with 500 μM Pae or DMSO, after 24 h the relative expression levels of miR-143 were analyzed by qRT-PCR. ** $P < 0.01$ versus control. (c, d) M14 and A375 melanoma cells were transfected with Anti-miR-143 or Anti-miR-NC, and were treated with 500 μM Pae or DMSO. After 24 h the relative expression levels of miR-143 were analyzed by qRT-PCR. ** $P < 0.01$ versus DMSO+Anti-miR-NC group. ## $P < 0.01$ versus Pae+Anti-miR-NC.

those from control group (Fig. 5a). The average size of xenografts of paeonol treatment group was 35% of that in control group (Fig. 5b). The average weight of tumors in paeonol treatment group was 26% of that in control group (Fig. 5c). We also examined miR-143 and WNT5B expression in the tumor xenografts. Consistent to our findings in cells, paeonol treatment dramatically induced miR-143 expression while suppressed WNT5B expression in tumor xenografts (Fig. 5d). Thus, paeonol not only inhibited the proliferation of melanoma cells *in vitro*, but also repressed melanoma development *in vivo*.

Discussion

The effects of paeonol on tumors include inhibition of cell proliferation, inducing apoptosis and attenuating metastasis (6, 9–11, 21). We indicated here that paeonol is capable of suppressing melanoma cell proliferation and inhibiting the growth of tumor xenografts, which is consistent with previous findings in other types of cancers. Given to the fact that paeonol is effective in a wide range of cancers, it is important to elucidate the underlying molecular mechanisms. The study from colorectal cancer cells suggests that paeonol exerts its anti-tumor function by reducing the expression of cyclooxygenase-2 and blocks prostaglandin E2 synthesis (21). In breast carcinoma, paeonol down-regulates B-cell lymphoma 2 and activates bcl-2-like protein 4, caspase 8 caspase 3 (10). Paeonol suppresses the phosphatidylinositol 3-kinase/protein kinase B pathway (22). In B16F10 melanoma cells, paeonol treatment blocks proinflammatory cytokines-mediated nuclear factor kappa-B and signal transducers and activators of transcription three pathways (11). In this study, we demonstrated that paeonol induces miR-143 expression in M14 and A375 cells. Although we cannot rule out the possibility that paeonol also regulates the other signaling pathways, our data from anti-miR-143 suggests that paeonol functions mainly through miR-143 in melanoma cells. Further studies are required to determine whether paeonol also controls miR-143 expression in other cancers.

miR-143 functions as a tumor suppressor in several types of cancers. As a microRNAs, miR-143 controls its target genes through the 3′ UTR element. Here, WNT5B was identified as the direct target of miR-143 in M14 and A375. The 3′ UTR of WNT5B is directly bound by miR-143. Therefore, the mRNA and protein levels of WNT5B are attenuated. Other target genes of miR-143 have also

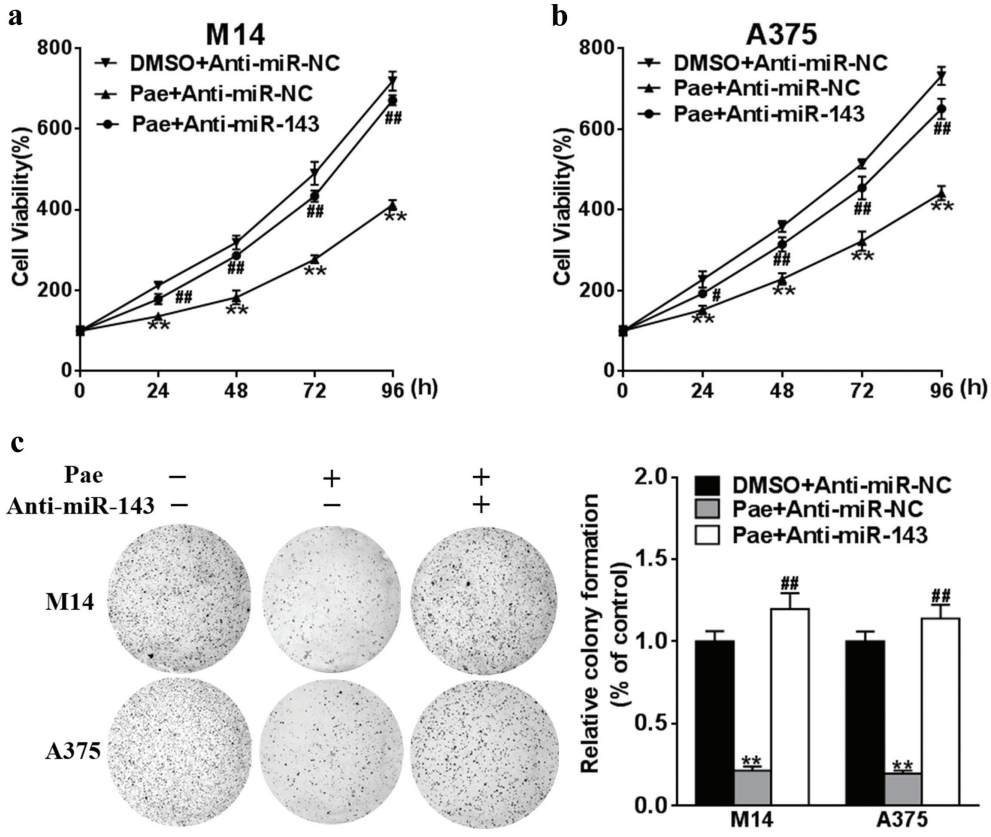

Fig. 3. Silence of miR-143 reverses the adverse effect of paeonol (Pae) on melanoma. M14 and A375 melanoma cells were transfected with Anti-miR-143 or Anti-miR-NC, and were treated with 500 μM Pae or DMSO. (a, b) CCK-8 assay was used to test cell proliferation. (c) Colony formation assay was employed to analyze the cell growth ability. ** $P < 0.01$ versus DMSO+Anti-miR-NC group. ## $P < 0.01$ versus Pae+Anti-miR-NC.

been identified. In bladder cancer T24 cells, miR-143 binds to extracellular-signal-regulated kinase 5 (ERK5) mRNA and therefore disrupts the expression of ERK5 (13). In colorectal cancer, the MACC1 is one of genes targeted by miR-143 (14). In non-small-cell lung cancer, miR-143 targets CD44v3 (15). In other melanoma cells, Sydecan-1 is also targeted by miR-143 (12). In esophageal squamous cell carcinoma, the quaking I-5 protein turns out to be the target gene of miR-143 (19). Taken together, it is very possible that miR-143 performs different functions by targeting distinct targets in distinct cells. Of note, although miR-143 is widely considered as a tumor suppressor, tumor-specific removal of miR-143/145 cluster in a mouse model of lung adenocarcinoma demonstrated that miR-143 expressed from the tumor microenvironment contributes to tumor development through its target calcium/calmodulin dependent protein kinase ID (23). Thus, more studies are needed to carefully characterize the targets of miR-143 in different types of cells.

Patients with malignant melanoma, especially those with late-stage metastatic melanoma suffer from the lack of effective and low-toxic therapeutic medicines (1, 24). Cytotoxic chemotherapy is the major treatment of metastatic melanoma, which has been used for over three decades. However, these chemotherapeutic agents only have modest anti-tumor efficacy in metastatic melanoma. To increase the anti-tumor efficacy, these drugs have been used alone or in combination (24). However, the combinations of cytotoxic agents do not extend survival significantly probably due to the greater toxicity. Paeonol has been used for hundreds of years in traditional Chinese medicine. Compared to cytotoxic agents, paeonol has the benefit of low toxicity. In addition, studies have shown that paeonol also contains anti-inflammatory and anti-oxidant activities (25, 26). Thus, paeonol does not only function as an anti-tumor agent but also serves as a systematic medicine when used in cancer patients. Moreover, paeonol has shown synergistic effects with cisplation on the treatment of hepatoma cells (2). We propose that the combination of cytotoxic agents and paeonol provides a new insight into the melanoma treatment. Further studies are on demand to find out the most efficient combination therapy for metastatic melanoma.

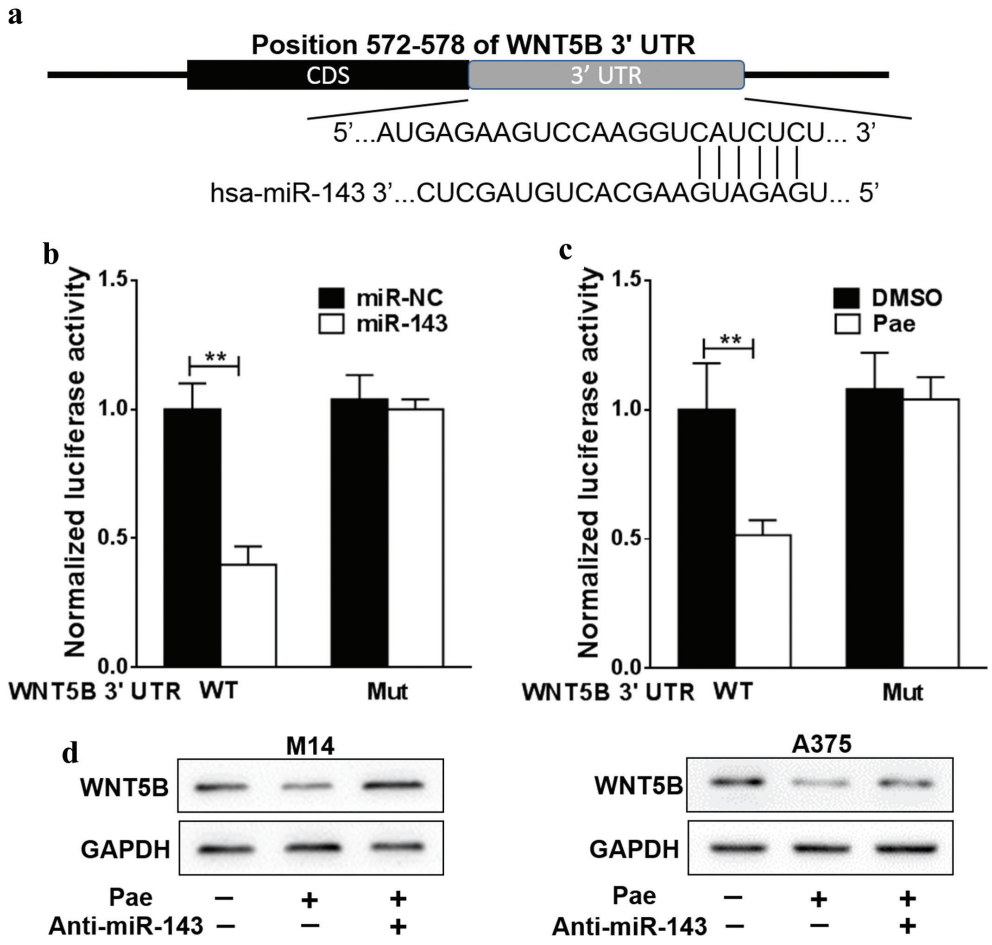

Fig. 4. WNT5B is the target of miR-143 and is suppressed by paeonol (Pae). (a) Putative seed-matching sites between miR-143 and 3′-UTR of WNT5B. (b) The WT and mut WNT5B 3′ UTR reporters were constructed and were co-transfected with miR-143 mimics or miR-NC mimics in M14 cells, after 24 h the luciferase activities were analyzed. (c) The WT and mut WNT5B 3′ UT reporters were transfected in M14 cells, and were treated with 500 μM Pae or DMSO. After 24 h the luciferase activities were analyzed. (d, e) M14 and A375 melanoma cells were transfected with Anti-miR-143 or Anti-miR-NC, and were treated with 500 μM Pae or DMSO, and western blotting were used to test the expression levels of WNT5B. ** $P < 0.01$ versus control.

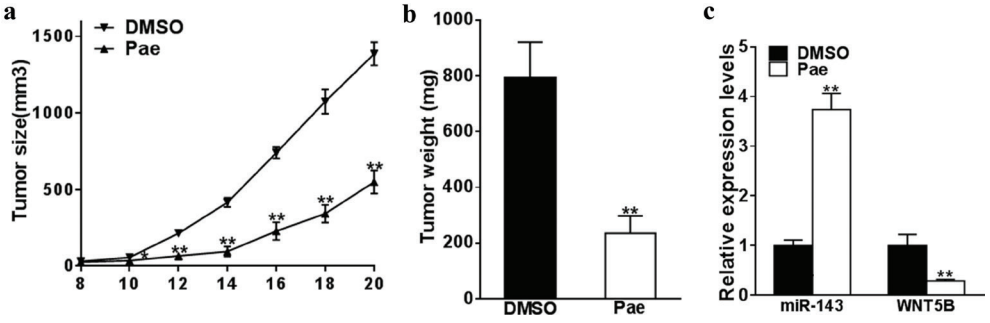

Fig. 5. Paeonol (Pae) suppresses tumor formation of melanoma *in vivo*. M14 cells were suspended in serum-free 1640 (1×10^6), and were injected in the posterior flank of the nude mice. After 8 days, the mice were treated with 100 mg/kg Pae or DMSO (each group with 4 mice) by tail intravenous injection. (a, b) The tumors were measured every 2 days until day 20, and were excised, photographed and weighed (Scale bars = 10 mm). (c) The expression levels of miR-143 and WNT5B of tumors were analyzed by qRT-PCR. ** $P < 0.01$ versus control.

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Conflict of interest and funding

None.

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