Suppression of cancer stem-like phenotypes in NCI-H460 lung cancer cells by vanillin through an Akt-dependent pathway

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Abstract. Cancer stem cells (CSCs) have been reported as a major cause of cancer metastasis and the failure of cancer treatment. Cumulative studies have indicated that protein kinase B (Akt) and its downstream signaling pathway, including CSC markers, play a critical role in the aggressive behavior of this cancer. In this study, we investigated whether vanillin, a major component in *Vanilla planifolia* seed, could suppress cancer stemness phenotypes and related proteins in the human non-small cell lung cancer NCI-H460 cell line. A non-toxic concentration of vanillin suppressed spheroid and colony formation, two hallmarks of the cancer stemness phenotype, *in vitro* in NCI-H460 cells. Western blot analysis revealed that the CSC markers CD133 and ALDH1A1 and the associated transcription factors, Oct4 and Nanog, were extensively downregulated by vanillin. Vanillin also attenuated the expression and activity of Akt, a transcription regulator upstream of CSCs, an action that was confirmed by treatment with the Akt inhibitor perifosine. Furthermore, the ubiquitination of Akt was elevated in response to vanillin treatment prior to proteasomal degradation. This finding indicates that vanillin can inhibit cancer stem cell-like behavior in NCI-H460 cells through the induction of Akt-proteasomal degradation and reduction of downstream CSC transcription factors. This inhibitory effect of vanillin may be an alternative approach in the treatment against lung cancer metastasis and its resistance to chemotherapy.

Introduction

Lung cancer has been reported as a major cause of mortality with a high metastasis rate compared with other types of cancers (1). Lung cancer cells contain a small population called tumor-initiating cells or cancer stem cells (CSCs) that have extremely tumorigenic, self-renewal and differentiating properties, resulting in a prolonged tumor status, resistance to chemotherapy and cancer relapse (2,3). Clinical observation has reported that this CSC subpopulation is found in cancer specimens and remains an obstacle for cancer treatment (4). *In vitro* and *in vivo* studies have revealed that lung cancer stem cells are extensively resistant to the first-line therapy cisplatin compared with neighboring cancer cells (5,6). These CSCs can maintain the cell survival signaling that provides their strength under severe environments (7). Therefore, the attenuation or removal of CSCs would be likely to improve patient outcome.

Like other types of stem cells, CSCs express specific surface markers, including octamer-binding transcription factor 4 (Oct4), Nanog, ATP-binding cassette subfamily G member 2 (ABCG2), and CD133. Oct4 and Nanog are transcription factors that are responsible for maintaining pluripotency, self-renewal proliferation and tumorigenicity in both normal stem cells and cancer stem cells (8,9). Several studies have reported that an elevation of Oct4 and Nanog is tightly related to a low survival rate and high incidence of cancer metastases (10,11). In lung cancer, both Oct4 and Nanog are required to maintain the CSC-like phenotype (12). Overexpression of Oct4 and Nanog enhances spheroid formation *in vitro* and significantly increases new tumor formation *in vivo*. Furthermore, CD133-positive lung cancer cells isolated from patients exhibited a high level of Oct4 and ABCG2, and displayed a clear resistance to chemotherapy and radiotherapy (13). Conversely, the attenuation of Oct4 and Nanog expression levels using RNA interference in CSCs led to the loss of the ability to form spheroids and enhanced the sensitivity of these cells to chemotherapy (12,14).

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Cumulative research has identified the mechanism regulating cancer stemness properties. Recently, the Akt pathway was demonstrated to be an upstream signaling pathway of Oct4, which is linked to the CSC-like phenotype (15). The phosphorylation of Akt promotes stemness through the upregulation of both the mRNA and protein levels of Oct4 and Nanog (15,16). Akt-positive cancers that express CSC markers can establish colonies *in vitro* and *in vivo* (17,18). In contrast, Akt-knockdown experiments using RNA silencing resulted in an extensive suppression of Oct4 as well as an impeded stemness behavior (19). The alteration of both Akt activity and expression may disrupt Oct4 and Nanog functions, and consequently, the CSC properties of cancer.

Several strategies to the suppression of the CSC mechanism have been intensively emphasized. Natural substances from plant sources, including vanillin, have gained increased interest in an alternative therapy due to their various pharmacological activities (20). Vanillin, 4-hydroxy-3-methoxybenzaldehyde (Fig. 1), is the main active ingredient found in the *Vanilla planifolia* seed. It is widely used as a flavoring agent in many products including food and cosmetics (21). Previous reports have demonstrated several biological activities of vanillin such as antimicrobial, hypotriglyceridemic, anti-inflammatory and antimutagenic activities in rodents and humans (22-24). Vanillin has been shown to inhibit cancer invasion and migration through the reduction of matrix metalloprotease activity and downregulation of nuclear factor-κB in hepatocellular carcinoma cells (25). Vanillin could also attenuate the formation of lamellipodia and angiogenesis in lung cancer via the suppression of PI3K activity and inducing the apoptosis of various cancer types, such as human cervical cancer and breast cancer (26-28). However, the pharmacological effect of vanillin and its mechanism on CSC-like properties in lung cancer has not been elucidated. In this study, we investigated the effects of vanillin on the CSC phenotypes in the non-small cell lung cancer (NSCLC) NCI-H460 cell line. We herein demonstrated for the first time that vanillin can suppress the stemness behavior of NCI-H460 cells, including spheroid formation, with a reduction in CSC markers. Furthermore, our data showed that Akt is the primary target of the vanillin-attenuating CSC behavior, suggesting that vanillin exhibits potential for further anticancer development.

Materials and methods

Cells and culture conditions. The human NSCLC cell line NCI-H460 was purchased from the American Type Culture Collection, ATCC (Manassas, VA, USA) and was cultured as monolayers in Roswell Park Memorial Institute-1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. Cell cultures were incubated at 37˚C in a humidified incubator filled with 5% CO_2. Cells were subcultured routinely with 0.25% (w/v) trypsin in 0.53 mM ethylenediamine tetraacetic acid (EDTA) and were seeded according to the manufacturer's instructions at ~70% confluence.

Reagents and antibodies. Vanillin (Fig. 1), as USP-secondary standard purity grade (>99.0% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The vanillin stock solution was freshly diluted with RPMI medium, and the treatment solution was prepared by dilution of the stock solution with culture media to the desired concentrations. Cell culture media were also used as the control solvent for the experiments. Glutamate, penicillin-streptomycin antibiotics, and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). Methanol, dimethyl sulfoxide (DMSO) and mouse monoclonal antibodies to Oct4 (1:1,000) and ubiquitin (1:1,000) were purchased from Sigma-Aldrich. Rabbit monoclonal antibodies to ABCG2 (1:1,000), β-catenin (1:1,000), Nanog (1:1,000) and phosphorylated Akt (P-Akt, (1:1,000) in Ser473 and Thr308 were from Cell Signaling Technology, Inc. (MA, USA). A goat monoclonal antibody against ALDH1A1 (1:1,000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit monoclonal antibody against CD133 (1:1,000) was purchased from United States Biological (MA, USA).

Cell viability and cell proliferation assays. Cell viability and proliferation assays were performed using the surrogate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, NCI-H460 cells were seeded onto 96-well plates at 10,000 cells/well and 2,500 cells/well for the cell viability and proliferation assays, respectively. After cell attachment, various concentrations of vanillin (0-400 μM) were added for 24 h for the cell viability assay. Cells pretreated with the same concentration of vanillin for 1 and 3 days were subcultured and incubated for 24, 48 and 72 h for the cell proliferation assays. At the end of each incubation time, the medium was removed and replaced with MTT solution (Life Technologies, Carlsbad, CA, USA) and incubated for 3 h at 37˚C. The MTT solution was then substituted with 100 μl of DMSO to dissolve the formazan crystals, and then the absorbance was measured at 570 nm using a microplate reader. The absorbance was calculated and represented as the % viability and % relative proliferation, respectively, compared with the untreated (control) cells that were set at 100%.

Determination of apoptotic and necrotic cell death. Hoechst 33342 and propidium iodide (PI) (Sigma-Aldrich) were used to stain the nuclei to identify necrotic and apoptotic cell death, respectively. Cells were treated with vanillin for 24 h and then were washed with PBS, followed by incubation with either 5 μM PI or 10 μM Hoechst 33342 at 37˚C for 30 min. Cells were visualized and captured under a fluorescence microscope at x10 magnification (Olympus IX5; 10X with DP70 digital camera system; Olympus, Tokyo, Japan). PI-positive necrotic cells and nuclear condensation of apoptotic cells were scored and reported as the % of all cells viewed.

Anchorage-independent growth assay. The NCI-H460 cells were pretreated with vanillin (0-50 μM) for 1 and 3 days before being subjected to the soft agar colony-formation assay to determine the anchorage-independent growth property of cancer stemness. Soft agar was prepared using a combination of complete media and 1% (w/v) agarose at a 1:1 ratio. This mixture was allowed to solidify in 24-well plates as a bottom layer. Next, pretreated cells suspended in RPMI complete
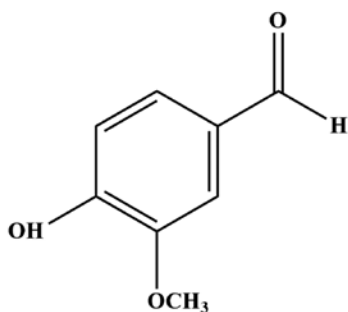
Figure 1. Chemical structure of vanillin (4-hydroxy-3-methoxybenzalde-hyde).

media were mixed with 0.3% (w/v) agarose, added onto the prepared bottom layer and then incubated at 37˚C. Further complete media were applied every 2 days to prevent the soft agar drying. After 2 weeks, the colony was examined under a phase-contrast microscope at x4 magnification (Olympus IX5; 4X with DP70 digital camera system; Olympus). The relative colony number and size were analyzed compared with those of the control group.

Spheroid formation assay. Vanillin-pretreated cells at a cell density of 2,500 cells/well were cultured in 24-well ultralow attachment plates in RPMI serum-free medium for 7 days. Next, the spheroids were disaggregated by trypsinization, resuspended in RPMI serum-free medium as a single cell suspension and then cultured onto 24-well ultralow attachment plates. After an additional 14 days, the presence of secondary spheroids was investigated by phase-contrast microscopy at x4 magnification (Olympus 1X51 with DP70). The relative spheroid number and size were analyzed compared with those of the control group.

Western blot analysis. Cells were lysed using lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10% glycerol, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), and a protease inhibitor cocktail (Merck Millipore, MA, USA), sonicated and incubated on an ice bath for 45 min. The protein content was measured using the BCA protein assay kit (Pierce™ Thermo Fisher Scientific Inc., IL, USA). An equal amount of denatured protein was separated by electro-phoresis [10% (w/v) acrylamide resolving gel] and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Each membrane was blocked with 5% (w/v) non-fat-milk in Tris-buffered saline solution containing 1% (v/v) Tween-20 (TBST) for at least 30 min and then incubated with the indicated primary antibody at 4˚C overnight. Thereafter, the membranes were washed three times with TBST and then incubated with secondary antibody at room temperature for 2 h. The antigen-antibody complexes were detected, after washing with TBST as above, using chemiluminescent solution (Pierce Biotechnology) and were exposed to film (Carestream Health, Inc., Rochester, NY, USA). The densitometry of the target protein was measured using the NIH ImageJ program and quantified as the relative expression level to that of the control group.

Immunoprecipitation assay. Protein interaction was examined by immunoprecipitation. Cells were incubated with 10 μM lactacystin for 1 h prior to treatment with vanillin to inhibit proteasome function while preserving the ubiquitin-protein complex. Treated cells were collected and lysed in TMN buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl and 0.5 mM $MgCl_2$) containing 10% glycerol, a protease inhibitor cocktail, 1% nonylphenylpolyethylene glycol (NP-40) and 1% PMSF for 30 min, followed by centrifugation at 12,000 rpm at 4˚C for 15 min. The supernatants were blocked with protein G agarose beads (GE Healthcare, Little Chalfont, UK) to remove non-specific binding. After centrifugation at 3,000 rpm at 4˚C for 5 min, the protein contents were measured. The supernatants were collected and stored at 4˚C as an input for immunoblotting where 300 μg of protein was incubated with anti-Akt antibody at 4˚C overnight and then incubated with protein G agarose beads for 2 h at 4˚C. The immunoprecipitates were collected, washed with TMN buffer and resuspended in 30 μl of 2X SDS sample buffer. The samples were boiled at 95˚C for 5 min and then were subjected to western blot analysis using antibodies against ubiquitin.

Statistical analysis. The data were collected from at least four independent experiments, normalized by the control groups and presented as the means ± standard deviation (SD). The significant difference among the groups was analyzed using one-way ANOVA followed by the post hoc test. Statistical analysis was performed using SPSS software (IBM Inc., NY, USA) and statistical significance was accepted at the p<0.05 level.

Results

Cytotoxicity and anti-proliferative effect of vanillin on NCI-H460 cells. To diminish the interference of the cytotoxic effect of vanillin on cancer stemness, a non-toxic concentration of vanillin was used on the NCI-H460 cells, as determined by the cytotoxicity assay. Cells were treated with various concentrations of vanillin for 24 h and were then subjected to the MTT cell viability assay. Fig. 2A demonstrates that 200 μM vanillin caused a significant (p=0.003) mortality with ~30% cell death, whereas at a concentration of <100 μM at least 80% viable cells remained. To confirm the cell death mechanism, cells were similarly treated with vanillin for 24 h and then were incubated with either Hoechst 33342 or PI, where apoptotic nuclei and necrotic bodies were clearly observed in the cells treated with a high (200 μM) dose of vanillin (Fig. 2B and C). This result is consistent with the cell viability testing, revealing that vanillin at concentrations of <100 μM are non-toxic to NCI-H460 cells, so these doses were used for subsequent experiments.

The effect of vanillin on NCI-H460 cell proliferation was also investigated. The cells were pre-treated with vanillin for 1 and 3 days, prior to measuring their proliferation after a further 24, 48 and 72 h. The proliferation of the cells pretreated with 100 μM vanillin for 3 days was significantly reduced as early as 24 h, whereas lower doses (<100 μM) had no significant effect at any detection time (Fig. 2E). Furthermore, pretreatment with vanillin for 1 day showed non-detectable
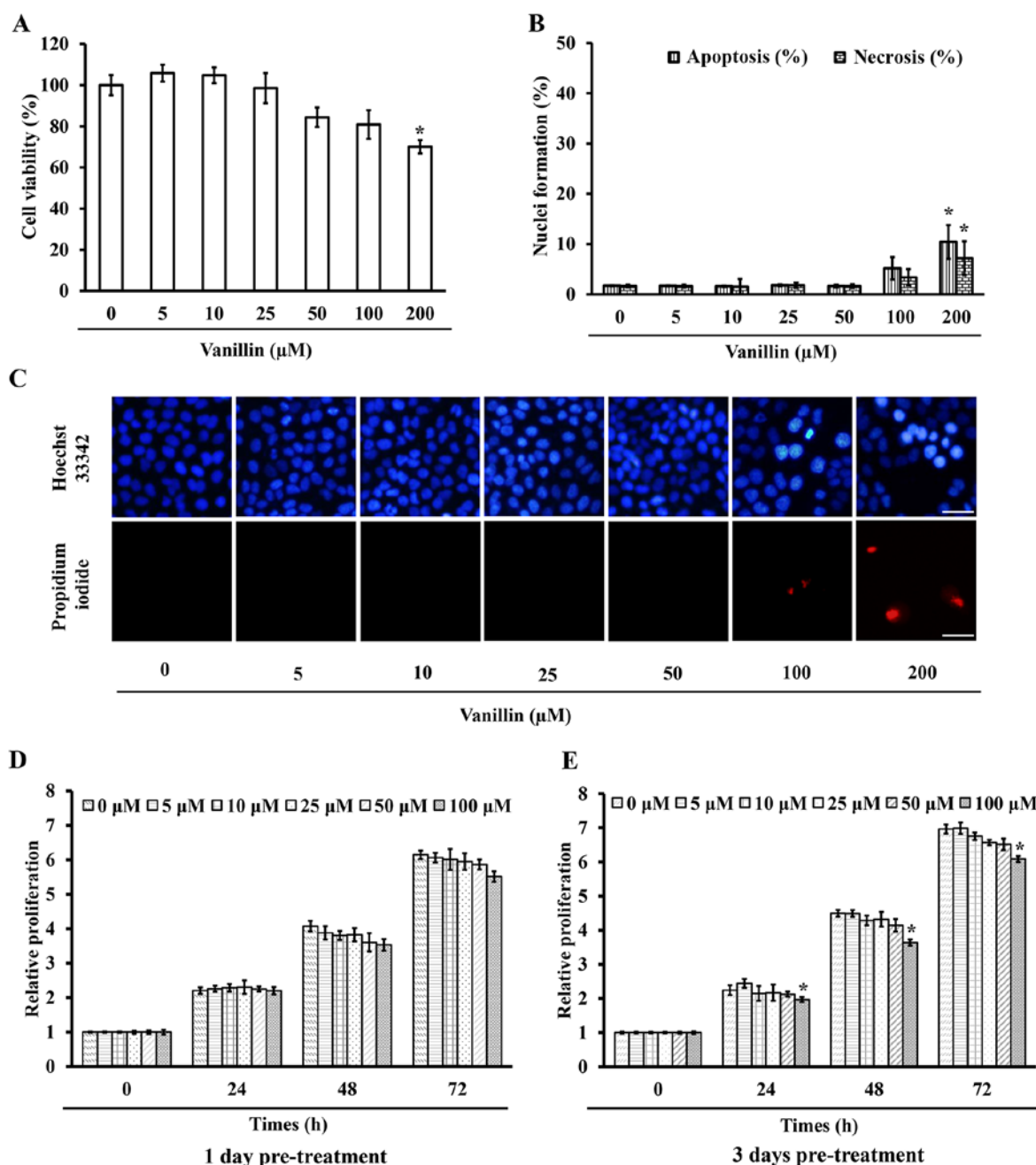
Figure 2. Cytotoxic and anti-proliferative effects of vanillin in NCI-H460 cells. (A) NCI-H460 cells were treated with vanillin at various concentrations (0-200 μM) for 24 h, and cell viability was measured by the MTT assay. (B and C) Apoptotic and necrotic cells were determined by staining with Hoechst 33342 (10 μM) and propidium iodide (5 μM), and examined under a fluorescence microscope (scale bar, 100 μm). Apoptotic nuclei and PI-positive necrotic cells were counted and reported as the percentage of the total cell number. NCI-H460 cells were pretreated with vanillin (0-100 μM) for 1 day (D) or 3 days (E). The proliferation of the cells was evaluated by the MTT assay for 24, 48 and 72 h, reported as the relative value to that of the control group at time 0 h. Data are presented as the means ± SD from at least four independent experiments. *p-value <0.05 versus the non-treated group.

changes compared with that in the control group (Fig. 2D). These results indicated that a low dose of vanillin (0-50 μM) neither induced cell death nor inhibited cell proliferation in the NCI-H460 cells. Cell growth and survival in the absence of extracellular matrix is one of the crucial behaviors of the cancer stem-like phenotype. Thus, to avoid any anti-proliferative effect of vanillin on cancer stem cell growth, vanillin doses that showed no such effect on NCI-H460 cells under the attachment condition were then used for the cancer stem-like phenotype characterization.

Inhibitory effect of vanillin on the anchorage-independent growth and spheroid formation of NCI-H460 cells. Because self-renewal and tumorigenicity are distinctive behaviors for the cancer stem-like phenotype, anchorage-independent growth and spheroid formation have been used to characterize these features. To investigate the suppressive effect of vanillin on colony and spheroid formation, NCI-H460 cells were pretreated with vanillin (0-50 μM) for 1 and 3 days, followed by examination of their anchorage-independent growth and spheroid formation. Fig. 3A shows representative images in
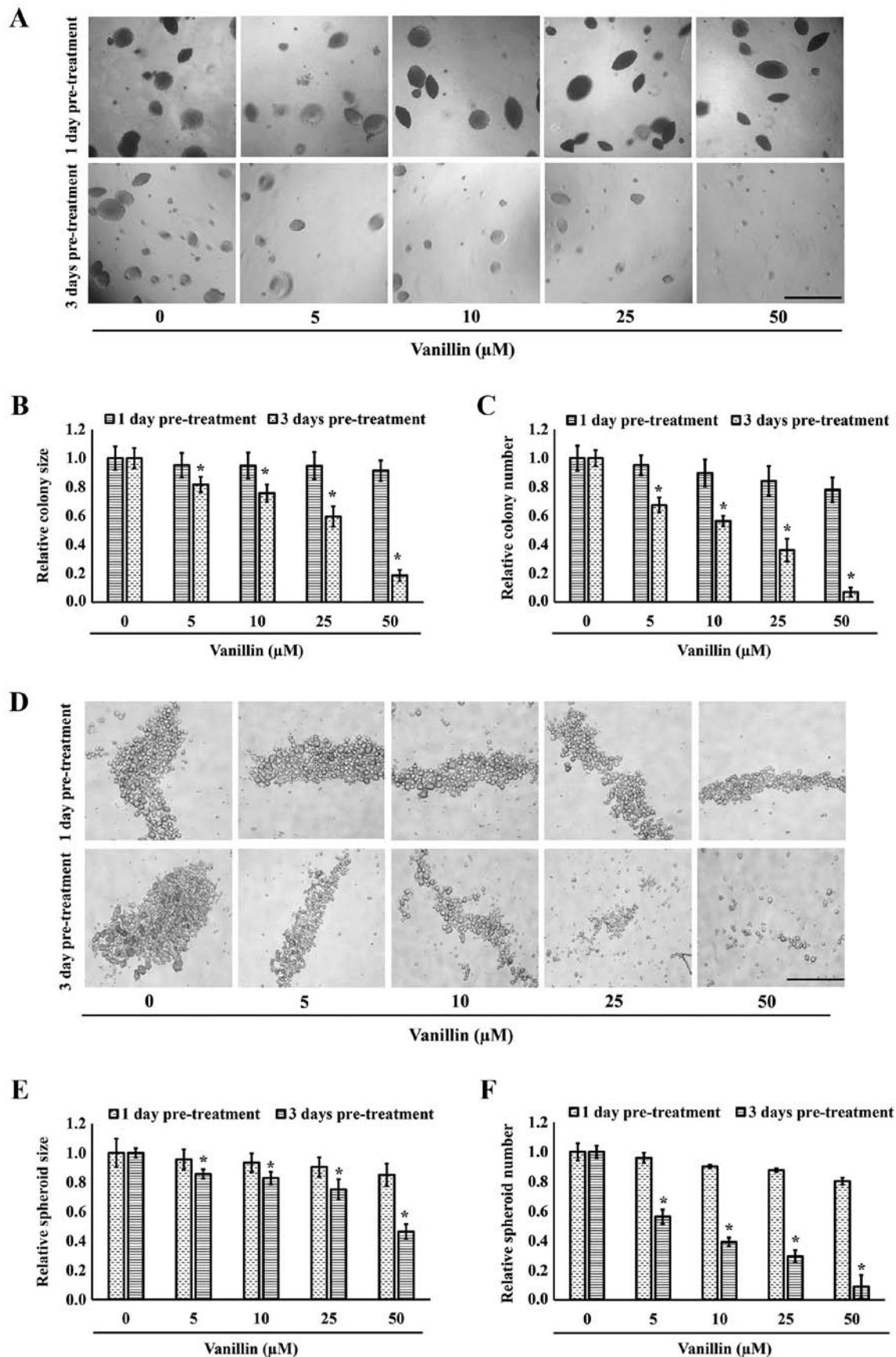
Figure 3. Vanillin suppresses anchorage-independent growth and spheroid formation of NCI-H460 cells. (A) NCI-H460 cells were pre-treated with vanillin (0-50 μM) for 1 and 3 days. Cells were then detached and cultured on soft agar as described in Materials and methods. Colonies were captured at 14 days after culture (scale bar, 200 μm). (B) The colony size and (C) number were measured and presented as the relative value to the control cells under each condition. (D) H460 cells were similarly pre-treated with vanillin (0-50 μM) for 1 and 3 days. Cells were detached and subjected to the spheroid formation assay. After 7 days, primary spheroids were dissociated and cultured on ultra-low cell culture plates for 14 days. The secondary spheroids were imaged (scale bar, 200 μm). (E) The spheroid size and (F) number were analyzed and presented as the relative value to the control cells of each condition. Data are presented as the means ± SD from at least four independent experiments. *p-value <0.05 versus the non-treated group.
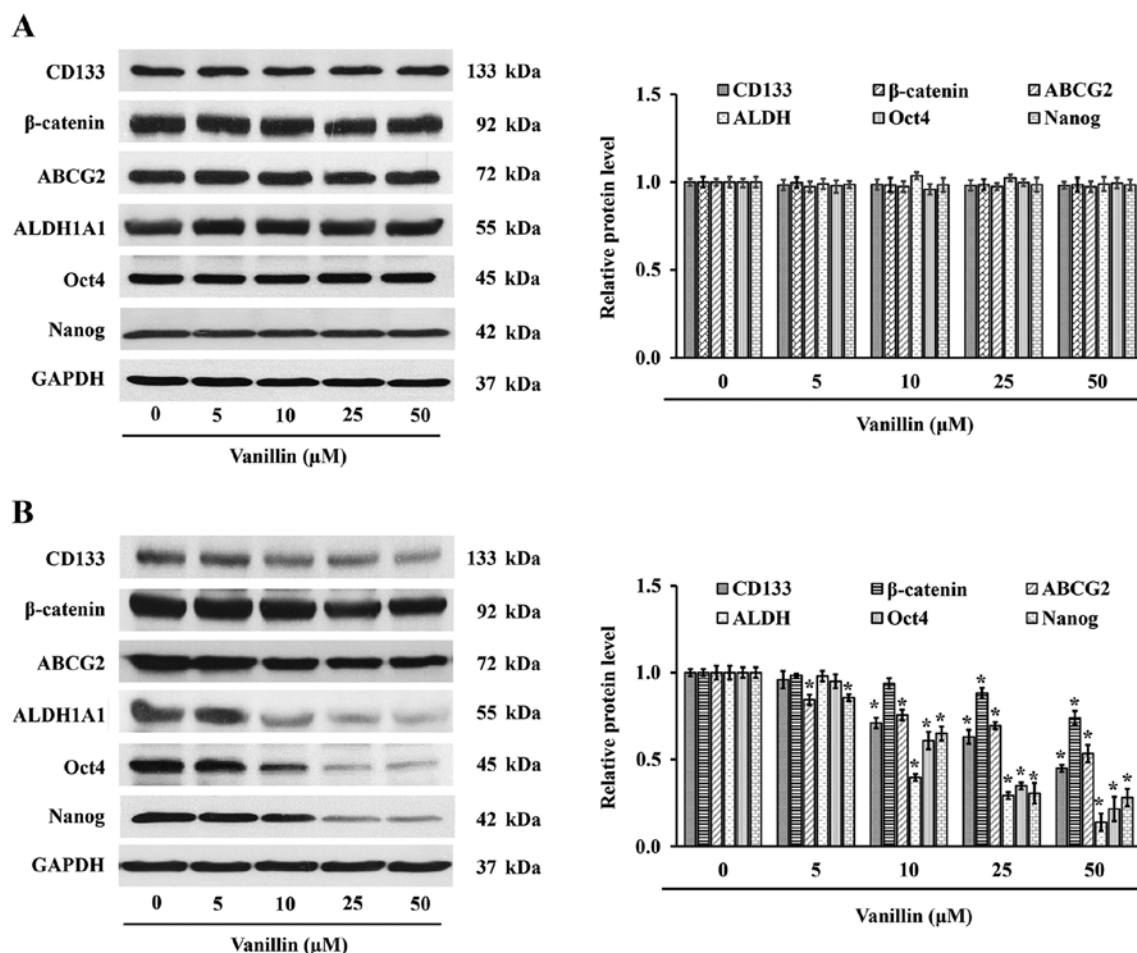
Figure 4. Vanillin downregulates lung cancer stemness markers. NCI-H460 cells were treated with vanillin (0-50 μM) for 1 day (A) or 3 days (B), and the expression levels of CD133, β-catenin, ABCG2, ALDH1A1, Oct4 and Nanog, were examined by western blotting using specific antibodies. The blots were reprobed with antibody against GAPDH to ensure equal loading and quantified densitometrically using ImageJ. Data are presented as the means ± SD from at least four independent experiments. *p-value <0.05 versus the non-treated group.

which both the colony size and number of cells pretreated with vanillin for 1 day were not different from those of untreated control cells. However, a significant and vanillin dose-dependent reduction in the cell size and number was observed when pre-incubated with vanillin for 3 days (Fig. 3B and C). Approximately 80 and 90% reduction in the colony size and number, respectively, were observed in the NCI-H460 cells pretreated with 50 μM vanillin for 3 days (p=0.027).

The effect of vanillin on the self-renewal property was evaluated by the spheroid formation assay. Primary spheroids, consisting of several types of cells, including progenitors, mature cells and stem cells (29), were first initiated for 7 days and then dissociated to single cells. The single cells were allowed to grow under detachment conditions to form secondary spheroids over 14 days, reflecting the pluripotency of CSCs. Interestingly, pretreatment with vanillin for 3 days markedly attenuated the number of secondary spheres formed under detachment conditions, with only ~0.2-fold of the number of sphere remaining after pretreatment with 50 μM vanillin for 3 days (p=0.022). In contrast, this effect was not found with a 1-day pretreatment with vanillin (Fig. 3D-F). These results suggest that vanillin can abolish the cancer stem-like phenotype of CSCs, in terms of both spheroid formation and anchorage-independent growth.

Vanillin downregulates stemness markers in NCI-H460 cells. Cancer stem cell markers are differentially expressed in different types of cancer, with CD133, ABCG2 and ALDH1A1 being distinctly expressed in lung cancer stem cells (3). Given the inhibitory effect of vanillin on the CSC phenotype, its effect on stemness cell markers was then evaluated in the NCI-H460 cells. NCI-H460 cells were incubated with vanillin (0-50 μM) for 1 and 3 days, and then the expression of CSCs markers was determined. Based on western blot analysis (Fig. 4), CSC protein marker expression was not significantly changed in response to the treatment with vanillin for 1 day (50 μM, p=0.124). However, the level of CSC markers, including those of CD133, ABCG2 and ALDH1A1, were markedly attenuated in a dose-dependent manner after treatment with vanillin for 3 days (Fig. 4B). Quantitative analysis demonstrated that a reduction in the expression level of these proteins was observed in cells treated with vanillin for 3 days at concentrations as low as 10 μM (p=0.021). Thus, vanillin suppresses not only the CSC phenotype but also CSC markers in NCI-H460 cells.

Vanillin suppression of Nanog and Oct4 expression is medi-ated by an Akt-dependent mechanism. The CSC phenotypes include not only an elevated expression of stemness markers, but also high levels of the related transcription factors,
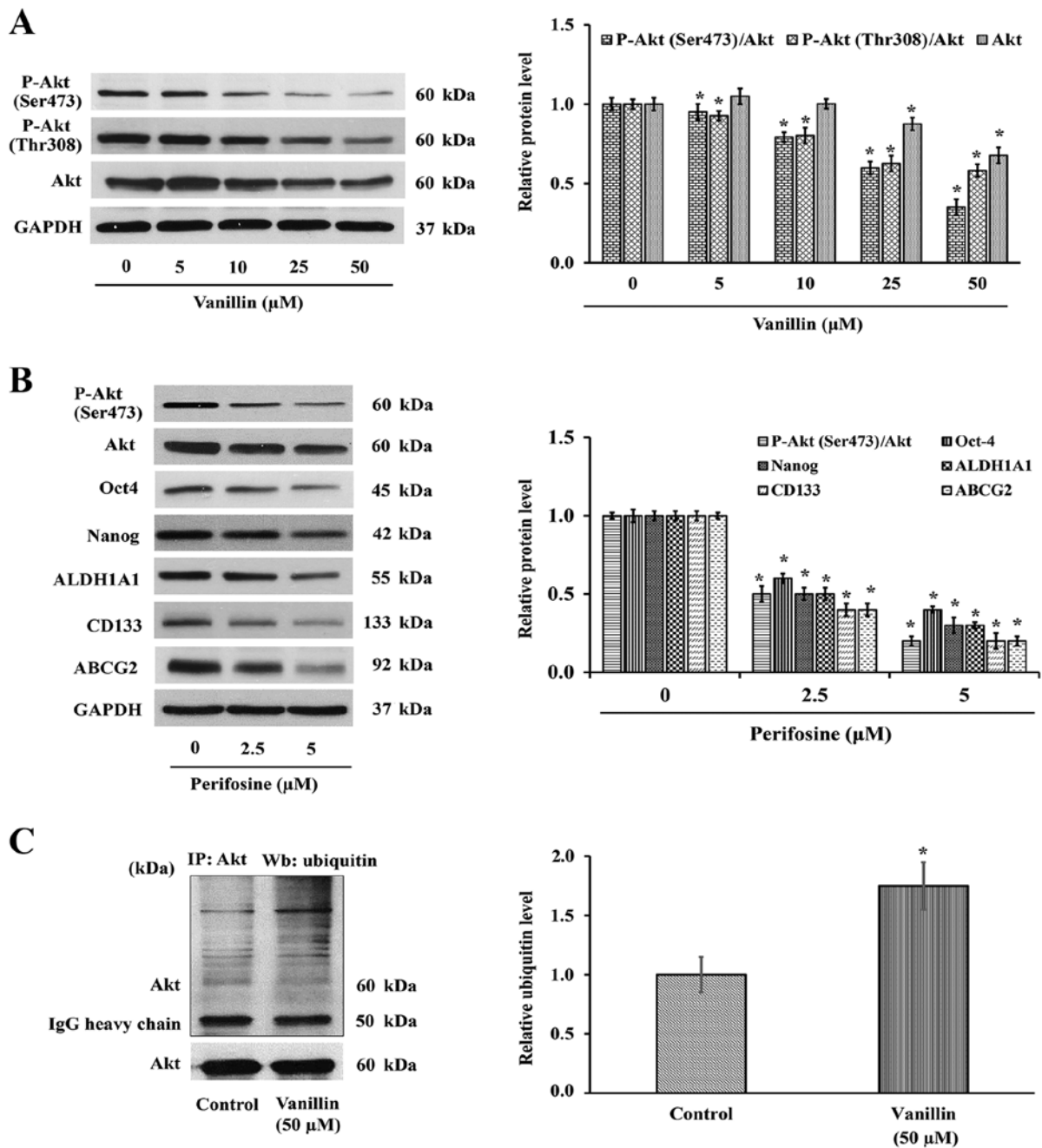
Figure 5. Vanillin mediates Akt degradation via a ubiquitin-proteasomal mechanism. (A) NCI-H40 cells were similarly treated with vanillin (0-50 μM) for 3 days, and the expression levels of Akt and its activated form (P-Akt, Ser 473 and Thr308) were determined by western blotting. (B) NCI-H460 cells were treated with perifosine (0-2.5 μM) for 3 days, and the protein expression was evaluated by western blot analysis. The blots were reprobed with antibody against GAPDH to ensure equal loading. Immunoblot signals were quantified using ImageJ and presented as the relative protein expression level normalized to the control group. (C) NCI-H460 cells were pre-treated with 10 μM lactacystin (Lac) prior to 50 μM vanillin for 3 h. The Akt was immunoprecipitated using an antibody against Akt and protein G agarose beads. The immunocomplex was analyzed by immunoblotting using antibody against ubiquitin. Data are presented as the mean ± SD, derived from at least four independent experiments. *p-value <0.05 versus the non-treated group.

including Nanog and Oct4 (10-12) that endow a self-renewal property to the cells (30). To assess whether the negative regulation of CSCs properties by vanillin involved these transcription factors, NCI-H460 cells were treated with various concentrations of vanillin for 1 and 3 days, and then protein expression levels of Oct4 and Nanog were evaluated. As shown in Fig. 4B, the Oct4 and Nanog protein expression levels were significantly decreased in the cells treated with vanillin for 3 days, but not in the cells treated for 1 day (Fig. 4A). These results illustrate a good correlation with the other stemness

phenotypes (Fig. 3 and 4), demonstrated by their suppression after treatment with vanillin for 3 days.

Because the expression levels of Nanog and Oct4, which contribute to the stemness behavior, are regulated directly by Akt (15), whether vanillin modulates Akt protein expression levels or its activity in terms of P-Akt expression was evaluated next. NCI-H460 cells were treated with vanillin for 3 days prior to determination of the Akt and P-Akt expression levels. Western blot analysis showed that the level of P-Akt (Ser473 and Thr308) or active Akt, was decreased (Fig. 5A),
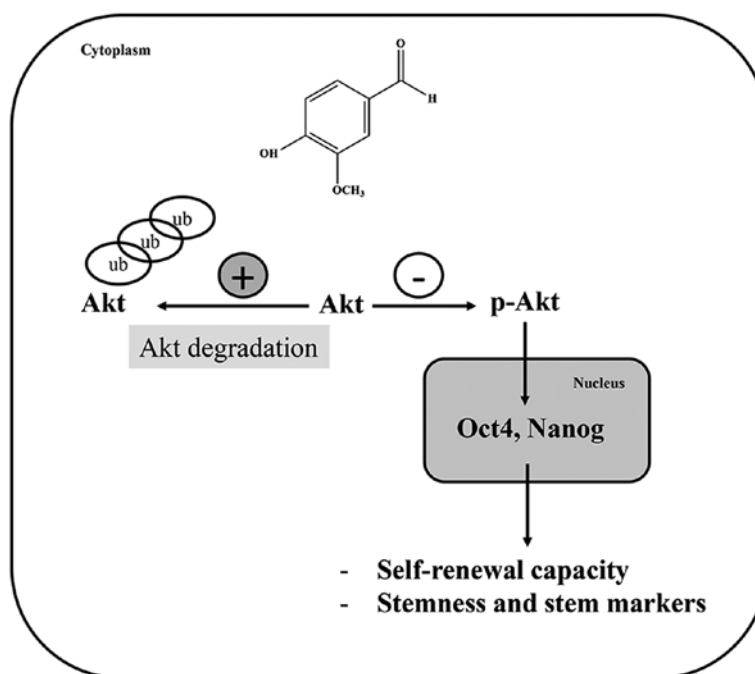
Figure 6. Schematic overview of the effect of vanillin on lung cancer stem-like phenotypes and its related pathway. Vanillin inhibits Akt activation and its expression by mediating Akt degradation through the ubiquitin-proteasomal pathway, downregulates the pluripotent transcription factors Oct4 and Nanog, and consequently suppresses self-renewal properties and cancer stem markers.

suggesting that the reduced CSCs phenotypes may result from an Akt-dependent mechanism. To verify whether the reduction of P-Akt by vanillin is a result of the suppression of P-Akt or total Akt expression levels, the total Akt expression level was also investigated. As shown in Fig. 5A, the total Akt level was clearly downregulated in a similar pattern. Densitometry analysis of P-Akt relative to total Akt was then analyzed to confirm the hypothesis that vanillin interferes with the Akt expression level. The quantitative results demonstrated that the ratio of P-Akt over its parental form are also changed, indicating the reduction of Akt expression may be, at least in part, a mechanism by which vanillin suppresses Akt activity.

To confirm the above finding that the downregulation of Nanog and Oct4 by vanillin is by an Akt-dependent pathway, perifosine (1,1-dimethylpiperidinium-4-yl octadecyl phosphate), an Akt inhibitor, was used as a positive control. H460 cells were treated with perifosine (0-5 μM) for 3 days, and the mentioned proteins were observed by western blotting as before. Interestingly, inactivation of Akt by perifosine caused a reduction of its downstream signaling, including Oct4 and Nanog (Fig. 5B), similar to that with vanillin (Fig. 4B). The CSC markers CD133, ABCG2 and ALDH1A1 were also downregulated corresponding to the suppression of Akt activity. Although the vanillin-blocked Akt activation occurs partly through downregulating the parental form, the total effect is similar to that by perifosine. These data indicated the Akt function that promotes cancer stemness property via Oct4 and Nanog, which was conversely inhibited by vanillin.

Vanillin mediates Akt degradation through the ubiquitin-proteasomal pathway. Emerging evidence has indicated that the existence of Akt is mainly governed by ubiquitin-proteasomal degradation via E3 ligase (31,32). The reduction of Akt

by the enhancement of this mechanism results in the interference of its downstream signaling mediators, including Oct4 and Nanog (11,15,16,33,34). Accordingly, we next examined whether downregulation of Akt by vanillin occurred through the ubiquitin proteasomal pathway. Protein degradation by this pathway requires the protein to bind with ubiquitin prior to recognition by the proteasome (35). NCI-H460 cells were pretreated with 10 μM lactacystin (Lac), a proteasomal inhibitor, prior to vanillin treatment (50 μM) for 3 h, and the presence of Akt-ubiquitin complex was determined by immunoprecipitation. Fig. 5C shows that the level of the Akt-ubiquitin interaction was markedly increased ~2-fold in the vanillin-treated group, which indicated that suppression of CSCs by vanillin may be a consequence of vanillin promoting Akt degradation through the ubiquitin-proteasomal pathway.

Discussion

Taken together, cancers are one of the most common non-accident related cause of deaths among human populations, but the successful rate of treatment is low due to the resistance to chemotherapy treatment, metastasis and the self-renewal properties of cancer cells. It has been reported that these aggressive behaviors are driven by CSCs (2), which are a small population found within tumors, especially in metastatic cancers (3). These CSCs exhibit an overexpression of specific markers such as CD133, ALDH1A1 and ABCG2 (2,36), and related proteins such as Akt and mTOR (37). Recently, research into drug discovery and development for cancer therapy has focused on exploring new natural compounds that target CSCs and related pathways. Several reports have suggested that many natural substances, such as curcumin, resveratrol and pomegranate extract, have the ability to inhibit CSCs (38-40).

Herein, we report for the first time that vanillin, an active component in *Vanilla planifolia*, suppresses CSCs in term of both their phenotype and related molecular mechanisms. A non-cytotoxic concentration of vanillin (50 μM) attenuated spheroid formation and anchorage-independent growth, which are the two prominent characteristics of CSCs (Fig. 3). The inhibitory effects of vanillin were clearly observed after treatment for 3 days, suggesting that in the early phase of treatment, a compensation mechanism by other kinase proteins, such as mitogen activated protein kinase (MAPK) or ERK may exist (41,42). Both PI3K/Akt and MAPK/ERK are known to play an important role in the maintenance of cell survival, differentiation and proliferation of cancer (43,44). A recent study reported that epidermal growth factor (EGF) could induce Akt activation through the MEK inhibitor (45). This phenomenon may occur during the 1-day treatment course of vanillin. An alternative possibility is that cellular signaling requires a longer activation time to induce the phenotypic changes. For example, the inhibitory effect of gigantol on CSCs was found only after treatment for at least 2 days (46). The ability of cancer cells to form neurospheres also indicates the pluripotency of CSCs (45), and our finding here demonstrated that vanillin greatly suppresses the *in vitro* formation of secondary spheroids consisting of NCI-H460 cells (Fig. 3D).

CD133, ALDH1A1 and ABCG2 have been reported to be specific markers in lung CSCs both *in vivo* and *in vitro* (47-49). Lung CSCs with CD133+ implanted in mice exhibited a self-renewal property, chemoresistance and a high tumorigenic capability. Like the *in vitro* study, NCI-H460 and NCI-A549, which express high levels of ABCG2 and CD133, can form spheroids, but this ability was attenuated after reduction of ABCG2 and CD133 expression (50). We further evaluated the expression of such markers in vanillin-treated cells. Corresponding to the phenotypic changes, vanillin downregulated these lung CSC markers (Fig. 4). As transcription factors, Oct4 and Nanog are frequently found in both CSCs and normal stem cells (49), and are responsible for maintaining the self-renewal and pluripotent properties of CSCs under regulation by the kinase activity of Akt (51). These transcription factors are upregulated in many cancers, especially lung cancer, and the reduction of Oct4 and Nanog expression can directly inhibit CSC phenotypes (11,30). Knockdown of these transcription factors was shown to decrease the proliferation and spheroid formation of cancer cells (15). Supporting our finding, Oct4 and Nanog were extensively decreased in response to vanillin treatment, and so likely contributed to the impedance of neurosphere formation and colony-formation capability of the NCI-H460 cells (Fig. 3).

Evidence has suggested that Akt plays a vital role in cell survival and proliferation (52), and drive cancer cells to CSC-like phenotypes including proliferation, migration and self-renewal properties, through the action of the transcription factors Oct4 and Nanog (16). Blockade of Akt was shown to inhibit the *in vitro* proliferation of spheroid formation and enhanced Oct4 degradation (33). In breast cancer, inhibition of PI3K or Akt activity led to pluripotency loss (37). Furthermore, in prostate cancer, the alteration of the PTEN/PI3K/Akt pathway interfered with the CSC phenotype (53). Likewise, in this study, vanillin was found to diminish Akt function and its expression, together with a decreased CSC behavior

and marker expression (Fig. 4). As a key protein catabolism pathway, the ubiquitin-proteasomal pathway is known to regulate several cellular signaling molecules, including Akt (54). Targeted proteins for degradation are poly-ubiquitinated by the ubiquitin ligase (E3) of the proteasome prior to recognition by ubiquitin-activating E1 and E2. The results of this study are consistent with the notion that vanillin drives Akt degradation through elevation of the ubiquitin-proteasomal pathway (Fig. 5). However, the mechanism that leads to ubiquitinized Akt is still unknown. Recent studies reported that multiple growth factor receptors such as EGFR and insulin-like growth factor 1 receptor, might be possible targets of natural compounds (55,56). Since the ubiquitination-proteasomal degradation of intracellular proteins is modulated by the downstream signaling of these receptors (57-60), it is possible that the mechanism of vanillin action on ubiquitinized Akt might involve vanillin-modulating of these receptors and their signaling pathways. However, the ubiquitin-proteasomal pathway is also regulated by the reactive oxygen species (61,62) and the exogenous compounds, that alter the oxidative status of the cells, affect this degradation pathway (63,64). Since vanillin has been demonstrated to have a dual effect as an oxidative stress scavenging and pro-oxidant activity, depending on the cell type and vanillin concentration (65-67), thus, it is also possible that vanillin may promote Akt degradation through this mechanism.

In conclusion, this study demonstrates that vanillin can suppress the CSC-like phenotype in NCI-H460 cells via the enhancement of the Akt-ubiquitin proteasome degradation pathway, resulting in the downregulation of the transcription factors Oct4 and Nanog (Fig. 6). This finding may provide a new strategy to overcome CSCs in lung cancer.

Acknowledgements

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