Sonic hedgehog pathway mediates genistein inhibition of renal cancer stem cells

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Abstract. Cancer stem cells (CSCs) have been implicated in the genesis, progression and recurrence of renal cancer. The sonic hedgehog (Shh) pathway serves a critical role in maintaining the stemness of CSCs. Genistein, a major isoflavone component extracted from soybeans and soy products, has been demonstrated to possess anticancer activity. However, the effects of genistein on renal CSCs and its underlying mechanisms remain to be fully elucidated. The aim of the present study was to investigate the role of the Shh pathway in genistein inhibition of renal CSCs. The results of the present study demonstrated that expression levels of renal CSC markers were markedly upregulated in the sphere-forming cells, which were isolated and enriched from 786-O and ACHN cells in a tumor sphere formation assay, and more cells were arrested at the G_0/G_1 phase instead of the S_1 phase compared with the adherent cells. Furthermore, the present study demonstrated that genistein could effectively diminish the activity of renal CSCs by suppressing tumor sphere formation, decreasing renal CSCs markers, inhibiting proliferation and inducing apoptosis. Additionally, the downregulation of Shh pathway activity could inhibit renal CSCs. Genistein exhibited an inhibitory effect on renal CSCs by attenuating the activation of the Shh pathway. In conclusion, the results illustrated the role of the Shh pathway in regulating renal CSC traits and the intervention of renal CSCs by genistein, which could provide novel insights into the molecular mechanisms of renal CSC intervention.

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Introduction

Renal cancer is a deadly disease with a global annual incidence of 143,000 cases of cancer-associated mortality, with the 9th highest incidence rate in men and 14th highest in women, as reported in 2012 (1). Renal cell carcinoma (RCC) is the most common form of renal neoplasm, accounting for >90% of cases in adults regardless of sex, worldwide (1). The high metastatic rate of RCC and its resistance to traditional chemotherapy and radiotherapy results in an unpredictable presentation as well as adverse clinical outcomes. Although surgical treatment remains the gold standard for treating primary RCC, 30-40% patients may undergo progression or recurrence following the initial radical nephrectomy, and the 5-year survival rate of metastatic patients was only 12% in 2015 (2). Therefore, there is an urgent need to investigate novel approaches for treating RCC.

Cancer stem cells (CSCs), a minor initiating cell subpopulation within solid tumors, are associated with unique characteristics, including clonogenesis, high self-renewal capacity and multilineage differentiation properties (3). Emerging evidence has demonstrated that CSCs are implicated in tumorigenesis, progression, chemoresistance and relapse of malignancies (3-5). To date, CSCs have been isolated and identified in various types of solid malignancy or established cell lines, including glioblastoma, breast cancer and renal cancer (6-8). Bussolati *et al* (9) isolated renal CSCs from the tumor samples of patients with clear cell RCC using fluorescence-activated cell sorting in 2008. Subsequently, Zhong *et al* (10) enriched CSCs from the RCC cell line SK-RC-42 using a sphere-formation assay. In addition, Huang *et al* (11) identified a cancer stem-like cell side population in the 769P cell line. Therefore, understanding the biology of renal CSCs for selective targeting can be an effective interventional strategy in preventing and treating renal cancer.

The sonic hedgehog (Shh) pathway has been considered as an oncogenic pathway, serving a crucial role in the maintenance of tissue polarity and CSC properties (12). Aberrant activation of the Shh pathway has been reported to be involved in various

types of human malignancy (13). The binding of hedgehog ligands, including Shh protein, to the transmembrane receptor patched 1 (PTCH) results in the activation of the Shh pathway. PTCH represses the activity of the transmembrane protein smoothened (Smo) in the absence of Shh protein, which can thereby activate an endocellular signal transduction cascade via the glioma-associated oncogene (Gli) transcription factor family, including Gli1, Gli2 and Gli3 (14). Gli1 acts as a transcription activator, while Gli2 and Gli3 can function either as repressors or activators depending on the context (14,15).

Recently, numerous studies have illustrated that dietary factors exert chemo-preventative functions and can decrease the risk of multiple types of cancer (16-18). Notably, certain botanical compounds can directly regulate CSC activity; compounds that have now been identified as promising anticancer and therapeutic agents. Among these compounds, genistein (4,5,7-trihydroxyisoflavone), a major isoflavone component that can be isolated from soybeans and soy products, possesses anticancer potential by targeting the CSCs of different tumors (19,20). Genistein exerts anticancer effects on various types of malignancy, including breast, colon and ovarian cancer (19,21,22). Additionally, it has been reported that genistein inhibits the pluripotency of prostate and gastric cancer cells by targeting the hedgehog-Gli1 pathway (20,23).

However, to the best of our knowledge, the anticancer properties of genistein against renal CSCs remain to be elucidated. Therefore, the aim of the present study was to evaluate the anticancer effects of genistein on renal CSCs, and to evaluate whether the Shh pathway may be involved in the genistein-induced anticancer activity. The findings of the present study may provide valuable avenues in the search for a potential interventional target of genistein in renal CSCs.

Materials and methods

Chemicals and reagents. Genistein and DMSO were purchased from Sigma-Aldrich; Merck KGaA. Vismodegib and purmorphamine were purchased from MedChemExpress and dissolved in DMSO at a stock solution concentration of 10 mM. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin were purchased from PeproTech, Inc.. In addition, 2% B27 was acquired from Gibco; Thermo Fisher Scientific, Inc..

The primary antibodies that were used in the present study, including Shh (cat. no. 20697-1-AP), Gli1 (cat. no. 66905-1-Ig), Gli2 (cat. no. 18989-1-AP), CD44 (cat. no. 15675-1-AP), CD133 (cat. no. 18470-1-AP), aldehyde dehydrogenase 1 family member A1 (ALDH1A1) (cat. no. 15910-1-AP), Oct4 (cat. no. 11263-1-AP), Nanog (cat. no. 14295-1-AP), cyclin D1 (cat. no. 60186-1-Ig), proliferating cell nuclear antigen (PCNA) (cat. no. 10205-2-AP), Bcl-2 (cat. no. 12789-1-AP), Bax (cat. no. 50599-2-Ig), and GAPDH (cat. no. 10494-1-AP) were purchased from ProteinTech, Inc.. Cleaved caspase 3 (cat. no. 9661), cleaved caspase 8 (cat. no. 8592) and cleaved caspase 9 (cat. no. 20750) were obtained from Cell Signaling Technology, Inc.. Smo (cat. no. DF5152) was obtained from Affinity Biosciences, Inc. Anti-rabbit (cat. no. SA00001-2) and anti-mouse (cat. no. SA00001-1) second antibodies were purchased from ProteinTech, Inc..

Cell culture and sphere formation assay. The renal cancer 786-O and ACHN cell lines were purchased from Type Culture Collection of the Chinese Academy of Sciences and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37°C with 5% CO_2.

The 786-O and ACHN cells were washed twice, seeded into a low-attachment plate at a density of 5,000 cells/well, and cultured at 37°C in a humidified incubator with 5% CO_2 in serum-free DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) containing 4 mg/ml insulin, 20 ng/ml EGF, 20 ng/ml bFGF, B27 (1:50 dilution) and 0.4% BSA (Beyotime Institute of Biotechnology). Half fresh medium was renewed and chemicals (genistein, vismodegib or purmorphamine) were added every other day. Tumor sphere formation was observed and images were captured using a light microscope (Nikon Corporation).

To investigate the effect of genistein and purmorphamine on the tumorsphere formation capacity of renal CSCs, various concentrations of genistein (0, 30, 60 and 90 μM) or purmorphamine (1 μM) were added to each well with 0.1% DMSO as the control. After 5 days treatment at 37°C in a humidified incubator with 5% CO_2, the number of tumor spheres in each group with a diameter >50 μm was counted.

Cell cycle analysis. Following culture in serum-supplemented medium (SSM) or serum-free medium (SFM) for 5 days, 786-O and ACHN cells were collected at a density of $1x10^6$/ml, and then fixed with 75% ice-cold ethanol at -20°C overnight. Subsequently, the cells were washed twice with PBS and treated with 20 mg/ml propidium iodide (PI; Invitrogen; Thermo Fisher Scientific, Inc.), 0.1% Triton X-100 (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.2 mg/ml RNase (PureLink; Thermo Fisher Scientific, Inc.) at room temperature in the dark for 15 min. Subsequently, flow cytometry (FCM) using a FACSCalibur™ flow cytometer (BD Biosciences) was used to analyze the cell cycle phase distribution and the percentage of cells in different phases. The data were analyzed using FlowJo software (v10.0.7; FlowJo LLC).

Western blotting. 786-O and ACHN tumor spheres were harvested and washed with ice-cold PBS. The cell precipitates were homogenized in RIPA buffer (Beyotime Institute of Biotechnology) containing complete protease inhibitors, followed by centrifugation at 15,000 x g for 30 min at 4°C. The supernatants were collected and denatured. BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.) was utilized to determine the concentration of each protein. The proteins (50 μg per lane) were separated via 10% SDS-PAGE and then transferred onto PVDF membranes (EMD Millipore). Subsequently, the PVDF membranes were treated with 5% skimmed milk at room temperature for 2 h and incubated with the primary antibodies against CD133 (1:500 dilution), Gli1 (1:500 dilution), Gli2 (1:500 dilution), CD44 (1:1,000 dilution), ALDH1A1 (1:1,000 dilution), CD44 (1:1,000 dilution), Oct4 (1:1,000 dilution), Nanog (1:1,000 dilution), Bcl2 (1:1,000 dilution), Bax (1:1,000 dilution), cleaved caspase 3 (1:1,000 dilution), cleaved caspase 9 (1:1,000 dilution), cleaved caspase 8 (1:1,000 dilution), cyclin D1 (1:1,000 dilution), PCNA (1:1,000 dilution), Shh (1:1,000 dilution), Smo (1:1,000

Table I. Oligonucleotide sequences of primers for reverse transcription-quantitative PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
CD133	TACAACGCCAAACCACGACTGT	TCTGAACCAATGGAATTCAAGACCCTTT
CD44	AGCAACCAAGAGGCAAGAAA	GTGTGGTTGAAATGGTGCTG
ALDH1A1	GCACGCCAGACTTACCTGTC	CCTCCTCAGTTGCAGGATTAAAG
Oct4	GACAACAATGAAAATCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA
Nanog	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
GAPDH	GACTGTGGATGGCCCCTCCGG	AGGTGGAGGAGTGGGTGTCGC

ALDH1A1, aldehyde dehydrogenase 1 family member A1.

dilution), and GAPDH (1:1,000 dilution) at 4˚C overnight. Membranes were rinsed and incubated with the corresponding peroxidase-conjugated secondary antibodies (1:5,000 dilution) for 1 h at 25˚C. Chemiluminescent detection was performed using an ECL kit (Beyotime Institute of Biotechnology). GAPDH was used as an internal control in order to measure the relative expression of each protein. ImageJ software (v1.46; National Institutes of Health) was used to quantify the protein bands of interest.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total cellular RNA was extracted from renal cancer adherent or tumor sphere cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and subsequently reverse-transcribed into cDNA using 5X All-In-One RT master mix (Applied Biological Materials, Inc.), according to the manufacturer's protocol, as follows: 25˚C for 10 min, 42˚C for 15 min for cDNA synthesis, then 85˚C for 5 min followed by chilling on ice. qPCR was performed using the Power SYBR Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a LC96 real-time PCR system. The amplification reactions were as follows: An initial hold step at 95˚C for 5 min and 45 cycles of a two-step PCR (95˚C for 15 sec, 54˚C for 30 sec and 72˚C for 30 sec). GAPDH levels were used as normalization controls. Fold changes in gene expression were calculated using a comparative threshold cycle (Cq) method using the formula $2^{-\Delta\Delta CT}$ (24). The forward and reverse primers used for qPCR are presented in Table I.

Detection of apoptotic cells by FCM. The apoptosis assay was performed using a fluorescein isothiocyanate (FITC)-Annexin-V apoptosis detection kit purchased from BD Biosciences. Following treatment with genistein at different concentrations (0, 30, 60 and 90 μM) for 5 days at 37˚C with 5% CO_2, 786-O tumor spheres were harvested and washed twice with PBS. Subsequently, cells were resuspended with 100 μl binding buffer (BD Biosciences) at a density of 1x10^6/ml, incubated with Annexin V-fluorescein isothiocyanate (5 μl) and PI (5 μl) at room temperature in the dark for 15 min, and then detected using FACSCalibur™ (BD Biosciences) flow cytometer within 1 h. The data were analyzed using FlowJo software (v10.0.7; FlowJo LLC).

Immunofluorescence staining. Following genistein treatment for 5 days at 37˚C with 5% CO_2, the tumor spheres were washed three times and fixed with 4% paraformaldehyde for 15 min at 25˚C. The tumor spheres were subsequently washed three times with PBS-Tween 20 (PBST) then blocked with 5% BSA (Beyotime Institute of Biotechnology) for 1 h at 25˚C, following which the tumor spheres were incubated with a rabbit CD44 or Smo (1:100 dilution) antibody at 4˚C overnight, followed by washing with PBST three times. Subsequently, tumor spheres were stained with Cy3-conjugated goat anti-rabbit secondary antibody (1:1,000 dilution; cat. no. P0173; Beyotime Institute of Biotechnology) for 2 h at 25˚C, followed by counterstaining with DAPI for 10 min at 25˚C. Images were obtained using a fluorescence microscope (Olympus Corporation) at a magnification of x100.

Statistical analysis. Each experiment was repeated three times independently. A two-tailed Student's t-test was used to analyze the statistical differences between two groups. One-way analysis of variance with Tukey post hoc test was utilized for comparisons among multiple groups. The results are presented as the mean ± standard deviation. All analyses were performed using SPSS version 11.0 software (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tumor sphere formation in renal cancer cells through culture in SFM. A number of studies have reported that CSCs have the ability to grow in suspension and to form spheres through culturing under SFM conditions (10,25,26). Therefore, a tumor sphere formation assay via culturing in SFM is an efficient way to isolate and enrich CSCs. In the present study, 786-O and ACHN cell lines were able to form stable cell spheres following culturing in SFM for 5 days (Fig. 1A). To further elucidate the CSC characteristics of the sphere-forming cells, mRNA and protein expression levels of renal CSC markers, including Nanog, Oct4, ALDH1A1, CD44 and CD133, were detected. Following culturing under SFM conditions for 5 days, the mRNA and protein expression levels of CSC markers were markedly upregulated compared with adherent cells cultured under SSM conditions (Fig. 1B and C). Furthermore, FCM analysis of the cell cycle distribution suggested that the sphere-forming cells were primarily arrested at G_0/G_1 phase instead of S_1 phase, in contrast to the adherent cells (Fig. 1D). These results illustrated the CSC characteristics of the cells cultured under SFM conditions.
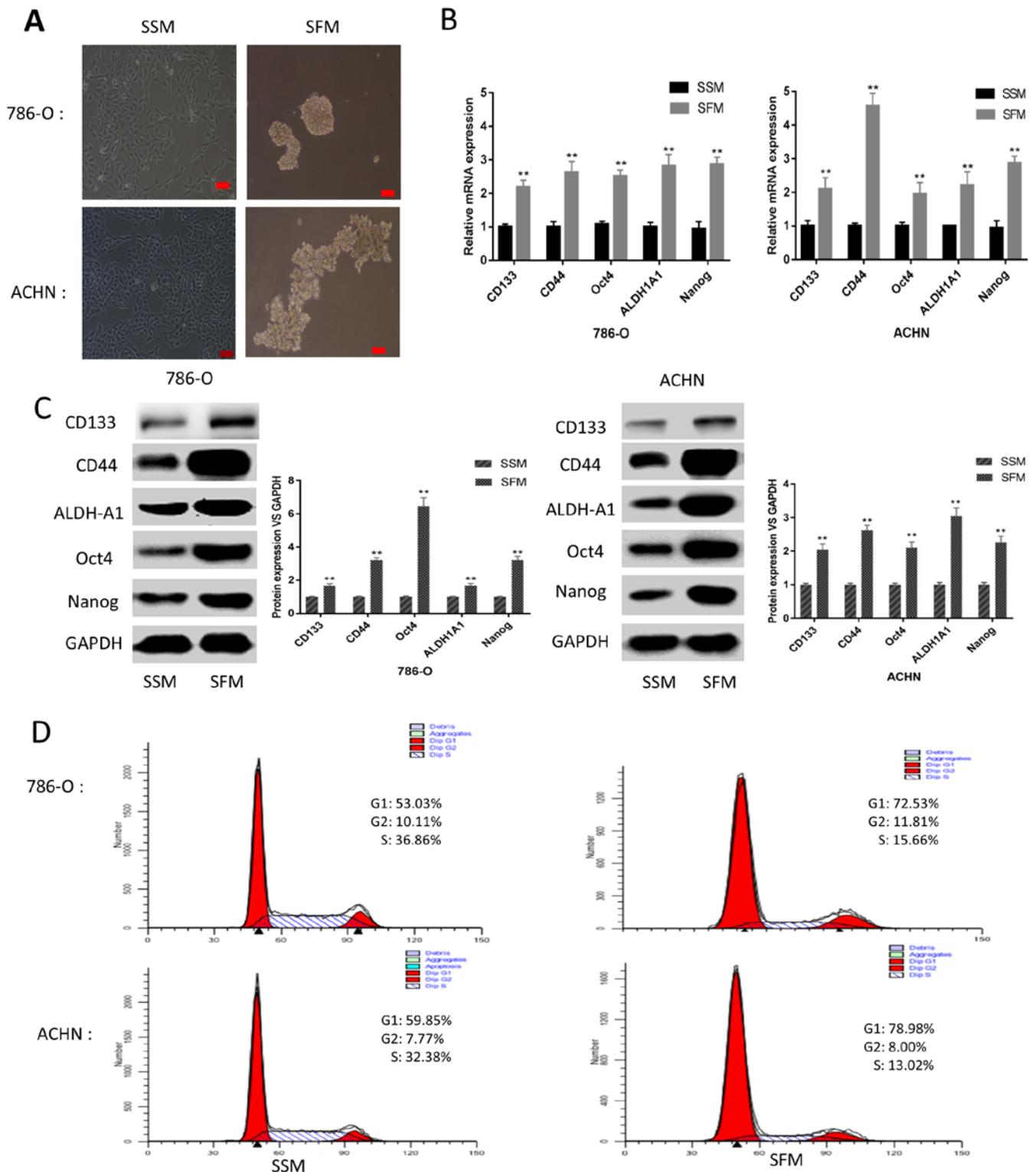
Figure 1. Tumor sphere-formation assay. 786-O and ACHN cells were cultured in SSM and SFM for 5 days. (A) Cell images were captured using a light microscope. Scale bar, 100 μm. (B) Reverse transcription-quantitative PCR was used to analyze the mRNA levels of renal CSC markers, including CD133, CD44, ALDH1A1, Oct4 and Nanog. (C) Western blotting was used to quantitatively measure the expression levels of corresponding renal CSC markers. (D) Flow cytometry was used to assess cell cycle phase distribution. Data are presented as the mean ± standard deviation from three independent experiments. **P<0.01 vs. SSM. ALDH1A1, aldehyde dehydrogenase 1 family member A1; CSC, cancer stem cell; SFM, serum-free medium; SSM, serum-supplemented medium.

Genistein diminishes the characteristics of renal CSCs. Genistein has been demonstrated to target CSCs in several types of malignancy (20,23,27). In the present study, 786-O and ACHN sphere-forming cells were treated with genistein at different concentrations (0, 30, 60 and 90 μM) for 5 days, in order to investigate whether genistein could exhibit its suppressive effect on renal CSCs. As presented in Fig. 2A and B, the tumor sphere formation assay revealed that genistein-treatment could, in a
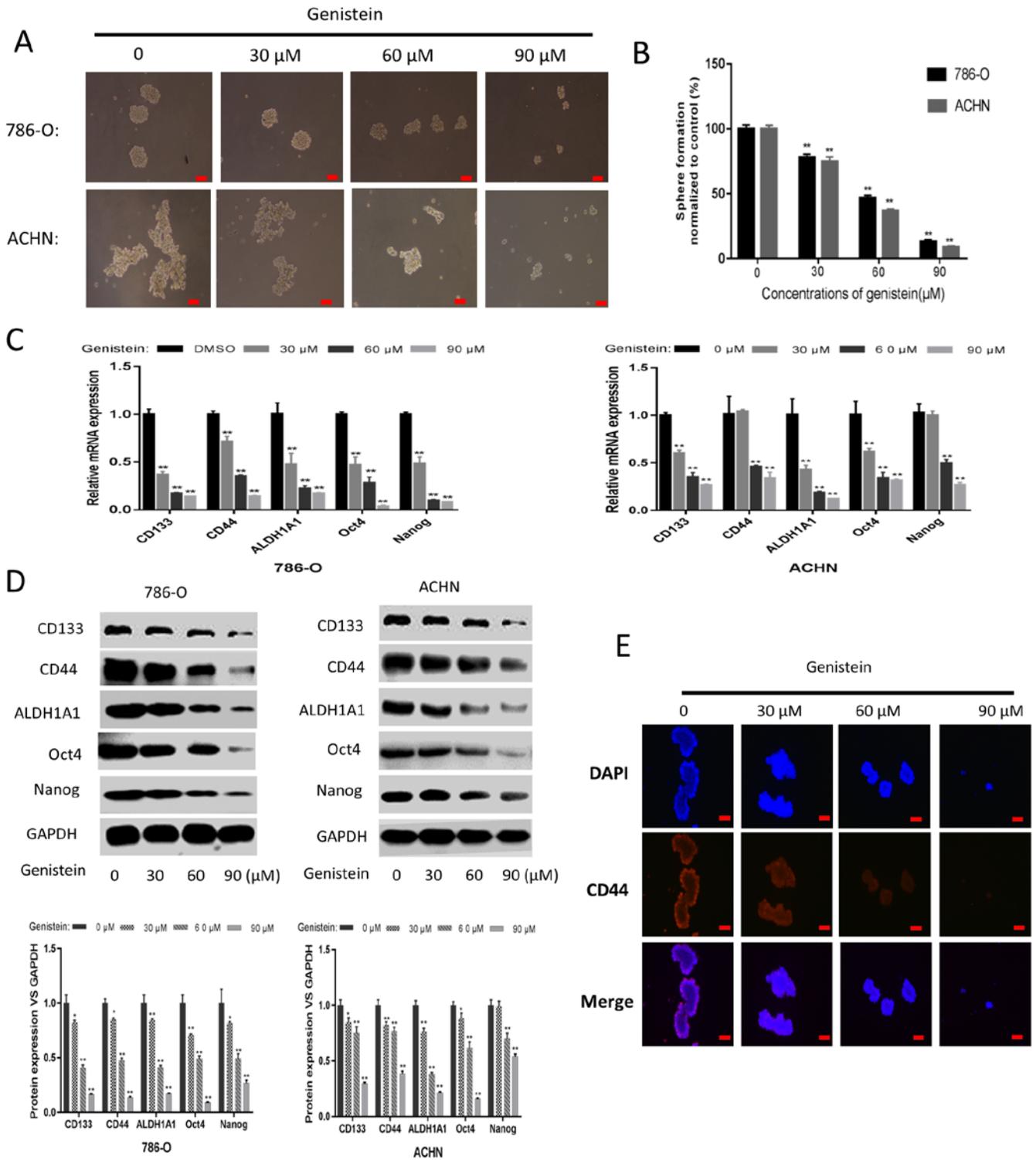
Figure 2. Genistein diminishes renal CSC characteristics. 786-O and ACHN sphere-forming cells were treated with different concentrations of genistein (0, 30, 60 and 90 µM) for 5 days. (A) Representative images. Scale bar, 100 µm. (B) Numbers of tumor spheres were counted and normalized to the control group. (C) mRNA expression levels of renal CSC markers were measured by reverse transcription-quantitative PCR. (D) Corresponding protein expression levels of renal CSCs markers were measured by western blotting. (E) Immunofluorescence staining images of 786-O tumor spheres were captured to determine CD44 expression. Scale bar, 100 µm. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01 vs. genistein 0 µM. ALDH1A1, aldehyde dehydrogenase 1 family member A1; CSC, cancer stem cell.

dose-dependent manner, significantly reduce the number and size of sphere-forming cells in the two cell lines. Following treatment with genistein for 5 days, the mRNA and protein expression levels of renal CSC markers were markedly downregulated in the sphere-forming cells of the two cell lines (Fig. 2C and D). In

addition, immunofluorescent staining indicated that the number of CD44-positive 786-O sphere-forming cells was decreased following genistein treatment in a dose-dependent manner (Fig. 2E). Overall, these data revealed that genistein could effectively diminish the characteristics of renal CSCs.
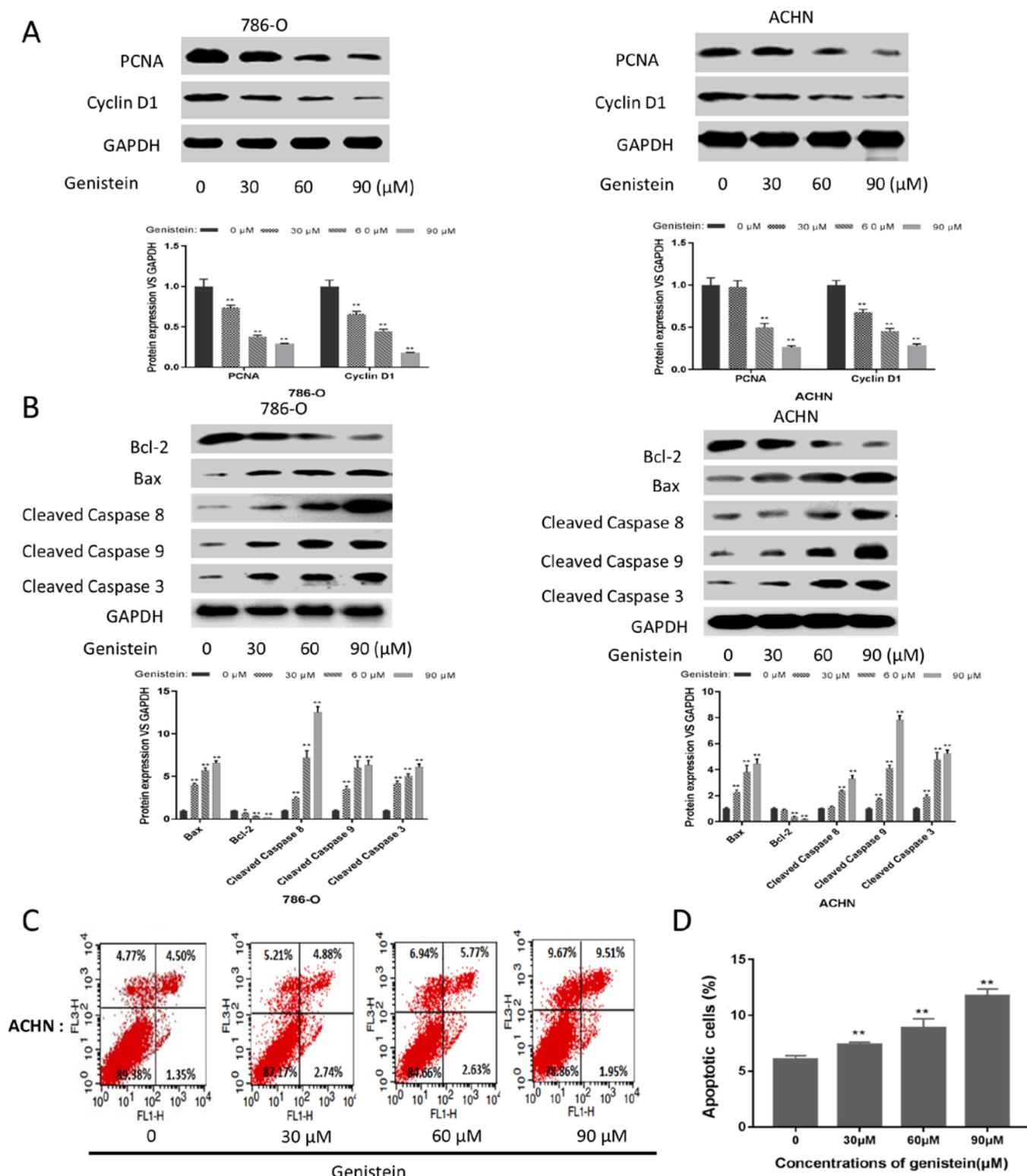
Figure 3. Genistein inhibits the proliferation and induces the apoptosis of renal CSCs. 786-O and ACHN sphere-forming cells were treated with different concentrations of genistein (0, 30, 60 and 90 μM) for 5 days. Western blotting was used to analyze the expression levels of various (A) proliferation-associated (PCNA and cyclin D1) and (B) apoptosis-associated (Bcl-2, Bax, cleaved caspase 8, cleaved caspase 9 and cleaved caspase 3) proteins. Apoptotic populations among ACHN sphere-forming cells were detected using flow cytometry. (C) Representative images. (D) Percentage of apoptotic cells among ACHN sphere-forming cells. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01 vs. genistein 0 μM. CSC, cancer stem cell; PCNA, proliferating cell nuclear antigen.

Genistein inhibits the proliferation and induces the apoptosis of renal CSCs. Furthermore, the effects of genistein on cell proliferation and apoptosis were investigated in the

present study. As presented in Fig. 3A, the expression levels of proliferation-associated proteins, including PCNA and cyclin D1, were markedly downregulated in the 786-O and
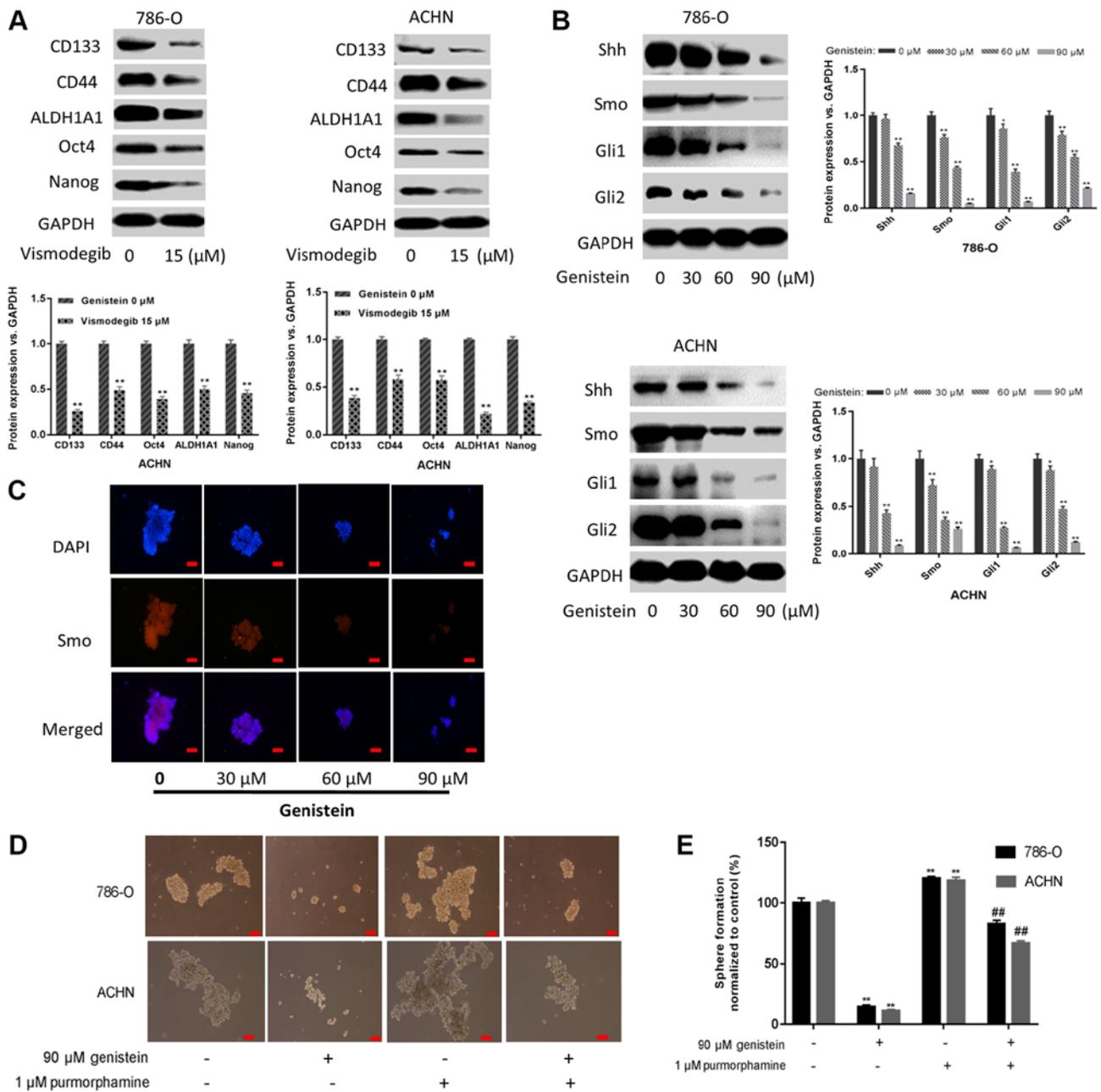
Figure 4. Genistein blocks the activity of the Shh pathway. (A) 786-O and ACHN sphere-forming cells were treated with the Smo inhibitor vismodegib (15 μM) for 5 days, and the expression levels of renal CSC markers were assessed by western blotting. (B) 786-O and ACHN sphere-forming cells were treated with different concentrations of genistein (0, 30, 60 and 90 μM) for 5 days. The expression levels of critical components of the Shh pathway were measured by western blotting. (C) Immunofluorescence staining images of 786-O tumor spheres were captured to determine Smo expression. Scale bar, 100 μm. (D) 786-O and ACHN sphere-forming cells were treated with 90 μM genistein in the presence or absence of 1 μM purmorphamine for 5 days. Representative images of sphere-forming cells. Scale bar, 100 μm. (E) Numbers of sphere-forming cells were counted and normalized to the control group (genistein 0 μM and purmorphamine 0 μM). *P<0.05, **P<0.01 vs. genistein 0 μM. ##P<0.01 vs. genistein 90 μM. ALDH1A1, aldehyde dehydrogenase 1 family member A1; CSC, cancer stem cell; Gli, glioma-associated oncogene; Shh, sonic hedgehog; Smo, smoothened.

ACHN sphere-forming cells following genistein treatment. Additionally, the expression level of the anti-apoptotic protein Bcl-2 was decreased, while the expression levels of the pro-apoptotic proteins Bax, cleaved caspase 8, cleaved caspase 9 and cleaved caspase 3 were markedly upregulated following genistein-treatment (Fig. 3B). In addition, FCM indicated that genistein could significantly induce apoptosis in ACHN sphere-forming cells (Fig. 3C and D). These results revealed that genistein could inhibit the proliferation and induce the apoptosis of renal CSCs.

Genistein diminishes renal CSC characteristics by suppressing the Shh pathway. The Shh pathway is understood to be an important regulator of renal CSCs (28-30). In the present study, it was demonstrated that sphere-forming cells treated with 15 μM vismodegib for 5 days, a Smo inhibitor that suppressed Shh activation, exhibited downregulated expression levels of renal CSC markers (Fig. 4A). Additionally, the present study investigated whether genistein could inhibit the Shh pathway in renal CSCs. As presented in Fig. 4B, genistein-treatment resulted in markedly decreased expression
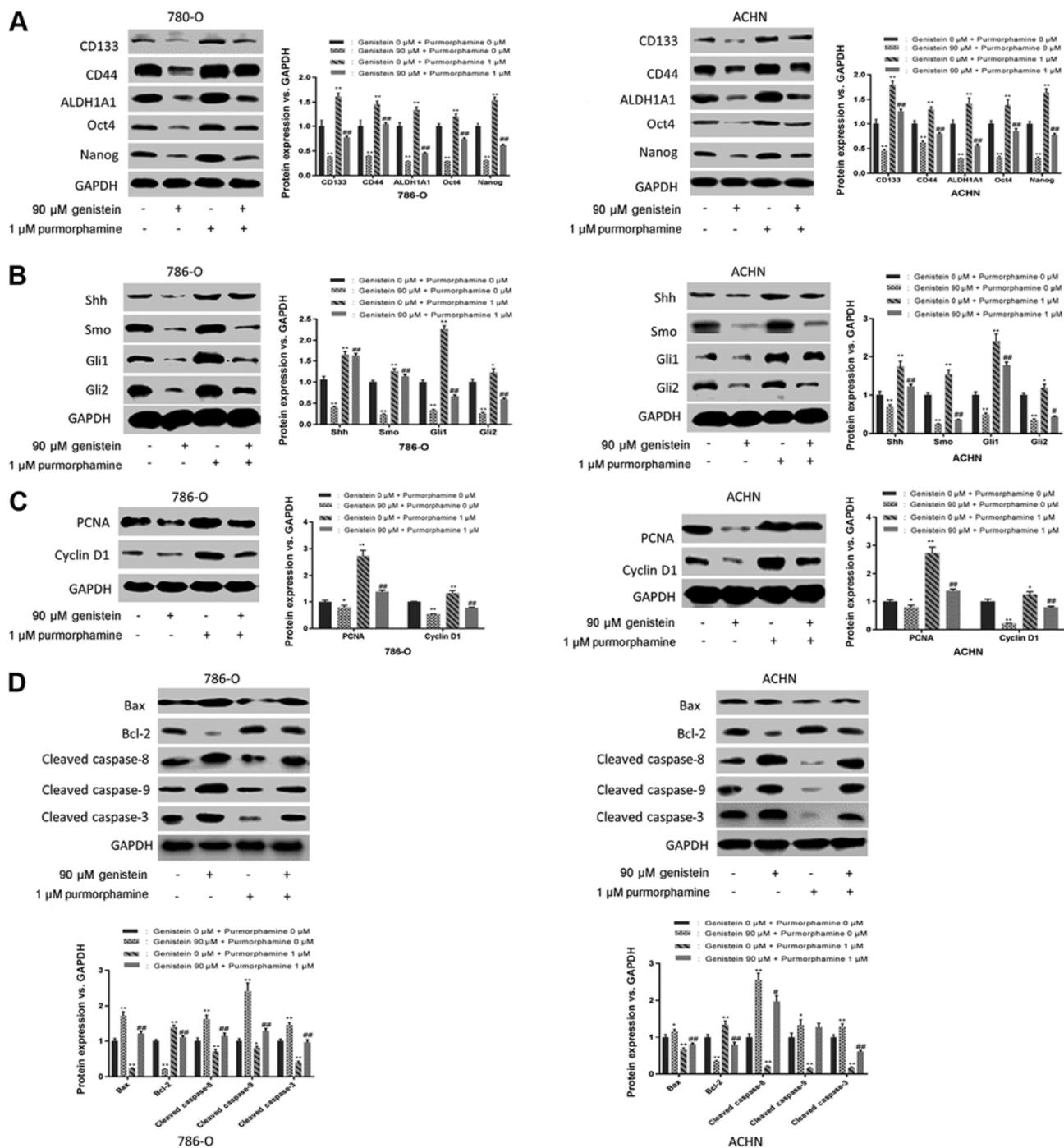
Figure 5. Genistein diminishes renal CSC characteristics by suppressing the Shh pathway. 786-O and ACHN sphere-forming cells were treated with 90 μM genistein in the presence or absence of 1 μM purmorphamine for 5 days. (A) Western blot analysis of renal CSC markers. (B) Western blot analysis of the critical components of the Shh pathway. Western blotting was used to analyze the expression levels of (C) proliferation-associated proteins (PCNA and cyclin D1) and (D) apoptosis-associated proteins (Bcl-2, Bax, cleaved caspase 8, cleaved caspase 9 and cleaved caspase 3). *P<0.05, **P<0.01 vs. genistein 0 μM. #P<0.05, ##P<0.01 vs. genistein 90 μM. ALDH1A1, aldehyde dehydrogenase 1 family member A1; CSC, cancer stem cell; Gli, glioma-associated oncogene; PCNA, proliferating cell nuclear antigen; Shh, sonic hedgehog; Smo, smoothened.

levels of the Shh pathway-associated proteins in 786-O and ACHN sphere-forming cells. Furthermore, immunofluorescent staining indicated that the number of Smo-positive 786-O sphere-forming cells decreased following genistein-treatment in a dose-dependent manner (Fig. 4C). These data indicate that genistein may inhibit the Shh pathway in renal CSCs.

In addition, purmorphamine, an activator of Smo that activates the Shh pathway, was used to further determine the role

of the Shh pathway in the suppressive effect exerted by genistein on renal CSCs. The results of the present study revealed that purmorphamine could reverse the suppressive effects of genistein on sphere formation (Fig. 4D and E). Additionally, purmorphamine-treatment increased the expression levels of renal CSC markers in 786-O and ACHN sphere-forming cells and reversed the inhibitory effect of genistein on the Shh pathway (Fig. 5A and B). Furthermore, the inhibitory effects of

genistein on cell proliferation and apoptosis-associated proteins were reversed following purmorphamine-treatment (Fig. 5C and D). In conclusion, these results indicate a suppressive effect of genistein on renal CSCs via the inhibition of the Shh pathway.

Discussion

It has been demonstrated that CSCs serve a vital role in cancer development, progression and drug resistance. The Shh signaling pathway has been implicated in maintaining the pluripotency of CSCs. Genistein, a major isoflavone component that is isolated from soybeans and soy products, has been reported to possess anticancer activity (31). The results of the present study suggested that genistein may inhibit the activities of renal CSCs by suppressing the Shh signaling pathway.

The formation of 3D spheres under non-adherent and serum-free conditions is considered to be one of the major characteristics of CSCs (32), and this trait provides a convenient and effective way to isolate and enrich putative CSCs. To date, several markers have been used to distinguish renal CSCs, including CD133, CD44, ALDH1A1, Nanog and Oct4 (6,33). CD133-positive progenitors contribute to tumor angiogenesis, which can notably enhance tumor development and growth when co-transplanted with renal carcinoma cells (9,34). CD44-positive populations derived from 786-O, Caki-1, ACHN and HEK293T renal cancer cells have self-renewal properties and sphere formation capabilities (35,36). In addition, clinical data have demonstrated that CD44 is associated with poor prognosis, cancer cell invasion and metastasis, as well as resistance to sunitinib-treatment (37,38). ALDH1-positive cells are associated with higher sphere-forming ability, self-renewal ability and tumorigenicity compared with their ALDH1-negative counterparts (39). Additionally, aberrant expression of Oct4 and Nanog is considered to be characteristic of the pluripotent stem cells of an embryo and embryonic stem cells, which are involved in maintaining the self-renewal ability and pluripotency of CSCs (40). In the present study, renal CSCs were enriched from the adherent renal cancer cell lines 786-O and ACHN, which exhibited sphere formation capabilities when cultured under SFM conditions. Additionally, the results of the present study indicated that sphere-forming cells cultured under SFM conditions exhibited markedly elevated expression levels of renal CSC markers, including CD133, CD44, ALDH1A1, Oct4 and Nanog. An increasing amount of evidence has suggested that the quiescent stem-like populations may contribute to the formation of CSCs (41,42). In line with the aforementioned findings, the present study also revealed that cells cultured under SFM conditions were more frequently arrested at the G_0/G_1 phase compared with the adherent cells cultured under SSM conditions. These results illustrated the CSC characteristics of these cells when cultured in SFM.

Genistein, a predominant isoflavone found in soybeans, has multiple anticancer effects in various different types of cancer, including lung, colon, head and neck squamous cell carcinoma, and hepatocellular carcinoma (21,43-45). A previous epidemiological study based on observations made in Asian countries, including China, demonstrated that the incidence rates of breast and prostate cancer are lower than those in the USA and Europe, where diets are lower in soy products (46). Accumulating evidence suggests that genistein

exhibits anticarcinogenic effects on CSCs *in vivo* and *in vitro*, including in prostate (27), gastric (20) and breast cancer (19). In line with the aforementioned studies, the results of the present study suggested that genistein could efficiently diminish the activity of renal CSCs by repressing tumor sphere formation, downregulating the expression levels of renal CSC markers, and inhibiting the proliferation and inducing the apoptosis of renal CSCs.

The Shh signaling pathway is a major regulator of cell differentiation, cell proliferation and tissue polarity (47). To date, a large amount of evidence has been accumulated that supports the view that the aberrant activation of Shh signaling is involved in tumorigenesis, tumor progression and therapeutic response in several types of cancer, including medulloblastoma, malignant glioma and leukemia, as well as lung, pancreatic, breast and renal cancer (48,49). Behnsawy *et al* (30) reported that treatment with Shh recombinant, the Shh signaling stimulator, could enhance RCC cell proliferation, whereas treatment with cyclopamine, a Smo inhibitor, could inhibit tumor growth. Qian *et al* (29) demonstrated that Shh signaling was involved in the stimulation of renal CSC pluripotency, induced by cigarette smoking. Therefore, targeting of the Shh pathway with phytochemicals may provide a potential strategy for intervention in renal cancer. In the present study, genistein could inhibit the activation of the Shh pathways in renal CSCs by downregulating the levels of Shh, Smo, Gli1 and Gli2. By contrast, the purmorphamine-triggered activation of the Shh pathway may abolish the inhibitory effects of genistein on tumor sphere formation, CSC marker expression, and the proliferation and apoptosis of renal CSCs. Collectively, these results demonstrated the interventional effect of genistein on renal CSCs via Shh pathway inhibition. However, the results of the present study are based on *in vitro* experiments, therefore further *in vivo* studies are required to validate the role of the Shh signaling pathway in renal CSCs inhibition by genistein. Moreover, additional *in vivo* studies are also warranted to explore whether genistein inhibits other renal CSC models, such as side population and chemotherapy enrichment.

In conclusion, the present study revealed that genistein exerts an interventional inhibitory effect on renal CSCs by suppressing the Shh pathway, which may provide novel insights into the molecular mechanism of renal CSCs, as well as valuable avenues for investigating potential interventional targets of genistein on renal CSCs.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

DY and CZ designed the study. EL, TZ, XS, YL and HG performed the experiments. EL analyzed the data and wrote the manuscript. All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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