Journal of the Formosan Medical Association (2016) 115, 1032–1038

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ORIGINAL ARTICLE

Chemotherapeutic effects of luteolin on radio-sensitivity enhancement and interleukin-6/signal transducer and activator of transcription 3 signaling repression of oral cancer stem cells

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Received 19 July 2016; received in revised form 18 August 2016; accepted 22 August 2016

KEYWORDS
cancer stem cells;
luteolin;
oral carcinomas;
radio-sensitivity

Background/Purpose: Previously, we successfully identified oral cancer stem cells (OCSC) displaying enhanced stemness and tumorigenic potentials. In the study, we investigated the chemotherapeutic effect of the flavonoid luteolin, commonly found in fruits and vegetables, on targeting OCSC.
Methods: Oralspheres was applied to isolate OCSC. aldehyde dehydrogenase 1 activity and CD44 positivity of OCSC with luteolin treatment were assessed by flow cytometry analysis. Radio-sensitivity of OCSC treated with luteolin was examined. Invasion and colony-forming

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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http://dx.doi.org/10.1016/j.jfma.2016.08.009
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assays were performed to assess oncogenicity in OCSC. The expression of interleukin-6 (IL-6)/ signal transducer and activator of transcription 3 (STAT3) was examined by enzyme-linked immunosorbent assay and western blot analysis.
Results: We showed that luteolin effectively inhibited the proliferation rate, self-renewal, aldehyde dehydrogenase 1 activity, and CD44 positivity of OCSC but did not cause significant cytotoxicity of normal epithelial cells. Moreover, luteolin restored radio-sensitivity in OCSC. Combined treatment with luteolin and radiation displayed synergistic effect on invasiveness and clonogenicity of OCSC. Mechanistically, treatment of luteolin resulted in inactivation of IL-6/STAT3 signaling.
Conclusion: These results suggest that combined treatment of luteolin and radiation therapy can attenuate tumorigenicity of OCSC through IL-6/STAT3 signaling inactivation.
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Introduction

The cancer stem cells theory postulates that a subpopulation of cells termed cancer stem cells (CSC) or tumor-initiating cells, drive tumor initiation, radio-resistance, progression, and high rate of relapse and metastasis; thus development of a novel approach targeting CSC is imperative.[1–5] Our group has previously identified a functional subset of oral CSC (OCSC) in oral squamous cell carcinomas (OSCC) marked by oralspheres,[6] CD133,[7] aldehyde dehydrogenase (ALDH),[3] membrane 78 kDa glucose-regulated protein (GRP78),[8] or side population.[9] We also demonstrated that these OCSC display enhanced tumorigenic potential *in vitro* and *in vivo*. Screening novel compounds for drug candidates that targets the CSC of OSCC specifically will be instrumental for future advanced OSCC therapy.

Luteolin (3′,4′,5′,7′-tetrahydroxyflavone), belonging to the flavone subclass, is found in plants such as chamomile tea, celery, perilla leaf, and green peppers.[10] Through a variety of experimental cancer models, luteolin has been found to possess pleiotropic antineoplastic activity including stimulation of cancer cell apoptosis, cell cycle arrest, repression of cancer cell proliferation, suppressing angiogenesis, and metastasis capacity.[11–13] Evidence suggests that luteolin inhibits proliferation and induces apoptosis of cancer cells via protein kinase B/Akt, p38, c-Jun N-terminal kinases, or nuclear factor-κB signaling.[14–17] Luteolin can reverse multidrug resistance in a variety of cancer cells.[16] Luteolin chemosensitizes ovarian cancer cells to paclitaxel through repression of epithelial–mesenchymal transition markers and traits.[18] Luteolin inhibits the metastasis of cancer cells *in vitro* and *in vivo* through inhibiting epithelial–mesenchymal transition, integrin β1, or focal adhesion kinase.[19] However, the efficacy of luteolin on the specific subset of OCSC has not been addressed. In this study, we investigated whether luteolin possesses anti-CSC effect and whether interleukin-6 (IL-6)/ signal transducer and activator of transcription 3 (STAT3) signaling is involved in luteolin-targeted CSCs in OSCC cells.

Materials and methods

Cell culture and reagents

Normal human gingival epithelioid S-G cells and OSCC cells lines (SAS and GNM) were cultivated as previously described.[20] Luteolin was purchased from Sigma–Aldrich (St Louis, MO, USA).

Cell proliferation determination by MTT assay

Cells were plated in wells of 96-well-plate as 1×10^4 cells/ well in 0.1% dimethyl sulfoxide (DMSO) or different concentrations of luteolin-containing medium and cultured at 37°C for 24 hours. Cell proliferation/survival was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The 570 nm absorbance of the DMSO-treated group was set as 100% and data are presented as percentage of DMSO control.[21]

Oralsphere-forming assay

OSCC cells will be dissociated and cultured as oralspheres in modified Dulbecco's modified Eagle medium/F-12 supplemented with N-2 (R&D, Minneapolis, MN, USA), 10 ng/mL epidermal growth factor (EGF; Invitrogen, Carlsbad, CA, USA), 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), and penicillin/streptomycin at 10^3 live cells/low-attachment six-well plate (Corning Inc., Corning, NY, USA), with the medium changed every other day until oralsphere formation was observed in about 2 weeks. For serial passage of oralsphere cells, single cells were obtained from Accutase (Sigma–Aldrich)-treated spheroids at a cell density of passage of 1000 cells/mL in the serum-free medium described above.[2]

Flow cytometry analysis for cancer stemness marker

The ALDEFLUOR kit (Stem Cell Technologies, Durham, NC, USA) was used to examine the ALDH enzymatic activity

according to the protocol described.[21] For CD44 analysis, cells were stained with anti-CD44 antibody conjugated to phycoerythrin (Miltenyi Biotech., Auburn, CA, USA), with labeling according to the manufacturer's instructions. Red (>650 nm) fluorescence emission from 10,000 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.[21]

Invasion and soft agar colony forming assay

Cell invasion and soft agar colony forming assays were performed as described previously.[5]

Western blot assay

The analysis was performed to detect the expression of p-Stat3 and glyceraldehyde 3-phosphate dehydrogenase as described previously.[3]

Statistical analysis

SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The presented results are representative of three independent experiments with similar results. Statistical differences were evaluated with the Student t test, and were considered significant at $p < 0.05$.

Results

Luteolin attenuated self-renewal ability in oral cancer stem cells

Initially, we examined the cytotoxic effect of luteolin to sphere-forming OCSC isolated from SAS and GNM. Luteolin treatment exerted an antiproliferation effect on OCSC in a dose-dependent manner, whereas the inhibition on normal oral epithelial cells (SG) proliferation was limited (Fig. 1A). As shown in Fig. 1B, luteolin treatment dose-dependently inhibited secondary sphere-forming ability, an index of self-renewal in CSCs, in OCSC.

Luteolin inhibited ALDH1 activity and CD44 expression in OCSC

Accumulated evidence indicates that the maintenance of cancer stemness in OCSC are via high CD44 expression

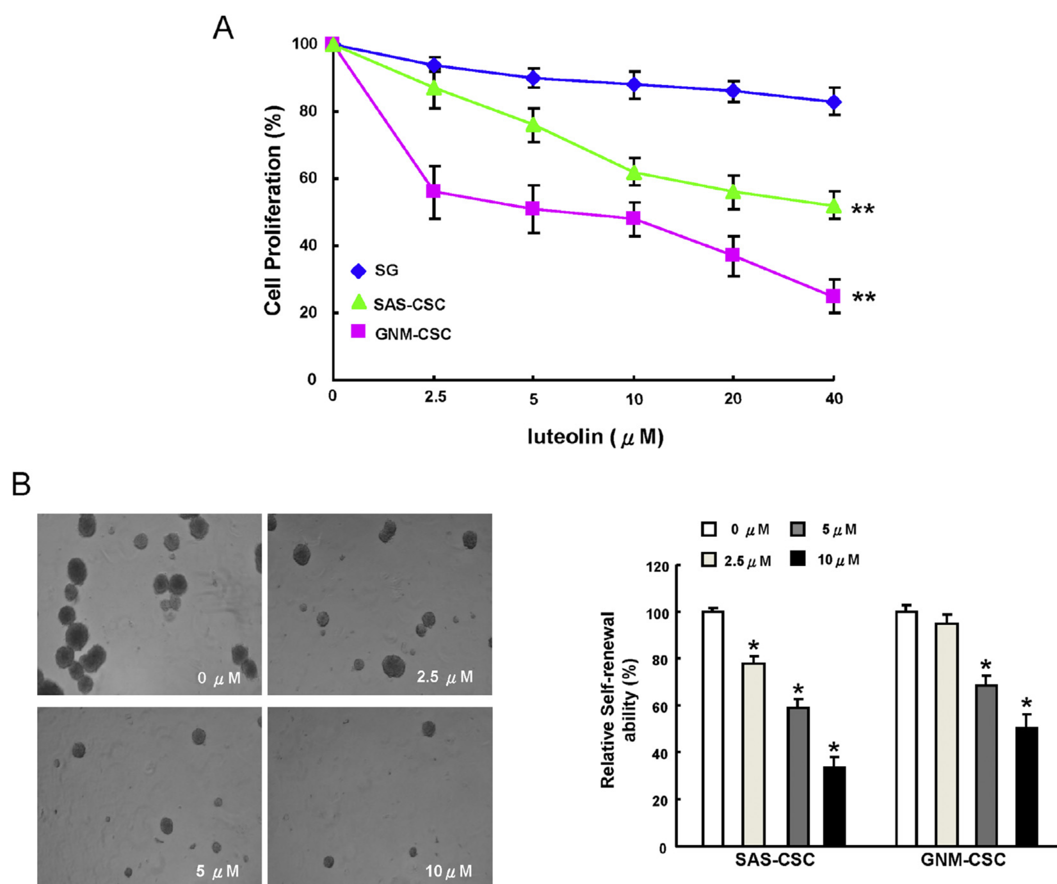

Figure 1 Diminished proliferation and self-renewal properties of oral cancer stem cells with luteolin treatment. (A) Luteolin treatment shows that concentration-dependent inhibition of cell viability in oral cancer stem cells by MTT assay. Cell survival was assessed and presented as percent survival relative to untreated cells. (B) Secondary sphere formation ability of luteolin-treated cells was examined. The bar graph shows quantification of secondary sphere number. The experiments were repeated three times and representative results are shown. Results are means ± standard deviation. * $p < 0.05$; ** $p < 0.001$.

and ALDH1 activity.[22] Elevated ALDH1 activity and CD44 expression also mediate tumorigenicity and radio-chemoresistance. To examine further the anti-CSC effect of luteolin on eliminating OCSC, we performed ALDH activity and flow cytometry assays to investigate luteolin's inhibition of CD44 positivity and ALDH activity of OCSC. By flow cytometry analysis, luteolin treatment significantly reduced CD44 population (Fig. 2) and ALDH1 activity (Fig. 3) in OCSC in a dose-dependent manner.

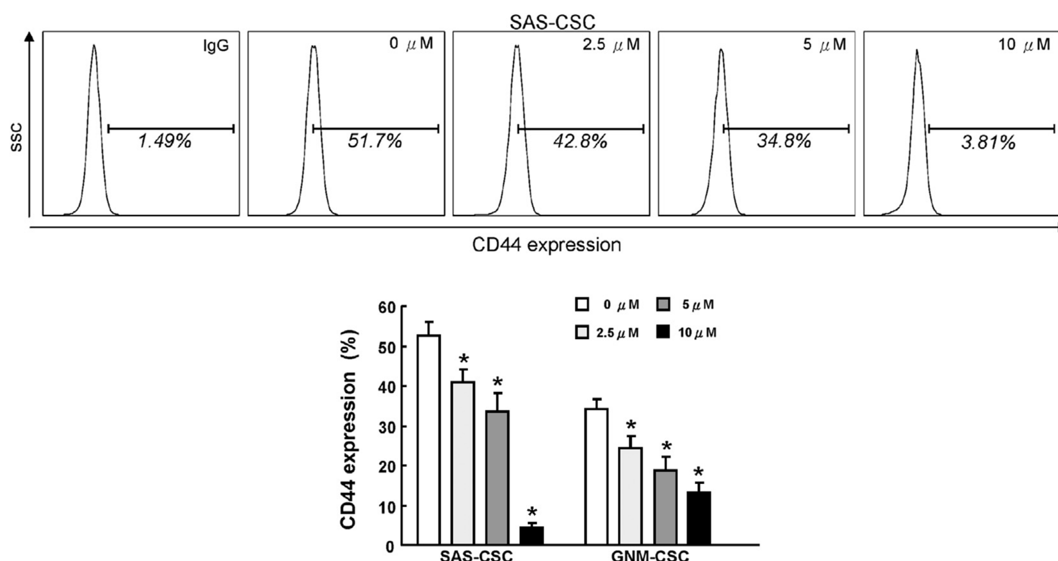

Figure 2 Reduced CD44 expression in luteolin-treated oral cancer stem cells. The CD44 positivity of oral cancer stem cells dose-dependently treated with or without luteolin was assessed by flow cytometry analysis. The bar graph shows quantitation of CD44-positive cells. The experiments were repeated three times and representative results are shown. Results are means ± standard deviation. * $p < 0.05$ versus control.

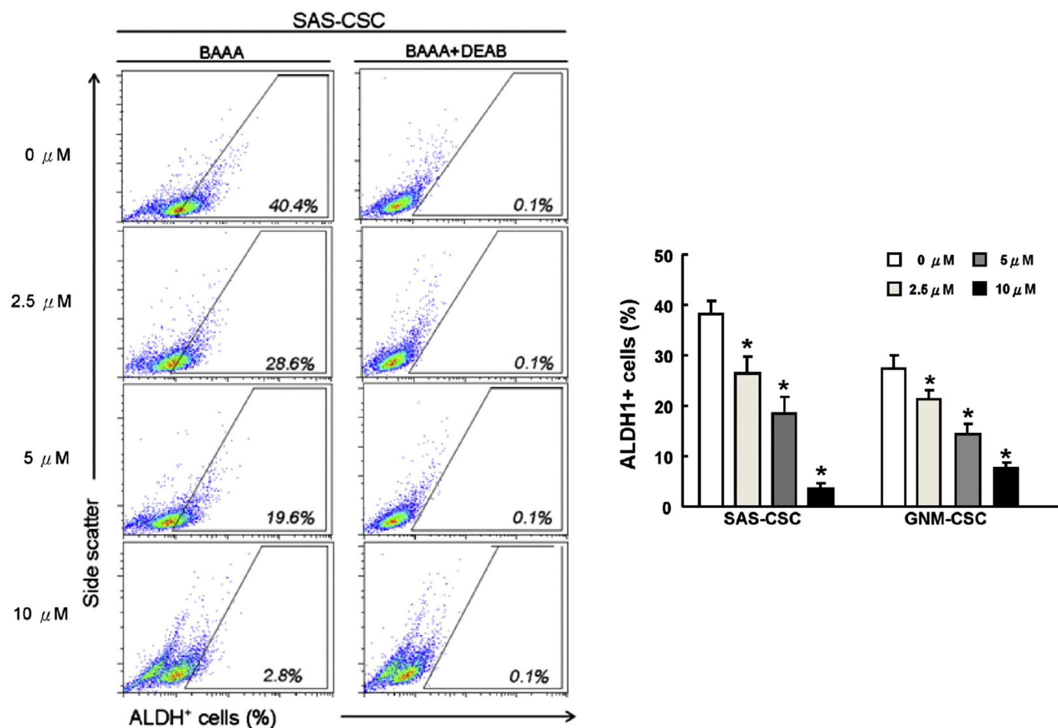

Figure 3 Luteolin dose-dependently represses aldehyde dehydrogenase (ALDH) 1 activity in oral cancer stem cells. Oral cancer stem cells were dose-dependently treated with luteolin for 24 hours, afterward; the intracellular ALDH activity was examined by ALDEFLUOR flow cytometry-based assay. N,N-diethylaminobenzaldehyde, a specific inhibitor of ALDH1 enzyme, was used as a negative control. The bar graph shows quantification of ALDH1-positive cells. The experiments were repeated three times and representative results are shown. Results are means ± standard deviation. * $p < 0.05$ versus control.

Luteolin enhanced radio-sensitivity of OCSC

OCSC have increased resistance to radiation treatment, which is commonly used for OSCC patients.[22] We therefore evaluated the effect of luteolin on the radio-sensitivity in OCSC. During MTT assay, OCSC displayed a radioresistant phenotype, and the combined treatment of luteolin enhanced the efficacy of radiation in OCSC (Fig. 4A). To determine whether combined treatment with luteolin and radiation would affect the malignancy of enriched OCSC *in vitro*, we analyzed the anchorage-independent growth potential and migration ability of OCSC combined treated with luteolin and radiation treatment. Moreover, OCSC treated with the combination luteolin and radiation treatment showed a synergistic effect in promoting elimination of invasion (Fig. 4B) and colony-forming potentials (Fig. 4C) of OCSC.

Inhibition of IL-6/Stat3 signaling in OCSC by luteolin

It was found that IL-6/Stat3 signaling is involved in maintaining CSC properties including OSCC.[23] To investigate further the mechanistic effect interfered by luteolin treatment to reduce the CSC properties, we examined the IL-6 secretion and STAT3/p-STAT3 expression of luteolin-treated OCSC. Enzyme-linked immunosorbent assay analysis showed that luteolin treatment decreased IL-6 secretion in OCSC by (Fig. 5A). Immunoblot analyses showed that the level of phospho-STAT3 protein was diminished in OCSC treated with luteolin (Fig. 5B). To elucidate further the involvement of IL-6 the anti-CSC effects of luteolin, luteolin-associated cancer stemness repression experiments with the treatment of IL6 in OCSC were performed. The anti-CSC effects, including self-renewal capability (Fig. 5C) and invasiveness (Fig. 5D), of OCSC were rescued by IL-6 treatment. These data suggest that luteolin-treatment impairs CSC properties through inactivating the IL-6/STAT3 signaling.

Discussion

Head and neck squamous cell carcinoma, including OSCC, is one of the most prevalent neoplasms worldwide.[24] The poor survival rate of OSCC could be attributable to either the late diagnosis of the disease or the potential resistance of OSCC cells to clinical therapeutic treatments.[24] Recent findings from our group have confirmed that OCSC probably impart resistance to conventional treatment modalities and promote tumorigenesis in OSCC.[25] Therefore, approaches that target CSCs for OSCC treatments may provide new way of clinical practices. In this study, we demonstrated that luteolin treatment reduced the proliferation and self-renewal ability in enriched OCSC (Fig. 1). We demonstrated that luteolin treatment reduced CSC markers such

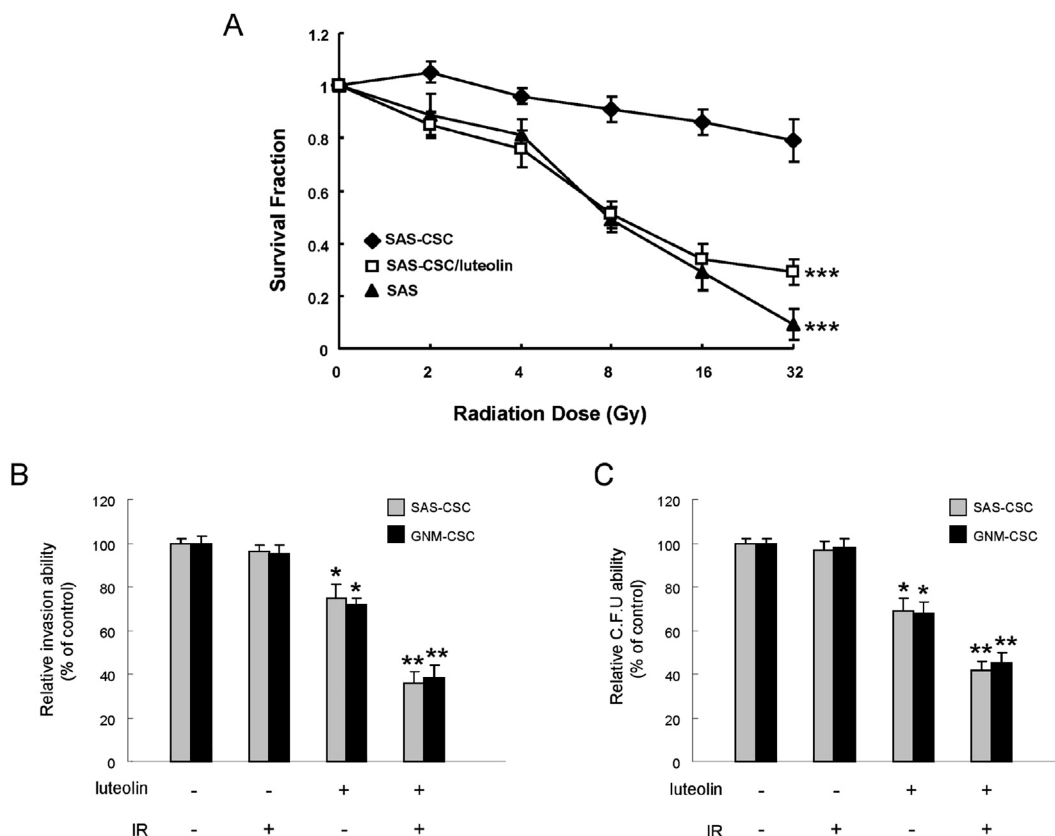

Figure 4 Restored radiosensitivity of oral cancer stem cells (OCSC) by luteolin treatment. After exposure to different doses of irradiation, the surviving cell fractions of the control or luteolin-treated OCSC were evaluated. (B) Invasion ability and (C) colony-forming ability in OCSC were examined after treatment with either luteolin or radiationtherapy or both. * $p < 0.05$ luteolin versus control; ** $p < 0.05$ luteolin + IR versus luteolin alone; *** $p < 0.001$. IR = infrared radiation.

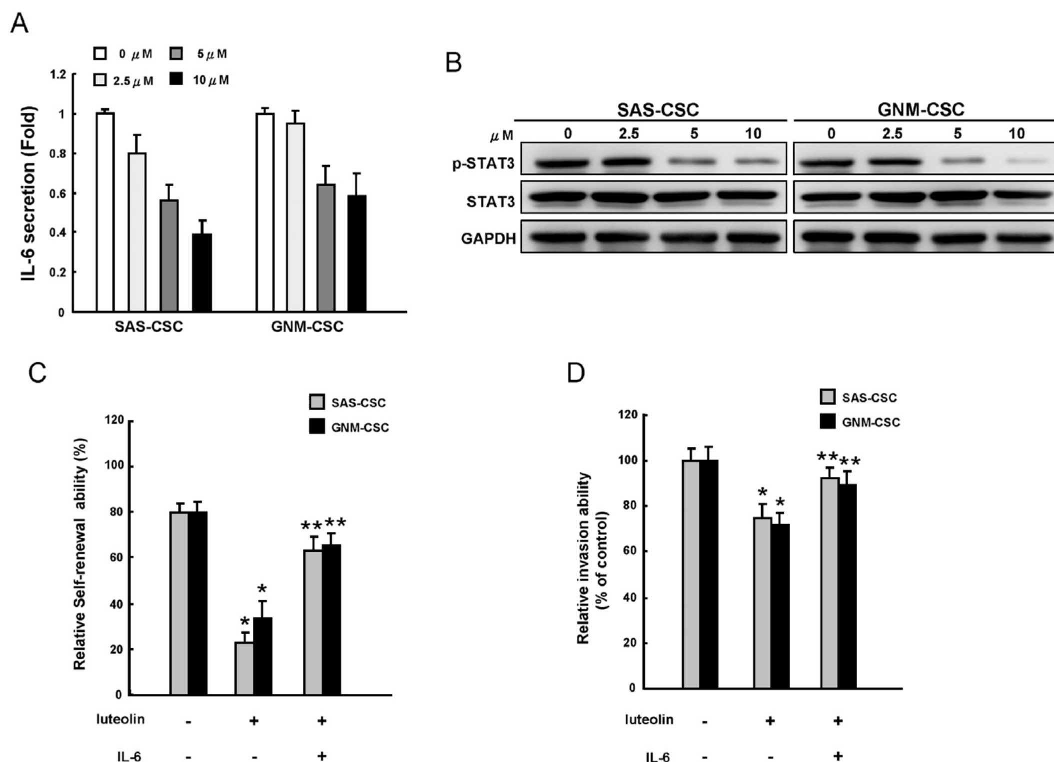

Figure 5 Inhibition of interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling of oral cancer stem cells (OCSC) by luteolin treatment. (A) IL-6 secretion level in luteolin-treated OCSC by enzyme-linked immunosorbent assay analysis; (B) cell extract proteins of luteolin-treated OCSC were collected and analyzed by immunoblotting against anti-p-STAT3, anti-STAT3, or antiglyceraldehyde 3-phosphate dehydrogenase antibodies as indicated. The immunoactive signal of glyceraldehyde 3-phosphate dehydrogenase protein of different crude cell extracts was referred as loading control; (C) self-renewal; and (D) invasion ability in OCSC were analyzed after treatment with either luteolin or luteolin combined with IL-6. * $p < 0.05$ luteolin versus control; ** $p < 0.05$ luteolin + IL-6 versus luteolin alone.

as ALDH+ and CD44+ cells (Figs. 2 and 3). Luteolin effectively restored the synergistic radiosensitivity to irradiation in OCSC (Fig. 4). To our knowledge, this is the first report inhibiting the cancer stemness and radioresistance properties of OCSC by luteolin.

Mechanistically, IL-6/STAT3 signaling is constitutively activated in various types of cancers including prostate cancer, breast cancer, leukemia, multiple myeloma, brain tumors, and OSCC.[26,27] Persistent oncogenic STAT3 activation is associated with poor prognosis of OSCC patients and promotes malignant progression.[28,29] In addition, IL-6/STAT3 is an important axis for maintaining cancer stem-like properties and tumorigenesis processes in several malignant carcinomas, including OSCC.[30–32] Korkaya et al[33] showed that the IL-6 pathway is responsible for the resistance to trastuzumab and cancer stem cells maintenance in breast cancer. The clinical treatment of stage 1 and 2 OSCC includes surgical resection or radiotherapy. For patients with advanced and metastatic OSCC, both radiation and/or chemotherapy are the major therapeutic measures. Radioresistance is the major cause of recurrence and metastasis as well as lethality in OSCC patients. Our previous studies have demonstrated that OCSC are more resistant to radiation therapy.[22] Notably, reports have shown that inhibition of STAT3 can significantly promote radiosensitivity and further suppress tumorigenicity.[34] Our present findings suggest that luteolin treatment decreases cancer stemness and malignancy through inhibition of IL-6 secretion and p-STAT3 activation (Fig. 5). These findings support the idea of using the IL-6/STAT3 axis as potential signaling for anti-CSC therapy, and the inhibition of IL-6/STAT3 signaling by luteolin treatment may be an important therapy when combined with radiotherapy.

Taken together, our data indicate that luteolin can inhibit oncogenicity and radioresistant properties of OCSC through targeting IL-6/STAT3 signaling. These findings provide a strong rationale for the potential use of luteolin as a radiosensitizer and anti-CSCs agent.

Acknowledgments

The present study was supported by grants (Grant No. R104-34) from the Ditmanson Medical Foundation Chia-Yi Christian Hospital and Chung Shan Medical University (CSMU-INT-102-03) in Taiwan.

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