RESEARCH ARTICLE

Impairment of tumor-initiating stem-like property and reversal of epithelial–mesenchymal transdifferentiation in head and neck cancer by resveratrol treatment

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Scope: Recent reports have demonstrated that head and neck cancer-derived tumor-initiating cells (HNC-TICs) presented high tumorigenic, chemoradioresistant, metastatic properties, and were coupled with gain of epithelial–mesenchymal transition (EMT) characteristics. The aim of this study was to investigate the chemotherapeutic effect and regulatory mechanisms of resveratrol on HNC-TICs.

Methods and results: We first observed that the treatment of resveratrol significantly down-regulated the ALDH1 activity and CD44 positivity of head and neck cancer (HNC) cells in a dose-dependent manner ($p < 0.05$). Moreover, resveratrol treatment reduced self-renewal property and stemness genes signatures (Oct4, Nanog, and Nestin) expression in sphere-forming HNC-TICs. Additionally, the repressive effect of resveratrol on in vitro malignant properties including invasiveness/anchorage-independent growth was mediated by regulating productions of EMT markers Slug, ZEB1, N-cadherin, E-cadherin, and Vimentin. Importantly, an in vivo nude mice model showed that resveratrol treatment to xenograft tumors by oral gavage reduced tumor growth, stemness, and EMT markers in vivo. Lastly, synergistic effect of resveratrol and conventional chemotreatment attenuated tumor-initiating cells property in HNC-TICs.

Conclusions: Our results demonstrated that resveratrol would be a valuable therapeutics clinically in combination with conventional chemotherapy treatment modalities for malignant HNCs by elimination of tumor-initiating stem-like and EMT properties.

Keywords:
Cancer Stem Cells / Epithelial-mesenchymal transition / Head and neck cancer / Resveratrol / Tumor-initiating cells

1 Introduction

Head and neck cancer (HNC) is one of the most common cancers in the world [1]. HNC is one of causes of cancer-related death due to conventional therapy resistance [1]. Therapeutic methodology of HNC patients usually consisted of more extensive surgery, radiotherapy, chemotherapy, or concurrent chemo-/radiotherapy [1]. Five-year survival rate of HNC is above 50% in general and less depending on the staging or lesion sites [2]. Despite improvements in the diagnosis and management of HNC, long-term survival rates have improved only marginally over the past decade [3]. Therefore, development for novel candidate compounds and understanding the molecular mechanisms of tumorigenesis are crucial to develop therapeutic approaches for HNC patient.

Mounting evidence has demonstrated that head and neck cancer tumor-initiating cells (HNC-TICs) with stem-like properties have been known to have the capacity to promote tumor progression and metastasis, and also contribute to radioresistance and chemoresistance [4–10]. HNC-TICs

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Abbreviations: **EMT**, epithelial–mesenchymal transition; **HNC**, head and neck cancer; **HNC-TICs**, head and neck cancer-derived tumor-initiating cells; **TICs**, tumor-initiating cells

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have been identified as a subpopulation of HNC cells with high CD44+ or intracellular aldehyde dehydrogenase activity (ALDH1+) marker [4, 9]. CD44, a transmembrane glycoprotein involved in many cellular processes, was shown to be a TICs marker in HNC [4]. ALDH1, a cytosolic isoenzyme, is responsible for oxidizing intracellular aldehydes and contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation [9]. ALDH1A1 is a marker of HNC that distinguishes malignant from premalignant cells and is also an essential epitope for developing ALDH1A1-based vaccines for HNC therapy [11]. Increased ALDH1 activity was also shown to be a putative marker of tumor-initiating cells (TICs) in breast cancer [12], hepatoma [13], colon cancer [14], and osteosarcoma [15]. Furthermore, some studies reported that HNC-derived ALDH+/CD44+ cells displayed tumor-initiating and chemoradioresistant properties [7, 16]. Nevertheless, an effective chemotherapeutic approach targeting HNC-TICs to improve HNC-related malignancies is urgently required.

Epithelial–mesenchymal transition (EMT), a dedifferentiation program that converts adherent epithelial cells into individual migratory cells, is thought to be a key step in the induction of tumor malignancy, oncogenic progression, and cancer metastasis [17]. The interplay between EMT and stemness signature has gained huge interest in the field of cancer research recently [18]. Researchers have shown that EMT could promote stemness properties and further generate cells with the features of breast TICs [19]. Later on, single or co-overexpression or knockdown of stemness factors, including Oct4 and Sox2, were associated with cancer metastasis [20]. These results provide a crucial link among metastasis, EMT, and stem cell properties.

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a natural polyphenolic polyphenol found mostly in red wine that possesses pharmacological properties including antitumor, anti-inflammatory properties, pharmacological antiaging activities, and lifespan extension capacity [21, 22]. Interestingly, resveratrol also inhibits tumor-initiating stem-like cells properties in breast [23], glioblastoma [24, 25], and pancreatic cancer [26]. However, the mechanisms of the resveratrol-mediated signaling pathways and possible therapeutic targets involved in cancer stem-like properties of HNC-TICs need to be further investigated. Therefore, the purpose of the present study was to demonstrate the chemopreventive and chemotherapeutic effect of resveratrol in the maintenance of stemness and tumorigenicity of HNC-TICs in vitro and in vivo and to explore the mechanisms by which the resveratrol is responsible for stem-like properties in HNC.

2 Materials and methods

2.1 Reagents

Resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in DMSO as a stock solution of 100 mM. Just before use, resveratrol was further diluted in culture medium to appropriate final concentrations [25].

2.2 Cell proliferation/survival determination by MTT assay

SG, SAS, or OECM1 cells were plated in wells of 96-well plate as 1×10^4 cells/well in 0.1% DMSO or different concentration of resveratrol-containing medium and cultured at 37°C for 24 h. Cell proliferation/survival was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The 570-nm absorbance of DMSO-treated group was set as 100% and data were presented as percentage of DMSO control.

2.3 Enrichment of tumor sphere-forming (HNC-TICs)

For enrichment of HNC-TICs, the two cell lines were cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL human recombinant fibroblast growth factor-basic (FGF), and 10 ng/mL epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN). The two cell lines were then cultured in tumor sphere medium consisting of serum-free DMEM/F12 medium (GIBCO), N2 supplement (GIBCO), 10 ng/mL human recombinant fibroblast growth factor-basic (FGF), and 10 ng/mL EGF (R&D Systems). Cells were plated at a density of 10^3 live cells/low-attachment six-well plate (Corning Inc., Corning, NY), and the medium was changed every other day until the tumor sphere formation was observed in about 2 weeks. For serial passage of spheroid cells, single cells will be obtained from accurtase-treated spheroids and the cell density of passage will be 1000 cells/mL in the serum-free medium as described above [6, 9].

2.4 Aldefluor assay

Aldefluor assay kit is purchased from StemCell Technologies, Inc. (Vancouver, BC, Canada) 1×10^5 cells will be suspended in 50 μL of assay buffer and added Aldefluor to final concentration of 1 μM. For ALDH1 inhibitor control, diethylaminobenzaldehyde (DEAB) will be added to final concentration of 150 μM. Cells will be then incubated at 37°C for 45 min and stained with 7-AAD on ice for further 5 min. After washing with PBS, green fluorescence positive cells in live cells (7-AAD) will be analyzed by flow cytometry (FACSCalibur™, BD Biosciences, San Jose, CA, USA) by comparing the fluorescence intensity of DEAB-treated sample and these cells will be represented as cells with high ALDH activity (ALDH+ cells) [6, 9].

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2.5 Quantitative real-time reverse transcriptase (RT)-PCR

Total RNA was prepared from cells or tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). qRT-PCRs of mRNAs were reverse transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems). Primer sequences are listed in Supporting Information Table S1.

2.6 Western blot

The extraction of proteins from cells and western blot analysis was performed as described. Samples (15 μL) were boiled at 95°C for 5 min and separated by 10% SDS-PAGE. The proteins were wet transferred to Hybond-ECL nitrocellulose paper (Amersham, Arlington Heights, IL). The following primary antibodies were used: rabbit anti-human Oct4, rabbit anti-human Nanog, rabbit anti-human Nestin, mouse anti-human Slug (Cell Signaling Technology, Beverly, MA, USA); mouse anti-human N-cadherin, mouse anti-human Vimentin (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-GAPDH (MDBio, Inc., Taipei, Taiwan);. Immunoreactive protein bands were detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences Co., Piscataway, NJ) [6, 9].

2.7 In vitro cell invasion analysis

The 24-well plate Transwell® system with a polycarbonate filter membrane of 8-μm pore size (Corning, UK) was employed to evaluate the invasion ability of cells. The membrane was coated with Matrigel™ (BD Pharmingen, NJ).The cancer cell suspensions were seeded to the upper compartment of the Transwell chamber at the cell density of 1×10^5 in 100 μL within serum-free medium. The lower chamber was filled with serum-free medium. or media with 10% serum After 24 h of incubation, the medium was removed and the filter membrane was fixed with 4% formalin for 1 h. Subsequently, the remaining cells of the filter membrane facing the lower chamber were stained with Hoechst 33258 (Sigma-Aldrich, San Louis, MO, USA). The migrated cancer cells were then visualized and counted from five different visual areas of 100-fold magnification under an inverted microscope.

2.8 Apoptotic assay

Apoptotic cells were detected with an Annexin V-APC kit (Calbiochem, Darmstadt, Germany) according to manufacturer's guidelines. After staining, the cells incubated with 20 μg/mL propidum iodide (PI) were analyzed by FACS Calibur apparatus (Becton Dickinson, San Diego, CA) [8].

2.9 Soft agar colony-forming assay

Six-well culture dish was coated with 2 mL bottom agar (Sigma-Aldrich) mixture (DMEM, 10% [v/v] FCS, 0.6% [w/v] agar). After the bottom layer was solidified, 2 mL top agar-medium mixture (DMEM, 10% [v/v] FCS, 0.3% [w/v] agar) containing 2×10^4 cells was added, and the dishes were incubated at 37°C for 4 weeks. Plates were stained with 0.005% Crystal Violet, then the colonies were counted. The number of total colonies with a diameter $\geq$100 μm was counted over five fields per well for a total of 15 fields in triplicate experiments [6].

2.10 Bioluminescence imaging measurement of tumor growth in nude mice

All procedures involving animals were in accordance with the institutional animal welfare guidelines of the Chung Shan Medical University. For the nude mice xenograft model, 5–6 weeks old immunodeficient nude mice (BALB/c nu/nu mice) weighing 18–22 g were used. The mice were housed with a regular 12 h light/12 h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO) and were kept in a pathogen-free environment at the Laboratory Animal Unit. SAS-derived sphere-forming HNC-TICs (1×10^5 cells/0.2 mL/mouse) were injected subcutaneously into the right front axilla. Eight days postimplantation, the mice were randomly divided into three groups ($N = 5$ for each group) and fed by oral gavage with saline (control) and resveratrol (20 and 40 mg/day/kg) suspended in saline. The day of cell implantation was designated day 0. Bioluminescence imaging (BLI) was performed using an IVIS50 animal imaging system (Xenogen Corp., Alameda, CA, USA). The photons emitted from the target site penetrated through the mammalian tissue and could be externally detected and quantified using a sensitive light imaging system. The image acquisition time was 1 min. The displayed images of the tumor sites were drawn around and quantified in photons per second using Living Image software (Xenogen Corp.) The volume was calculated (according to the following formula: ([length $\times$ width^2]/2), and then analyzed using Image-Pro Plus software. Body weight was assessed daily after cell injection. After 20 days, the animals were euthanized, and the primary tumors were isolated and weighed.

2.11 Immunohistochemistry

Tissue samples were spotted on glass slides for immunohistochemical staining. After deparaffinization and rehydration, tissue sections were processed with antigen retrieval by 1× Trilogy diluted in H_2O (Biogenics, Napa, CA, USA) with heating. The slides were immersed in 3% H_2O_2 for 10 min and washed with PBS three times. Tissue sections were blocked with serum (Vestastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min, then incubated with the

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primary antibody anti-human Oct-4, anti-human Nestin, anti-human E-cadherin, and mouse anti-human Vimentin (Cell Signaling Technology) in PBS solution at room temperature for 2 h. Tissue slides were washed with PBS and incubated with biotin-labeled secondary antibody for 30 min, then incubated with streptavidin-horseradish peroxidase conjugates for 30 min, and washed with PBS three times. Tissue sections were then immersed with chromogen 3-3′-diaminobenzidine plus H_2O_2 substrate solution (Vector® DBA/Ni substrate kit, SK-4100, Vector Laboratories) for 10 min. Hematoxylin was applied for counterstaining (Sigma Chemical Co.). Finally, the tumor sections were mounted with a cover slide with Gurr® (BDH Laboratory Supplies, UK) and examined under a microscope. Pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis.

2.12 Statistical analysis

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL) was used for statistical analysis. Student's *t*-test was used to determine statistical significance of the differences between experimental groups; *p* values less than 0.05 were considered statistically significant. The level of statistical significance was set at 0.05 for all tests.

3 Results

3.1 The cell viability effect of resveratrol in normal epithelial gingival and head and neck cells

We first determined whether resveratrol has any cytotoxic effect to normal head and neck gingival epithelial cells (SG) and head and neck cells (SAS and OECM1). As shown in Fig. 1, resveratrol could suppress cell proliferation of SAS and OECM1 HNC cells in a dose-dependent manner by MTT assay. The effect of resveratrol on normal gingival epithelial SG cells revealed that this compound did not have any significant cytotoxicity on these cells (Fig. 1).

3.2 Resveratrol effectively eliminates ALDH1 and CD44 positivity in HNC cells

Both intracellular ALDH1+ and CD44+ cells have been proposed to exhibit TICs stem-like properties and have been used as TICs functional markers for HNC-TICs from HNC cells [4, 7]. Ginestier et al. used ALDH1+/CD44+ cells to selectively isolate CSCs from breast cancer patient samples and found that only 20 CD44+CD24-Lin-B38+ALDH1+ cells were required to regenerate a new tumor [12]. In previous studies, ALDH1 and CD44 could be used as potential markers for the isolation of HNC-TICs that possess CSC-like

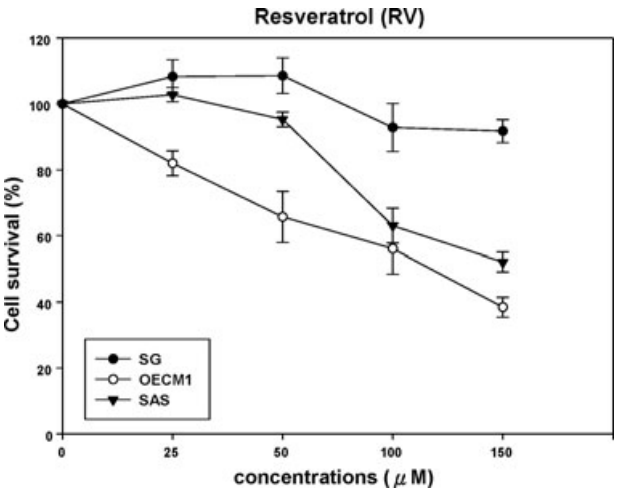

Figure 1. The cytotoxic effect of resveratrol in normal head and neck epithelial and HNC cells. Normal epithelial gingival cells SG and head and neck cancer cells (OECM1 and SAS) cells were plated as 1×10^4 cells/well in 96-well plate and treated with different concentration of resveratrol or 0.1% DMSO for 24 h. Cell proliferation/survival was determined by MTT assay. The 570-nm absorbance of DMSO-treated group was set as 100% and data were presented as percentage of DMSO control.

properties and are resistant to chemoradiotherapeutic treatment [7, 16]. Next, we examined the effects of resveratrol on ALDH activity in established HNC cancer cell lines (SAS and OECM1) by Aldefluor assay. Our data suggested resveratrol treatment significantly decreases ALDH1 activity of both HNC cells (SAS and OECM1) in a concentration-dependent manner (Fig. 2A and 2B). Similarly, the expression of CD44 was also decreased in HNC cells with dose-dependent resveratrol treatment (Fig. 2C). In summary, downregulation of both ALDH1 and CD44 suggests resveratrol treatment as potential compound on targeting TICs in HNC.

3.3 Inhibition of self-renewal capacity and stemness gene signatures in HNC-TICs under resveratrol treatment

Successful sphere formation phenotypes after serial passages of culture, one of indexes for evaluating the persistent self-renewal property of TICs, showed the self-renewal capacity of all HNC-TICs [6, 9]. To investigate the effect of resveratrol in targeting self-renewal or cancer stem-like properties of HNC-TICs, we evaluated the sphere-forming ability with resveratrol treatment in primary tumor sphere-forming HNC-TICs. In HNC-TICs, dose-dependently treated with resveratrol, the sphere-forming ability among the primary secondary and tertiary spheres was consistently impaired (Fig. 3A and 3B). To further determine whether the reduction in tumor sphere formation efficiency with resveratrol treatment was due to decreased stemness markers expression, stemness genes

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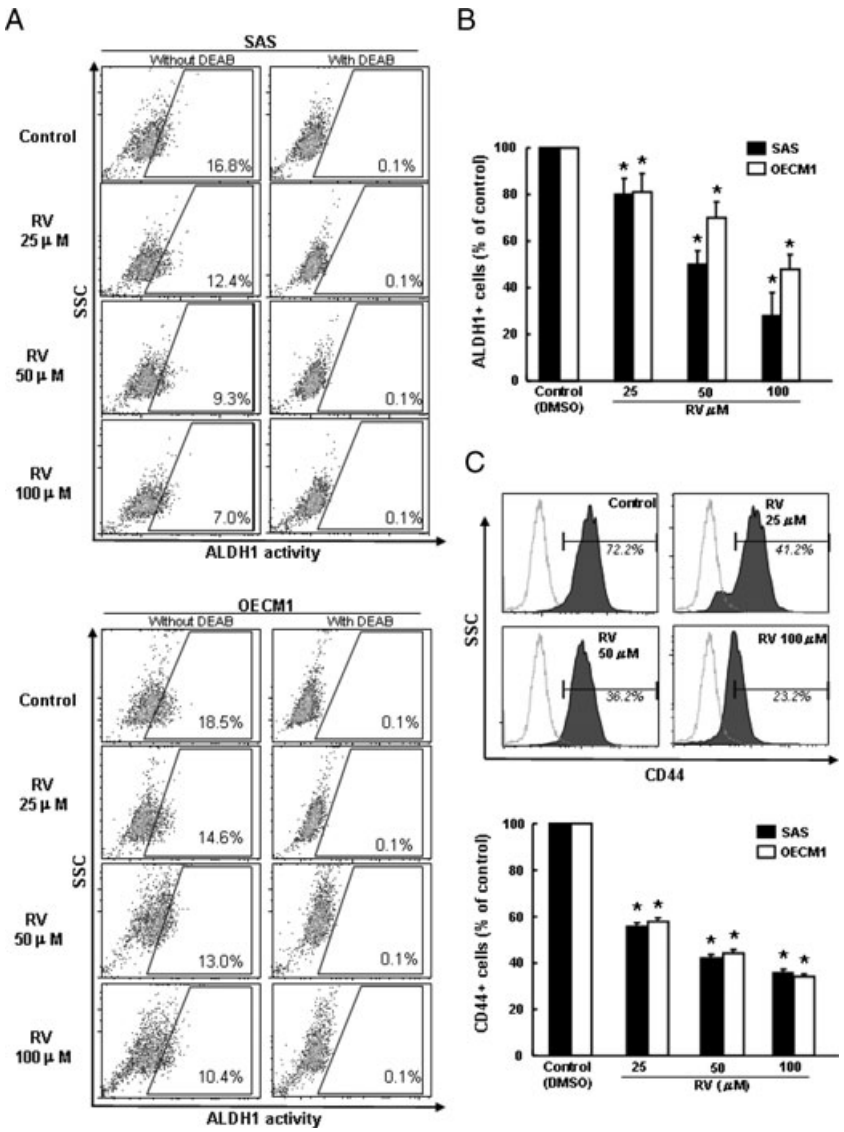

Figure 2. Resveratrol efficiently eliminates ALDH1-positive cells in SAS and OECM1 cells in dose-dependent manner. (A) HNC cells (SAS and OECM1) were seeded as 2×10^5 cells/well in six-well plate and treated with different concentration of resveratrol or DMSO control. ALDH+ cells were determined by Alderfluor assay and viable cells (7-AAD negative) were used for analysis. DEAB-treated cells were used as negative control. (B) The quantification results were shown in bar graph. (C) The expression of CD44 positivity of control and different concentration of resveratrol-treated HNC cells was determined by flow cytometry analysis. Data shown here are the mean ± SD of three independent experiments. $*p < 0.05$ versus control.

(Oct-4, Nanog, and Nestin) of HNC-TICs with control (DMSO), and different concentration of resveratrol treatment were determined by real-time PCR and western blot analysis. The results confirmed that resveratrol-treated HNC-TICs markedly reduced the mRNA and protein expression level of Oct-4, Nanog, and Nestin in both HNC-TICs (Fig. 3C and Fig. 3D).

3.4 Resveratrol abrogated metastatic capacity of HNC-TICs by downregulation of mesenchymal transformation marker

Since TICs appear to play a significant role in tumorigenesis and metastasis [27], we sought to measure the effects of resveratrol on anchorage-independent growth and invasion ability of HNC-TICs. Single cell suspension of control or resveratrol-treated HNC-TICs was used for analysis of their metastatic capacity in vitro as described in Materials and Methods section. Overall, our data indicate that resveratrol inhibits tumor-initiating activity including invasion and colony formation abilities of HNC-TICs in vitro (Fig. 4A and 4B). HNC epithelial cells can acquire mesenchymal traits that facilitate migration and invasion through EMT process [28]. It is known that EMT can give rise to cells with stem cell properties, and tumor stem cells that have undergone EMT are therefore more motile and metastasized [18]. Since we have found the effect of resveratrol on migratory ability in HNC, we keep on exploring whether the resveratrol-mediated TICs depends on EMT pathway. Real-time PCR analysis and western blotting demonstrated downregulation of mesenchymal-like protein (Slug, ZEB1, N-cadherin, and vimentin) and upregulation of epithelial protein (E-cadherin) was seen in HNC-TICs with resveratrol treatment (Fig. 4C

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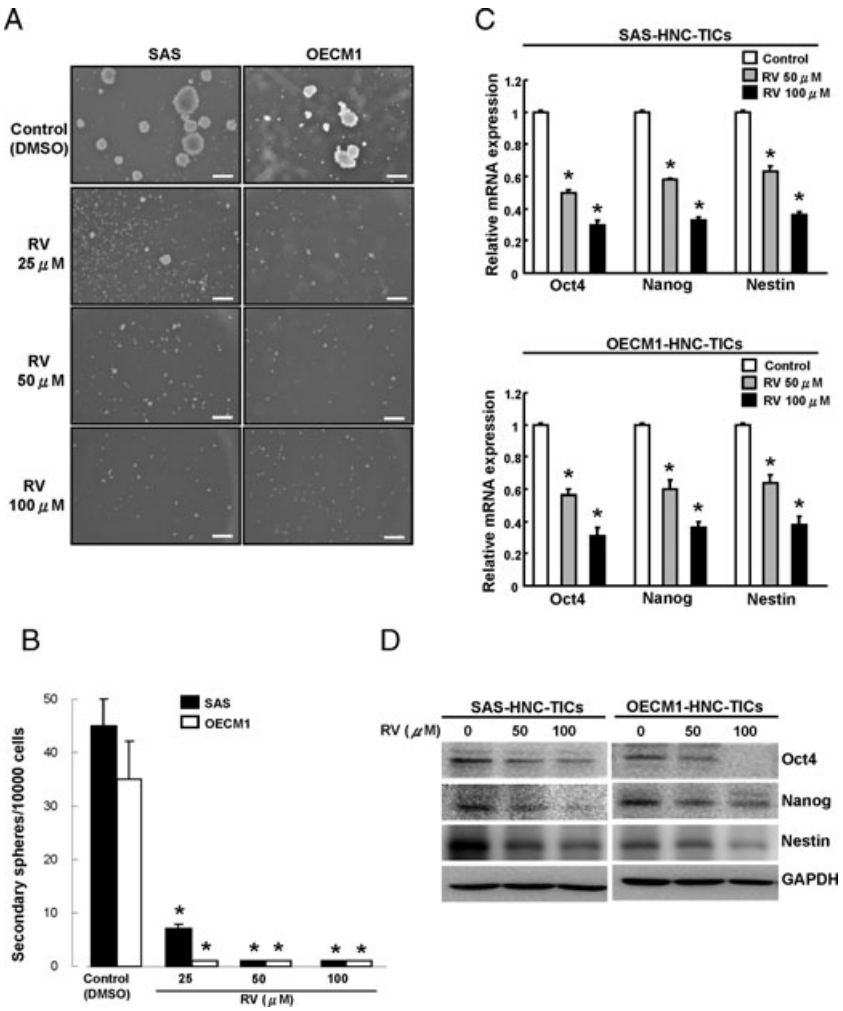

Figure 3. Self-renewal sphere-forming ability and stemness markers were inhibited by resveratrol in dose-dependent manner. SAS or OECM1 was suspended as 1×10^4/mL in sphere culture medium as described in Materials and Methods section, plated into low attachment plate in presence of 0.1% DMSO or indicated concentration of resveratrol and cultured for 1 week allowing the formation of primary spheres. (A) For self-renewal analysis, single cell suspension was obtained from primary spheres by accutase digestion and secondary sphere formation capacity was determined with primary sphere culture procedure except the plating cell density as 10 000 cells/mL. The experiments were repeated three times and representative results were shown. Results are presented as means ± SD. (B) SAS or OECM1-derived HNC-TICs treated with 0.1% DMSO or indicated concentration of resveratrol for further 24 h and analyzed transcripts and protein level of Oct-4, Nanog, and Nestin by real-time RT-PCR (C) and immunoblotting analysis (D), respectively. $*p < 0.05$ versus control.

and 4D). In Summary, our results suggested that resveratrol showed switch regulating TICs properties, in which it regulated mesenchymal state.

3.5 Enhanced apoptosis capability of HNC-TICs by resveratrol treatment

To further determine whether the reduction in TICs efficiency with resveratrol treatment is due to decreased HNC-TIC survival, we determined the percentage of apoptotic cells using Annexin V staining. SAS- and OECM1-derived HNC-TICs under resveratrol treatment increased the percentage of apoptotic Annexin V-positive cells in a dose-dependent manner (Fig. 5A). The caspase 3 activity in HNC-TICs was also enhanced by resveratrol treatment in a dose-dependent manner (Fig. 5B). Furthermore, resveratrol treatment dose dependently suppressed the transcript expression of Bcl-2 and cyclin D1 by real-time RT-PCR anaysis (Fig. 5C). Accordingly, the western blotting data showed that resveratrol treatment downregulated protein levels of Bcl-2 and cyclin D1 and upregulated caspase 3 expression (Fig. 5D). These data suggest that resveratrol induce a cell death program in HNC-TICs through apoptosis.

3.6 Enhanced chemosensitivity in HNC-TICs by resveratrol treatment

Resistance to chemotherapies treatment is a major clinical criteria for characterizing TICs in malignant tumors including HNC cancer [29]. The findings that resveratrol diminished TICs properties suggested resveratrol as chemoadjuvant in modulating the chemoresistant properties of HNC-TICs. As expected, HNC-TICs were more chemoresistant compared to the parental HNC cells. Importantly, cell viability assays showed that resveratrol ameliorated the drug resistance of HNC-TICs to cisplatin or fluorouracil (5-FU) treatment (Fig. 6A). Notably, coadministration of resveratrol and cisplatin led to the maximal inhibition of colony-forming ability in HNC-TICs (Fig. 6B). Similar synergistic effect of resveratrol delivery and chemotreatment was also observed in

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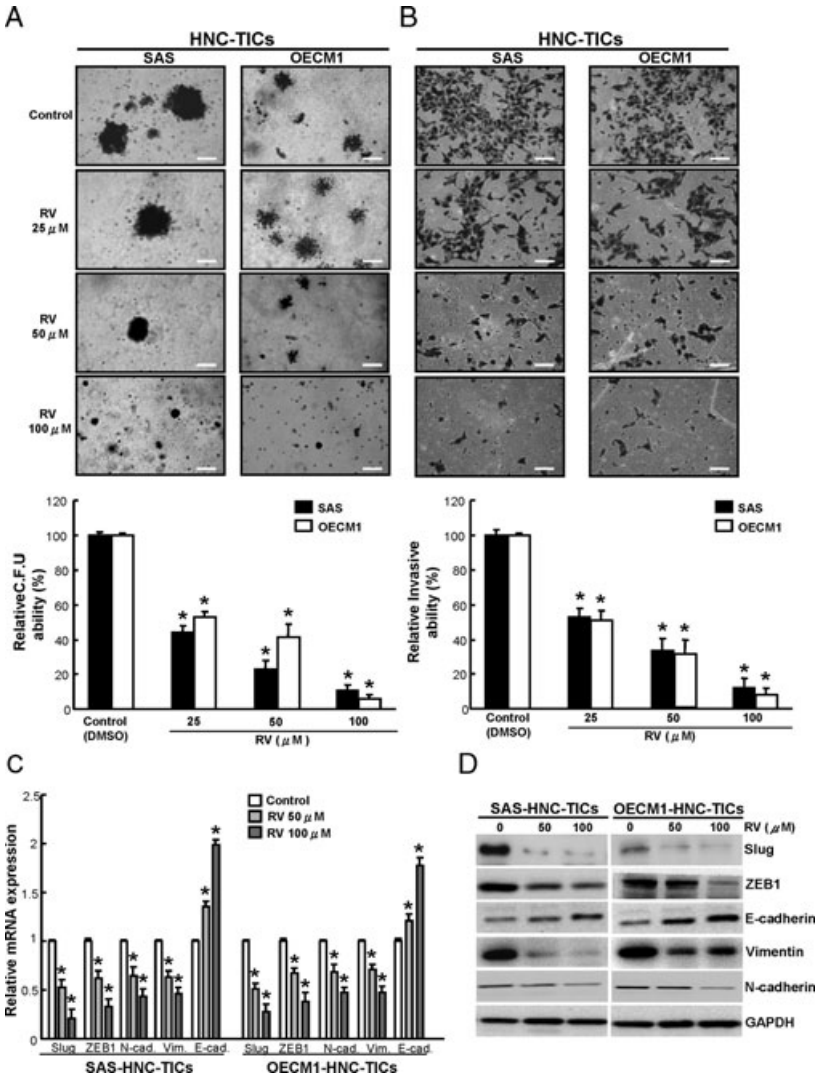

Figure 4. Resveratrol treatment inhibited invasiveness and clonogenicity in HNC-TICs cells by downregulation expression of mesenchymal markers. Control HNC-TICs (SAS and OECM1) and different concentration resveratrol-treated HNC-TICs were subjected to soft agar colony formation assay (A) and matrix invasion assay. (B) Quantitative RT-PCR (C) and immunoblotting analysis (D) of EMT-related markers (Slug, ZEB1, E-cadherin Vimentin, and N-cadherin) in control and resveratrol-treated HNC-TICs was determined. The amount of GAPDH protein of different crude cell extracts was referred as loading control for further quantification. The quantification results were shown in bar graph. $*p < 0.05$ versus control.

cell invasion assay (Fig. 6C). The observation of resveratrol-mediated regulation of the TICs population and its properties suggested their involvement in modulating the chemoresistance of HNC-TICs. Taken together, resveratrol delivery exhibited a prominent therapeutic effect in enhancing the sensitivity of chemotherapy in HNC through elimination of TICs.

3.7 Chemotherapeutic delivery of resveratrol in HNC-TICs-transplanted mice by oral gavage attenuated tumor progression in vivo

To verify the antitumor effects of resveratrol against HNC-TICs in vivo, HNC-TICs-bearing nude mice were treated with saline or resveratrol by oral gavage. Notably, tumor formation in all recipients was reduced following xenotransplantation of sphere-forming HNC-TICs that received oral gavage resveratrol treatment on day 20 as compared to control animals (Fig. 7A). Moreover, by day 20, resveratrol feeding (40 mg) induced a reduction in tumor volume (Fig. 7B) and tumor weight (Fig. 7C) and without any apparent signs of toxicity as evidenced by body weight monitoring (Fig. 7D) throughout the experiment. Histochemical analysis of the pathologic sections of these tumors showed that resveratrol-treated tumor had low level of Oct4, Nestin, Vimentin and high levels of E-cadherin compared to control HNC-TICs tumors (Fig. 7E). These data suggest that resveratrol treatment impaired tumor-initiating activity, stemnness signature, and EMT properties in vivo.

4 Discussion

HNC bearing dismal prognosis is a lethal cancer with clinical, pathological, phenotypical, and biological heterogeneity [1]. Most patients, however, relapse within months after radiochemotherapy [2]. The prognosis of HNC patients under

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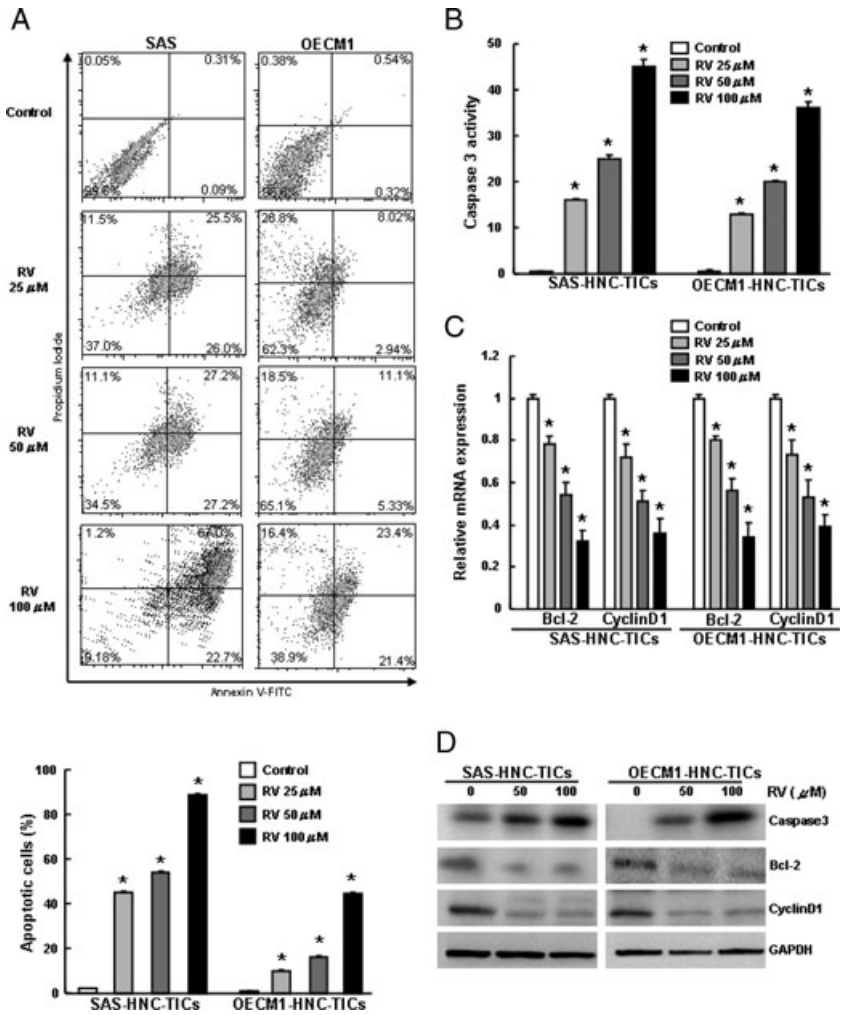

Figure 5. Induction of apoptosis in HNC-TICs after resveratrol treatment. (A) The expression Annexin V positive staining of apoptotic cells in HNC-TICs treated with different concentration of resveratrol or DMSO control was assessed, respectively, by flow cytometry. (B) ELISA analysis of caspase 3 activity was determined in HNC-TICs after treatment with the resveratrol or DMSO control. Transcript and protein expression level of Bcl-2 and cyclinD1 in HNC-TICs treated with different concentrations of resveratrol was examined by real-time RT-PCR (C) and western blotting (D), respectively. The experiments were repeated three times and representative results were shown. Results are means ± SD. $*p < 0.05$ versus control.

current treatment protocols remains poor and more than 50% of patients die of this disease or related complications within 5 years [2]. Tumor is composed of a heterogeneous population of cells, and it has been observed that a subpopulation of cells, so called cancer stem cells (CSCs) or TICs, within tumor tissues have stemness properties [27, 29]. TICs are considered to be responsible for the initiation, propagation, and metastasis of tumors [27, 29]. Importantly, the existence of TICs might explain cancer recurrences, even after clinical treatment with either radiotherapy or chemotherapy on cancer patients [27, 29]. Therefore, searching the novel compound targeting TICs that hopefully improve therapeutic efficacies and increase the patient survival rate has become a prospective direction for cancer therapy development [30]. Recent studies have shown that resveratrol-induced apoptosis not only inhibited tumor growth but also acted as a radiochemosensitizer for anticancer therapy [31]. Importantly, resveratrol showed the potential to block constitutive Src/STAT3-axis signaling in malignant cancer cells as well as suppress the expression of phosphorylated STAT3 (p-STAT3) and downstream genes (survivin, cyclin D1, Cox-2, and c-Myc) in cancer lines [25, 32].

In the present report, we first showed resveratrol provided a chemotherapeutic effect in HNC-TICs by inhibiting the TICs-like and EMT properties of HNC, such as the self-renewal, stemness signature, and in vitro and in vivo tumorigenecity. A further understanding on the effect of resveratrol on regulating EMT and stemness signaling may update our current knowledge on the development of therapeutic treatments for malignant cancers in the future.

The EMT process disrupts E-cadherin-mediated cell–cell adhesion during embryonic development, and changes the cell phenotype into a more loosely mesenchymal-like cell, leading to the invasion of extracellular matrix [17]. Intensive studies revealed that transcriptional factors, such as Snail, Slug, and Twist regulate EMT process [33]. The key signaling pathways of EMT include receptor tyrosine kinases, transforming growth factor (TGF)-β superfamily, Wnt, Notch, hedgehog pathway, and NF-κB [33]. In addition to development roles, EMT occurs in both physiological and pathological conditions [33]. For example, keratinocytes at wound tissues display migratory behavior to accelerate wound healing. From a tumor in situ to an invasive carcinoma, epithelial

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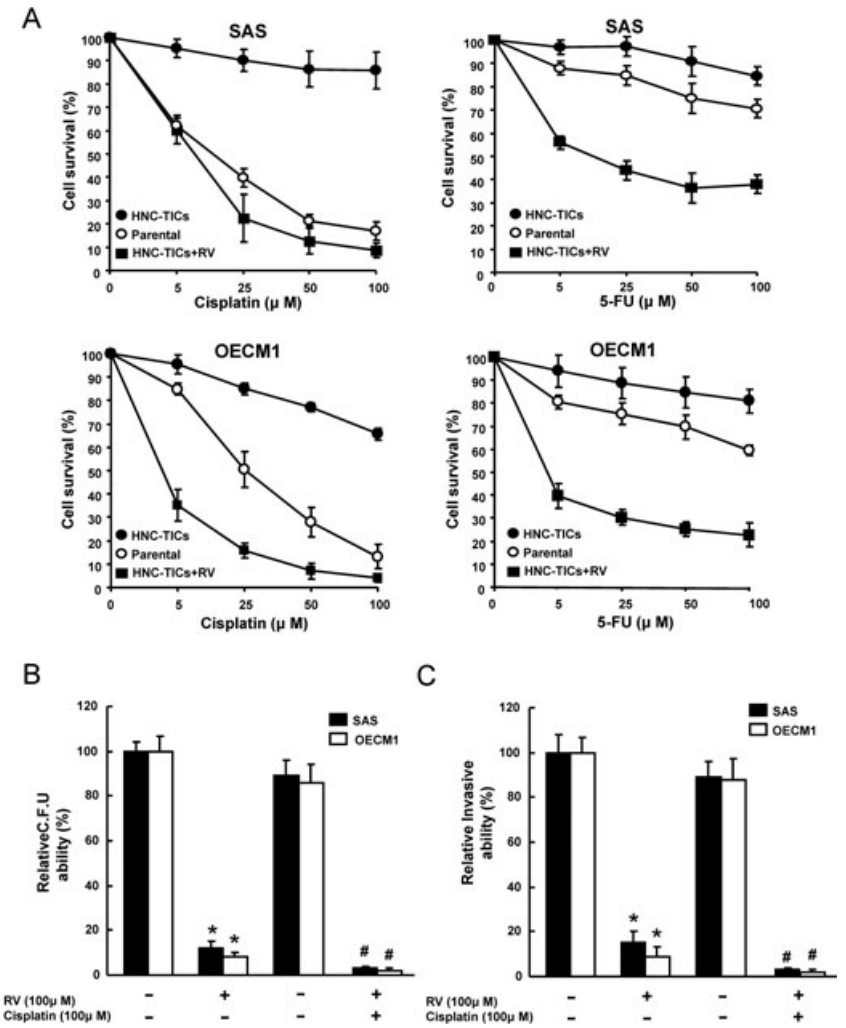

Figure 6. Resveratrol restored chemosensitivity in HNC-TICs. Parental HNC cells or HNC-TIC cells with control or resveratrol were subjected to treatment with different concentrations of cisplatin or 5-FU. Cell viability was determined by MTT assay. (A) Colony-forming ability (B) and invasion ability (C) was assessed in HNC-TIC cells synergetically treated with resveratrol combined with cisplatin chemotherapy. $*p < 0.05$ RV versus HNC-TICs; $\#p < 0.05$ RV + Cisplatin versus RV alone.

tumor cells will lose the contact from their neighbors with decreased expression of E-cadherin through EMT program [17, 33]. Furthermore, EMT program also governs the development of tumor-initiating stem cells, a subpopulation of cancer cells that is responsible for cancer initiation, drug resistance, and metastasis [18, 19]. Aberrant enhanced EMT characteristic is associated with poor overall and metastasis-free survival in patients with HNC [28]. Recently, a chemical screening for compounds that selectively kill breast TICs using knockdown E-cadherin expression of immortalized mammary epithelial cells is conducted and identified salinomycin could also efficiently suppress the metastatic ability of breast cancer cells in both mouse breast cancer and human xenograft [34]. The discovered roles of stemness signature in EMT process have gained huge interest in the field of cancer research as it indicates that misplaced stemness properties contribute to tumor metastasis and recurrence, making cancer difficult to be tackled. However, numerous phytochemicals have been identified as blockers of EMT and tumor-initiating stem cells, including synthetic molecules and plant polyphenols. Inhibition of TICs characteristics in pancreatic and prostate cancer by epigallocatechin-3-gallate (EGCG) synergizes with quercetin treatment [35]. Curcumin, piperine, and sulforaphane have also been found to inhibit the cancer stem cells property in breast cancer cells through Wnt pathway [36, 37]. Lupeol effectively eradicates TICs in liver cancer cells by PTEN regulation. Genistein represses sphere-forming ability in breast TICs [38]. Moreover, a better understanding of the regulatory networks among EMT, stemness signature, and CSC putative markers may enhance the development of therapies for malignant cancers. These findings make natural dietary agents as a switch worth further investigation in reversal EMT and antimetastasis of tumors.

However, resveratrol treatment did not completely eradicate tumorigenesis of HNC-TICs in vivo (Fig. 6). It is possible that EMT signaling is among one of the molecular mechanisms in regulation of HNC-TICs. With the increasing awareness of the importance of miRNAs in tumorigenicity, accumulating evidence has been reported supporting the involvement of miRNAs in CSC-like TICs properties. For example,

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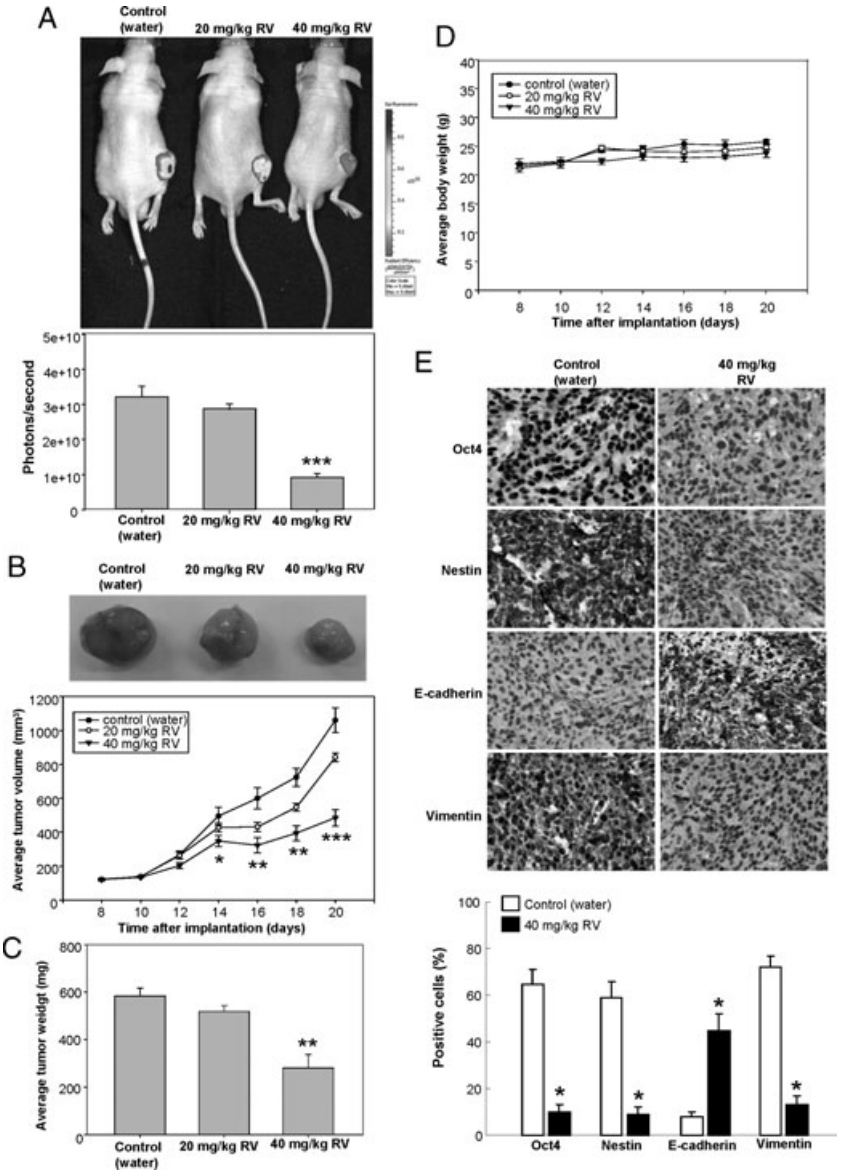

Figure 7. Resveratrol attenuated tumor progression of HNC-TICs-derived xenograft tumors in vivo. After subcutaneous implantation of HNC-TICs, HNC-TICs-derived xenograft was treated with control (water), 20 mg/kg/day resveratrol, or 40 mg/kg/day resveratrol by oral gavage and then analyzed for (A) IVIS imagining system, (B) tumor volume, (C) average tumor weight, (D) and average body weight. (E) HNC-TICs-derived xenograft were removed and sectioned. The tissue samples were stained with Oct4, Nestin, E-cadherin, and Vimentin by immunohistochemistry analysis, respectively (upper panel). The quantitative result of IHC results was determined (lower panel). The values represented the means (SD). Comparisons were performed by Student's *t*-test. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.01$).

ectopic induction of let-7, miR200c, miR34a, or miR145 suppresses the tumorigenicity of TICs [39–43]. Iliopoulos and colleagues reported that miR200b regulates CSC properties through directly targeting Suz12, a subunit of a polycomb repressor complex [44]. Thus, more studies are needed to investigate the potential cross-linking of resveratrol signaling with microRNAs epigenetic regulation in HNC-TICs in the future.

Collectively, the present report showed that the resveratrol effectively suppressed self-renewal, tumor-initiating, and chemoresistance properties of HNC-derived TICs in vitro and in vivo partially through EMT modulation. The promising therapeutic prospect of resveratrol for treating TICs may render it a potential approach to improve current head and cancer treatments, especially for those tumors that have developed a resistance to conventional therapeutic methods. Further in-depth evaluation of the therapeutic possibilities of resvertrol in HNC or other solid tumors is greatly needed clinically, especially in cancers that are resistant to conventional therapies.

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