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Limonin enhances the radiosensitivity of nasopharyngeal carcinoma cells via attenuating Stat3-induced cell stemness

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ABSTRACT

The inhibitory effects of limonin have been disclosed in various tumors, however, its roles in nasopharyngeal carcinoma (NPC) progression are never been revealed. In the current work, we collected NPC cells with a higher stemness compared with bulk cells through isolating the side population (SP) cells. It was found that limonin exhibited a stronger inhibitory effect on SP cells than that in bulk cells, which was evident by a lower IC50 value. Additionally, limonin attenuated the stemness and migration ability of SP cells with the higher stemness, characterized as decreasing the spheroid formation ability, expression of stemness markers and migration ability. Moreover, the proportion of SP cells in G0 phase was remarkably higher than that in bulk cells. Notably, upon limonin treatment, the proportion of SP cells in G0 was decreased and S/G2/M increased. Furthermore, limonin enhanced the radiosensitivity of NPC cells. The mechanistic studies based on RNA-sequencing analysis revealed that limonin inhibited the gene transcription driven by Stat3 (signal transducer and activator of transcription 3) and an activator of Stat3 (Colivelin or IL-6) rescued the inhibitory effects of limonin. Therefore, these results demonstrate that limonin could reduce the stemness of NPC cells and thus the radiosensitivity through suppressing Stat3 transcriptional activity.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern and southeastern of China [1]. In clinic, most of them are poorly differentiated squamous cell carcinomas or undifferentiated carcinomas [2]. NPC is prone to distant metastasis, and owing to the complex of nasopharyngeal cavity, it is difficult to operate, thus chemotherapy and radiotherapy have become the main methods for NPC treatment, especially radiotherapy is the first choice [3]. However, the resistance to radiotherapy is an important factor affecting the efficacy of NPC treatment [3]. Therefore, it is an urgent need to find the critical contributors for the resistance of radiotherapy in NPC.

Cancer stem cells (CSCs) are a special subset of cells latent in cancer cells, which have the characteristics of continuous self-renewal and multidirectional differentiation potential [4]. CSCs have been regarded as the root of the resistance of conventional therapy in cancers, such as chemotherapy and radiotherapy [5]. CSCs could exist in a resting and poorly differentiated state for a long time, these traits are similar with NPC, most of which are poorly differentiated or undifferentiated [6]. Thus, targeting CSCs have been explored to treat NPC. For example, our previous work showed that the long non-coding RNA (lncRNA) THOR attenuates the stemness and cisplatin resistance of NPC cells via directly binding to YAP and suppressing its translocation from nuclear to cytoplasm [7]. The transcription factor interferon regulatory factor 6 (IRF6) suppresses the stemness of NPC cells via directly targeting the ABCG2 gene [8]. Notably, some potential drugs have been revealed to reduce the stemness of NPC cells. Such as, resveratrol attenuates the stemness, epithelial-mesenchymal transition (EMT) and metabolic reprogramming through activating the p53 pathway in NPC cells [9], and longikaurin A, a natural ent-kaurane, suppresses stemness and thus enhances the efficacy of radiotherapy and chemotherapy in NPC cells [10]. However, these works did not perform *in vivo* experiments. Therefore, it is still important to find novel drugs attenuating the stemness of NPC cells, which may facilitate NPC treatment.

Limonin is a triterpenoid compound, which is an important secondary metabolite with high biological activity in plants [11]. Recent studies have shown that it has anti-tumor, neuroprotection, antioxidant and other biological activities [12–14]. A previous study has demonstrated that limonin inhibits the expression of P-glycoprotein in human colon and leukaemia cell lines, which displays a high level in CSCs [15]. This suggests that limonin may target CSCs, which is confirmed by the recent two studies showing that limonin attenuates the stemness of cervical carcinoma cells by promoting YAP nuclear-cytoplasmic translocation [16], and breast cancer cells via suppressing MIR216A

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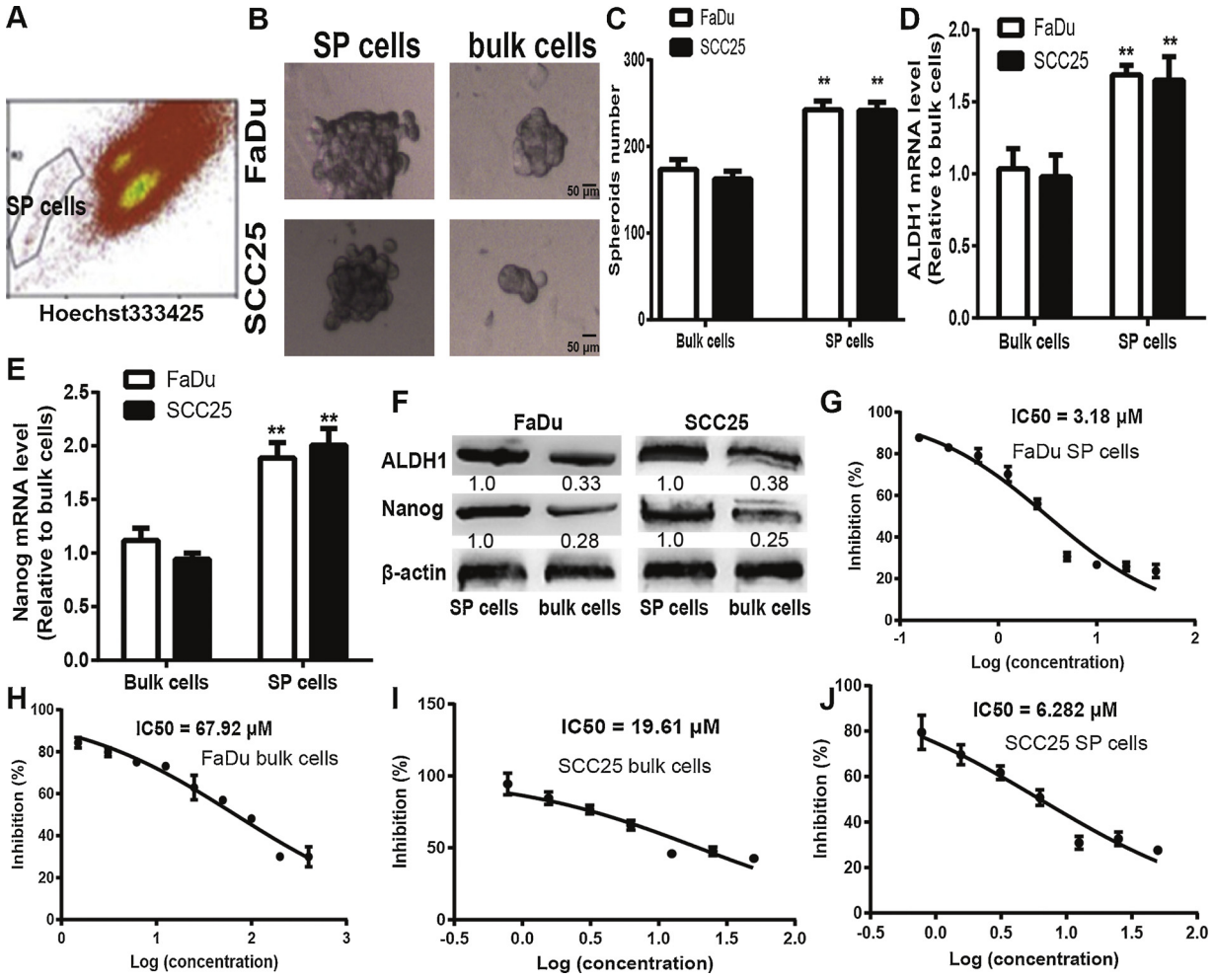

Fig. 1. Limonin significantly and specifically reduces the SP cell viability of NPC cells. (A) A representative picture of SP cells. (B and C) The spheroid formation ability was evaluated in NPC bulk and SP cells through measuring spheroid size and number. (D and E) The mRNA levels of stemness markers (ALDH1 and Nanog) were detected in NPC bulk and SP cells. (F) The protein levels of stemness markers (ALDH1 and Nanog) were examined in NPC bulk and SP cells. (G–J) Cell viability was determined in NPC bulk and SP cells with limonin treatment. Data are presented as the mean ± s.d, **P < 0.01 vs. Bulk cells.

methylation [17]. However, the roles of limonin in NPC cell progression are never been disclosed.

In the current study, we speculate that limonin could also attenuate the stemness of NPC cells. Firstly, we collected NPC cells with a stronger stemness than the bulk cells via isolating the side population (SP) cells, which had been confirmed to hold CSCs-related traits [18]. We found that limonin exhibited a stronger inhibitory effect on SP cells than that in bulk cells. Additionally, limonin attenuated the stemness of SP cells, characterized as decreasing the spheroid formation ability, expression of stemness markers, migration ability and provoking SP cells entry into cell cycle. Furthermore, limonin enhanced the radiosensitivity of NPC cells. RNA-sequencing analysis showed that Stat3 signaling was mostly enriched in the upregulated pathways, which was further confirmed by western blot analysis. Importantly, an activator of Stat3 (Colivelin and IL-6) rescued the inhibitory effects of limonin.

2. Material and methods

2.1. Cell culture and drugs

NPC cell lines FaDu and SCC25 were purchased from the Chinese Academy of Sciences Cell Bank. FaDu cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) medium (Gibco,USA), and SCC25 cells were cultured in 1640 medium supplemented with 10% FBS (Fetal bovine serum) (Gibco), 80 U/ml penicillin and 0.08 mg/ml streptomycin at 37 °C under humidified atmosphere with 5% CO_2. Limonin was purchased from Selleck Chemicals (Houston, TX, USA). Colivelin (2 μM) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The concentrations of 2 μM, 4 μM and 8 μM for limonin were used for research in this work. Human interleukin-6 (IL-6) was purchased from MedChem Express (Monmouth Junction, NJ, USA) and the concentration of 100 ng/ml was used in the current study.

2.2. Isolation of side population (SP) cells

NPC cells were digested as the single cell suspension and stained with Hoechst33342 with the final concentration of 6 μg/ml. The SP cells were separated using BD FACSAria II (BD Pharmingen, San Diego, CA, USA) using 355 nm UV excitation source, 610 nm two-color short-pass reflector filter, 450 nm and 675 nm edge long-pass filter. The percent of SP cells in NPC cells was about 5.4%.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagents (Invitrogen, MA, USA) according to the manufacturer's recommendation. cDNA was reversely transcribed using a reverse transcription kit (Vazyme, China). Then the levels of mRNA were detected using SYBR Green qPCR Master Mix (QIAGEN, Germany). GAPDH was used as an internal control. Primers sequences were shown as below: Nanog,

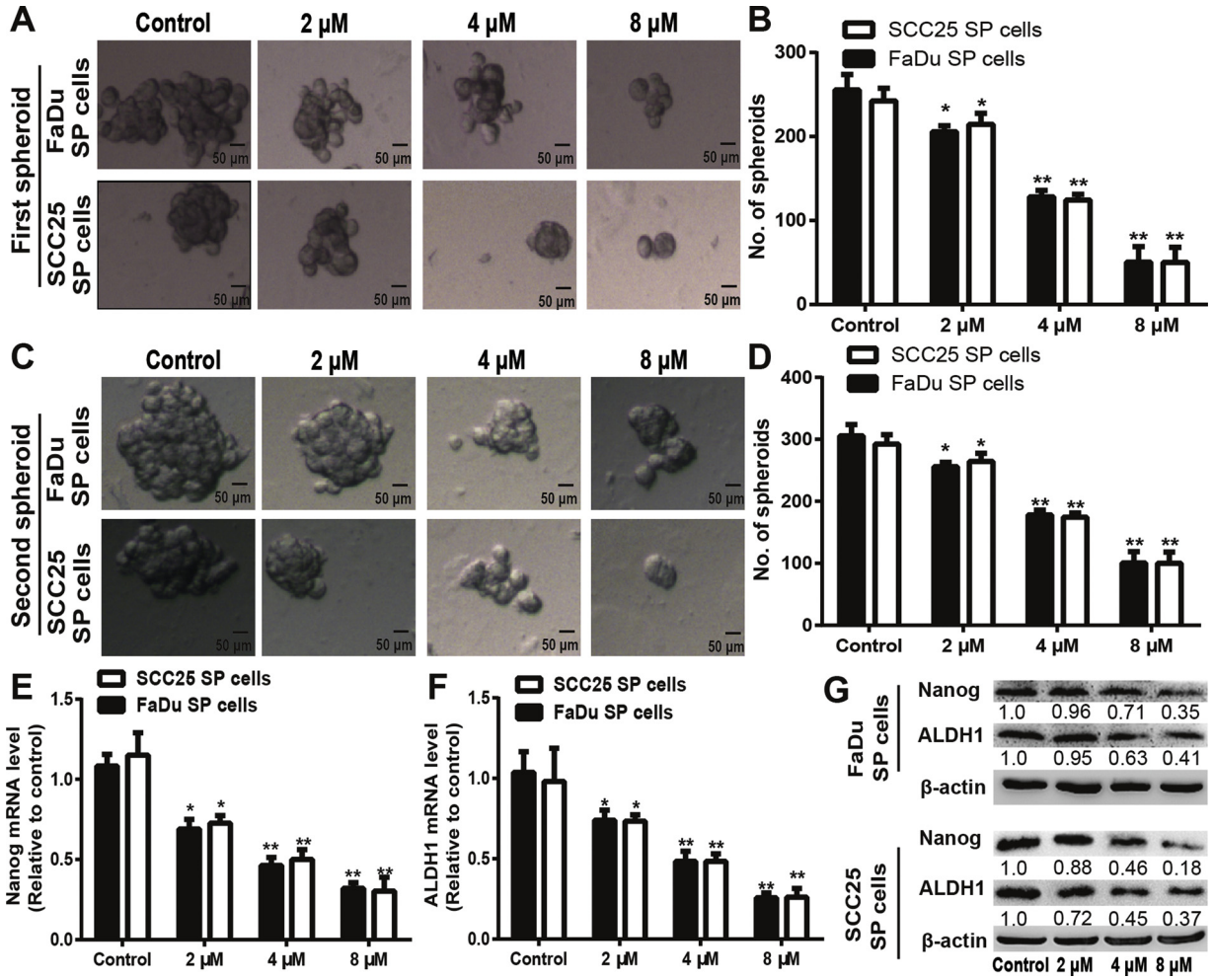

Fig. 2. Limonin attenuates the stemness of NPC SP cells. (A and B) The first spheroid formation capacity was evaluated in NPC SP cells with different concentration of limonin treatment. (C and D) The second spheroid formation ability was detected in cells digested from the first spheroids. (E–G) The expression of stemness markers (ALDH1 and Nanog) was examined in NPC SP cells with different concentrations of limonin treatment. Data are presented as the mean ± s.d, *P < 0.05, **P < 0.01 vs. Control group (solvent).

forward, 5′- CACGCCAGACTTACCTGTCCTACT-3′, reverse, 5′ - TGTCA ACATCCTCCTTATCTCCTT-3′; ALDH1, forward, 5′- GCAGGTATGGGT TCATAGAAGGGC- 3′, reverse, 5′ - TGTGAGTGTCTGGTAGCAGGGATT-3′; CD18, forward, 5′- GCAGGAGGGCCCAGCGACGCCGCCG- 3′, reverse, 5′ - CGGCGGCGTCGCTGGGCCCTCCTGC- 3′; CD64, 5′ - GATTG GCTTGCTTGTGCTTTGTAA- 3′, 5′ - TGATGGTCTCTGAGTGGCTGTGTG-3′; CD11c, forward, 5′- TTCGGGCTGGTGACAGGGAAGACA- 3′, reverse, 5′ - TTTGCGGGACAAAGGGCAAGATTT- 3′. GAPDH, 5′ - GACACCCAC TCCTCCACCTTTGAC- 3′, 5′ -TCTCTCTTCCTCTTGTGCTCTTGC- 3′. The levels of mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot

Nuclear protein was separated and extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific). Separated cytoplasmic and total proteins were extracted from cells with RIPA lysis, and then the concentration was measured using BCA Protein Assay Kit. 30 μg of protein was separated on 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 10% non-fat milk, incubated with primary antibodies at 4 °C overnight, washed with TBST and then incubated with HRP-conjugated secondary antibody. ECL reagent was used to develop the blots. The primary antibodies are listed below: ALDH1 (Cat.No. 15910-1-AP, proteintech, Wuhan, China, 1:1000), Nanog (Cat.No. 67255-1-Ig, proteintech, 1:1000), p-STAT3 (Tyr705) (ab76315, Abcam,1:1000), p-STAT3 (Ser727) (ab32134, Abcam, 1:1000), STAT3 (Cat.No. 10253-2-AP, Proteintech, 1:1000), Histone H2A (Cat.No. 16441-1-AP, Proteintech, 1:1000) and β-actin (Cat.No. 66009-1-Ig, Proteintech, 1:1000).

2.5. Cell viability assay

Cells were seeded in 96-well plates at the density of 5×10^3 cells/well. 24 h later, cells were treated with ionizing radiation (IR) (8 Gy) using an RS2000 X-ray irradiator (Rad Source Technologies, FL, USA). And cell viability was detected with CCK8 assay 24 h, 48 h and 72 later by measuring the absorbance at 570 nm with a microplate (BioTek, VT, USA).

2.6. Transwell migration assay

Cells were seed into 24-well transwell chambers at the density of 8×10^4 cells/well evaluate cell migration. The upper chambers were filled with medium without FBS, and the lower-chamber medium contained 10% FBS. 16–20 h later, the non-migrating cells were gently removed with a cotton swab, the chambers were stained with 0.1% crystal violet and then eluted with 33% acetic acid. The migrated cells were captured and counted in five randomly fields with microscope. Subsequently, the absorption at 570 nm was measured using a microplate, which indicates the number of migrated cells.

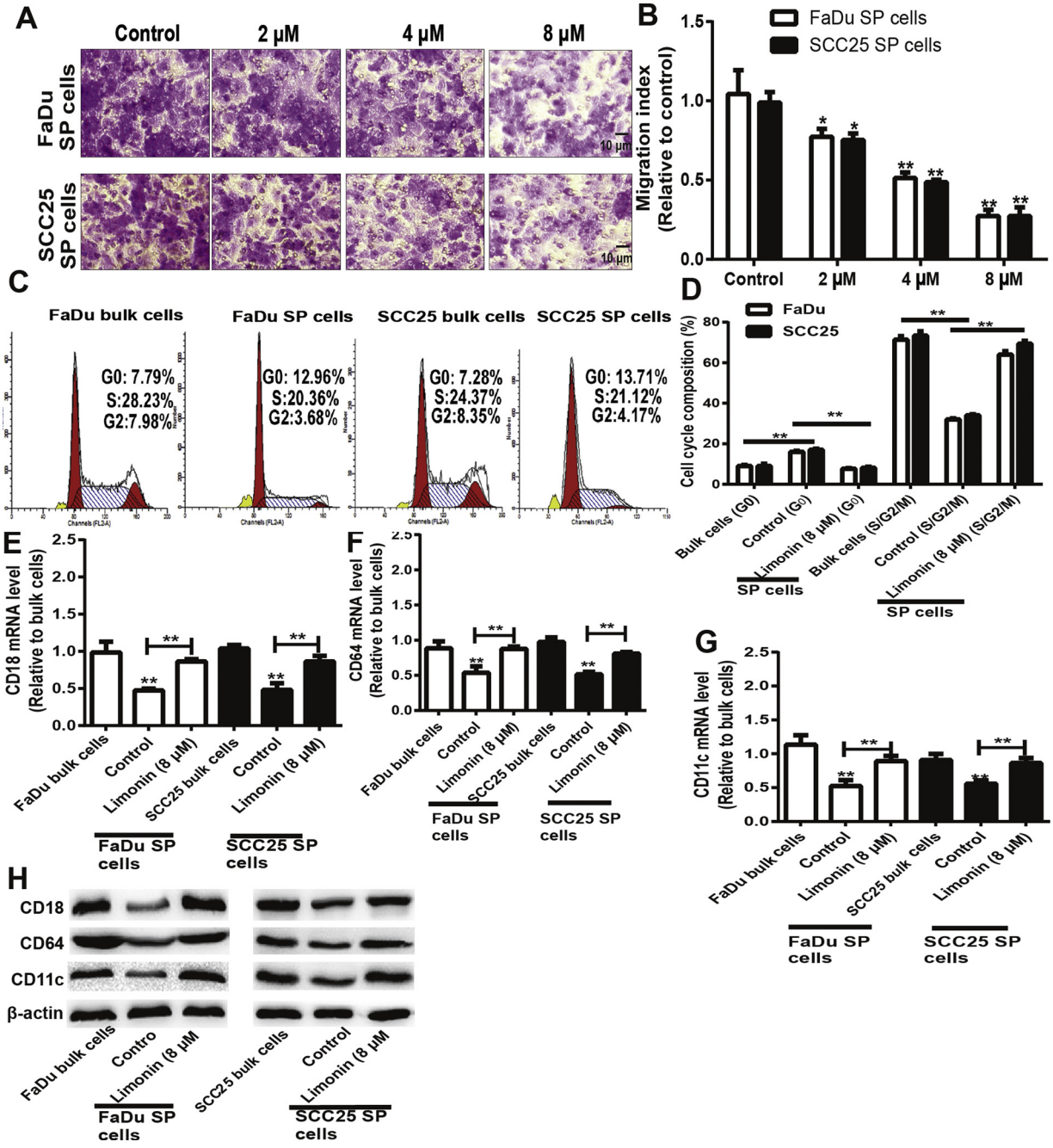

Fig. 3. Limonin reduces the migration ability of NPC SP cells and provokes them entry into cell cycle. (A and B) The migration ability of NPC SP cells was evaluated in NPC SP cells with different concentrations of limonin treatment. (C) Representative FACS plot of cell cycle for bulk and SP cells. (D) Cell cycle assay was performed in NPC and SP cells with or without limonin treatment. (E–H) The mRNA levels of differentiation markers (CD18, CD64 and CD11c) were measured in NPC and SP cells with or without limonin treatment. Data are presented as the mean ± s.d, *P < 0.05, **P < 0.01 vs. bulk cells.

2.7. Spheroid formation assay

Cells were seeded in ultra-low-attachment 6-well plates (Corning Incorporated, Corning, NY, USA). The medium was formulated with serum-free DMEM/F12 medium, 20 ng/ml EGF, 1% B27 and bFGF. The number of spheres was counted 14 days later, and sphere-forming efficiency was calculated based on the number of initially seeded cells.

2.8. Cell cycle assay

Cells were harvested 24 h after IR and used for cell cycle analysis by flow cytometry. Briefly, 10^5 cells were collected and fixed in 70% ethanol for 24 h. Subsequently, the cells were washed, suspended in PBS containing 50 μg/ml PI and incubated for 15 min at room temperature in the dark. Then the cells were determined using flow cytometry (Beckman, CA, USA).

2.9. RNA-sequencing analysis

RNA-sequencing and related analysis was performed by GENEWIZ (Suzhou, Jiangsu, China). The detailed procedure was referred to the previous work [18]. Briefly, RNA-seq libraries were acquired using TruSeq PE Cluster Kit V4 and sequenced by HiSeq 2000 sequencer. We used Cuffdiff to estimate fragments per kilobase of transcript per million (FPKM) values for known transcripts and analyze differentially expressed transcripts. $p < 0.05$ was considered as significant. Heatmap

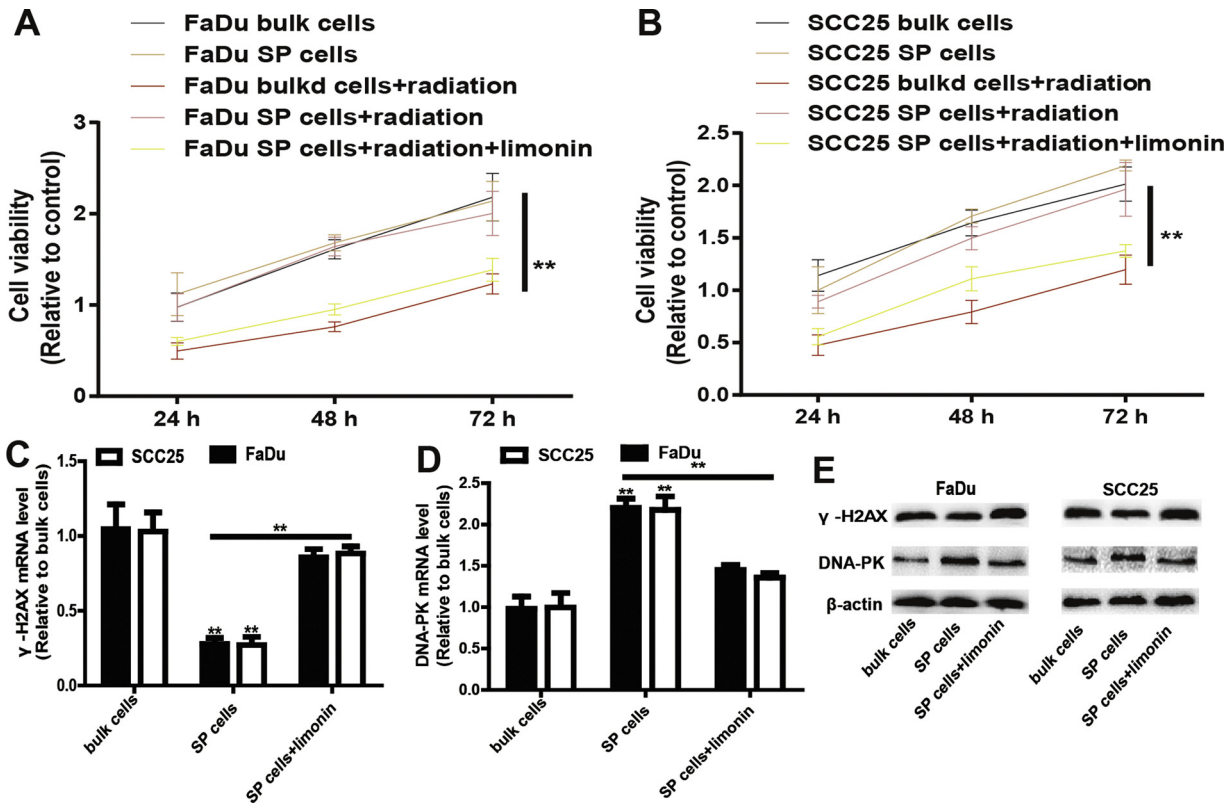

Fig. 4. Limonin enhances the radiosensitivity of NPC SP cells. (A and B) NPC bulk and SP cells were treated with radiation plus limonin treatment or not, and subjected to cell viability analysis. (C–E) The expression levels of DNA-damage and –repair proteins were examined in NPC bulk cells and SP cells with or without limonin (8 μM) treatment. Data are presented as the mean ± s.d, *P < 0.05, **P < 0.01 vs. bulk cells.

of gene expression was generated based on log2 (FPKM) using HemI 1.0.3.7 (http://hemi.biocuckoo.org/down.php). GO analysis of gene expression changes was performed using GO-TermFinder. KEGG enrichment analysis was performed according to Rich factor, Qvalue, and numbers of enriched genes. The richer factor is bigger, and the degree of enrichment is bigger; the Qvalue is smaller, and the degree of enrichment is more significant.

2.10. Statistical analysis

All results were denoted as Mean ± SEM and analyzed using Graphpad Prism (Version X; La Jolla, CA, USA). For pairwise comparisons, a Mann-Whitney non-parametric test was used unless otherwise indicated. $P < 0.05$ or less was considered statistically significant.

3. Results

3.1. Limonin significantly and specifically reduces the SP cell viability of NPC cells

We firstly isolated the NPC cells with a stronger stemness than the bulk cells via collecting SP cells using technologies of FACS. As expected, SP cells (Fig. 1A) exhibited a stronger stemness than the bulk cells, evident as the increase of spheroid formation capacity (Fig. 1B and C) and stemness marker expression (Fig. 1D–F). Then the effects of limonin on the viability of SP and bulk cells were evaluated. As shown in Fig. 1G–J, limonin exhibited a strong inhibitory effect on SP cell viability than that on bulk cells, evident by the remarkable decrease of IC50 value (3.182 μM vs 67.92 μM in FaDu cells, 6.288 μM vs 19.61 μM in SCC25 cells).

3.2. Limonin attenuates the stemness of NPC SP cells

Then we detected the effects of limonin on the stemness of SP cells. It was found that limonin reduced the spheroid formation ability of SP cells in concentration dependent manner, which was shown as the decreased spheroid size and spheroid number (Fig. 2A and B). Notably, to evaluate the effects of limonin on the self-renewal ability of SP cells, the second spheroid formation ability was further determined and the consistent results were obtained (Fig. 2C and D). Additionally, the expression of stemness markers (Nanog and ALDH1) was decreased in a concentration-dependent manner (Fig. 2E–G).

3.3. Limonin reduces the migration ability of NPC SP cells and provokes them entry into cell cycle

We further determined the effects of limonin on the migration ability of SP cells. As shown in Fig. 3A and B, the migration ability of SP cells was reduced by limonin in a concentration-dependent manner. Since CSCs enter a quiescent stage after a standardized therapy responsible for tumor recurrence [19,20], the proportion of cell cycle in SP and bulk cells was detected. Hereinafter, the biggest concentration (8 μM) of limonin was chosen for research. As shown in Fig. 3C and D, SP cells displayed the increased proportions of quiescent (G_0) cells and decreased $S/G_2/M$ cells compared to bulk cells, which was rescued by limonin treatment. Additionally, the expression of differentiation markers (CD18, CD64 and CD11c) was inhibited in SP cells compared to bulk cells, which was increased by limonin treatment (Fig. 3E–H).

3.4. Limonin enhances the radiosensitivity of NPC SP cells

Since CSCs are resistant to conventional therapy, such as chemotherapy and radiotherapy, we explored whether limonin could

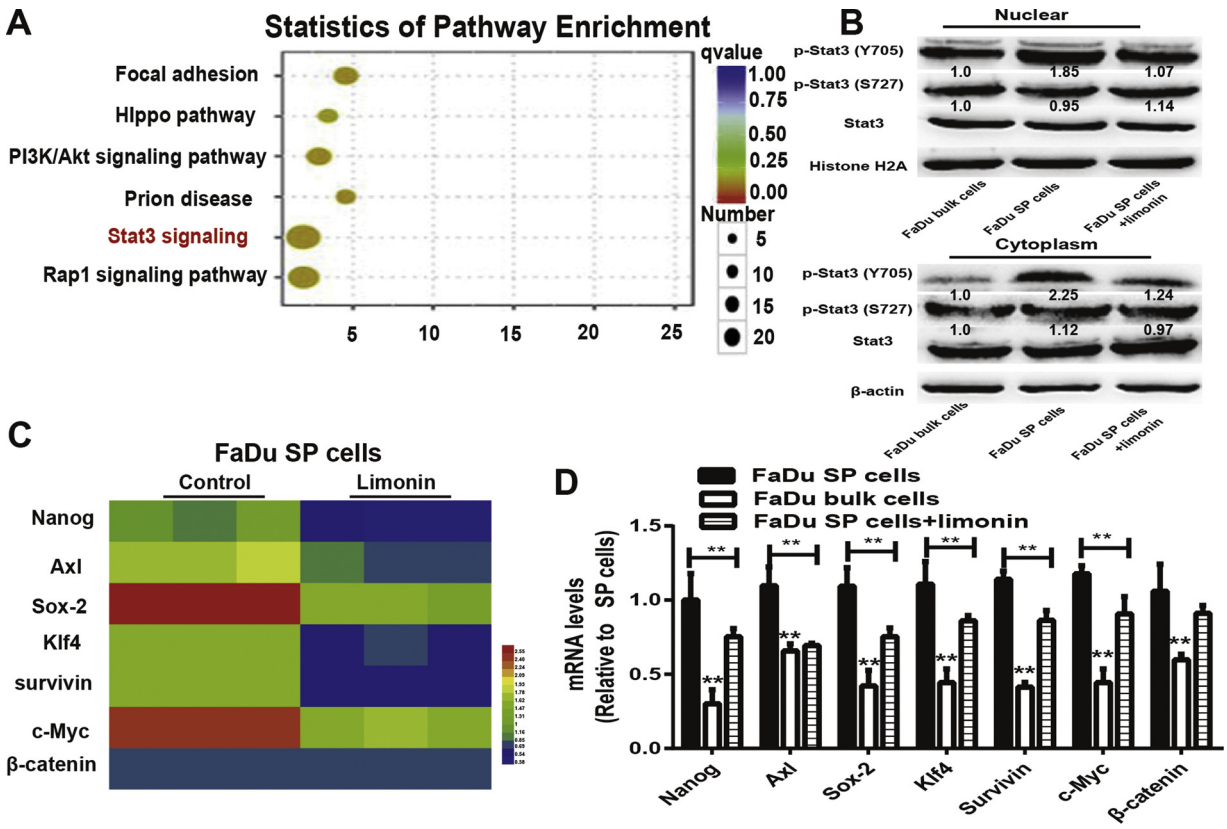

Fig. 5. Stat3 signal was targeted by limonin in NPC SP cells. (A) KEGG pathway enrichment was analyzed based on RNA-sequencing analysis in FaDu SP cells with or without limonin treatment. Number means the number of enriched genes. (B) The protein levels of p-Stat3 (Y705), p-Stat3 (S727) and total Stat3 were detected in the nuclear and cytoplasm of FaDu SP cells. (C) The expression of downstream effectors of Stat3 was examined in FaDu SP cells by RNA-sequencing analysis. (D) The mRNA levels of the downstream effectors of Stat3 were determined in FaDu bulk and FaDu SP cells with or without limonin treatment. Data are presented as the mean ± s.d, *P < 0.05, **P < 0.01 vs. bulk cells.

regulate the radiosensitivity of NPC cells. As shown in Fig. 4A, SP cells exhibited an extent of resistance of radiotherapy compared to bulk cells. Notably, limonin attenuated the radioresistance of NPC SP cells (Fig. 4B). Additionally, the expression levels of DNA damage protein γ-H2AX and DNA damage-repair protein DNA-PK were decreased and increased in SP cells relative to bulk cells [21], respectively, while this effect was partially reversed by limonin treatment (Fig. 4C–E). Thus, these results indicate that limonin could enhance the radiosensitivity of NPC cells.

3.5. Stat3 signal was targeted by limonin in NPC SP cells

Following this, we explored the mechanisms contributing to limonin-mediated effects on the stemness of NPC cells. An integrated transcriptome analysis based on RNA-sequencing data was employed in SP cells with or without limonin treatment. The significantly enriched pathways were identified and it was found that Stat3 signaling was mostly enriched (Fig. 5A), which is engaged in CSCs progression [22]. STAT3 is activated by phosphorylation at Tyr705 (Y705) (p-Stat3) which translocates into the nuclei to induce the transcription of several target genes implicated in cancer cell malignancy [23]. Then we defined the expression and location of p-Stat3 in bulk and SP cells, and found that the expression of p-Stat3 (Y705) in the cytoplasm and nuclei was remarkably increased in SP cells, which was reduced by limonin, but the expression of p-Stat3 (S727) and total Stat3 was unaffected (Fig. 5B). Furthermore, the expression of the downstream effectors (Nanog, Axl, Sox-2, Klf4, survivin, c-Myc, and β-catenin) of Stat3 was increased in SP cells [24], which was also decreased by limonin (Fig. 5C and 5D). Taken together, these data indicate that limonin decreases p-Stat3 (Y705) into the nuclei and thus attenuates the activation of Stat3 signaling activity.

3.6. Activation of Stat3 signaling partially reversed the inhibitory effects of limonin on NPC SP cell stemness

Finally, we evaluated whether limonin reduces the stemness of NPC cells dependent on Stat3 signaling. Stat3 was activated using colivelin and IL-6, both of which are Stat3 activators. As shown in Fig. 6A, colivelin and IL-6 indeed increased the p-Stat3 level and the expression of stemness markers in NPC bulk cells. Meanwhile, limonin-mediated inhibition on the expression of stemness markers and p-Stat3 (Y705) was rescued by colivelin and IL-6 in NPC SP cells (Fig. 6B). Additionally, colivelin and IL-6 not only enhanced the spheroid formation ability of NPC bulk cells (Fig. 6C and D), but also decreased the inhibitory effect of limonin on the spheroid formation ability of NPC SP cells. Collectively, our work demonstrates that limonin attenuates the stemness of NPC cells through targeting Stat3 signaling.

4. Discussion

NPC is a common head and neck malignant tumor in southern China [2]. Radiotherapy is the main treatment, but at present, radiotherapy and chemotherapy can not completely eradicate tumors [3]. With the in-depth study of the pathogenesis of NPC, it has been found that NPC CSCs can produce heterogeneous tumor cells, have a strong self-renewal ability, can promote the occurrence and development of tumors, and are closely related to drug resistance, recurrence and metastasis of NPC [25]. Previous studies have indicated that Wnt/β-catenin, Notch, Hedgehog, NF-kappa B and mTOR signaling pathways play important roles in NPC CSC progression, however, targeting these signaling does

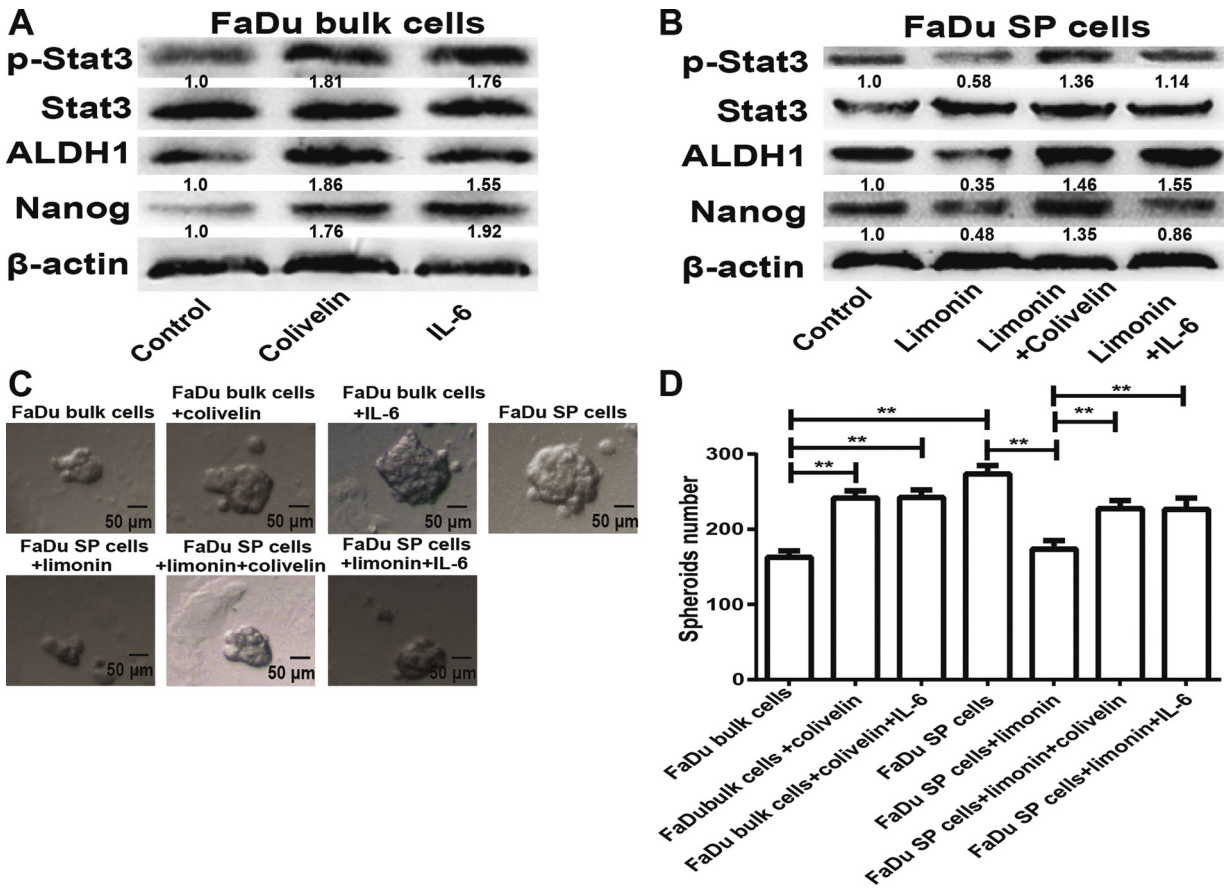

Fig. 6. Activation of Stat3 signaling partially reversed the inhibitory effects of limonin on NPC SP cell stemness. (A) The protein levels of p-Stat3 (Y705) and stemness markers (ALDH1 and Nanog) were detected in FaDu cells with or without colivelin and IL-6 treatment. (B) The protein levels of p-Stat3 (Y705) and stemness markers (ALDH1 and Nanog) were examined in FaDu SP cells with limonin plus colivelin or IL-6 treatment. (C and D) The spheroid formation ability was evaluated in the cells described in (B) through measuring spheroid size and number. Data are presented as the mean ± s.d, **P < 0.01 vs. bulk cells.

not achieve better therapeutic effects [26]. Therefore, there may be other signaling responsible for NPC CSC progression which should be validated and targeted for NPC treatment.

The transcriptional factor Stat3 is a cytokine closely related to cancer progression [27]. Also, recent studies have shown that Stat3 is a critical factor which is responsible for CSCs and normal stem cells development, for example, Stat3 promotes muscle stem cell oxidative metabolism and differentiation through regulating Fam3a activity [28]. Shiraiwa K. et al showed that the JAK/Stat3 signaling plays important roles in anaplastic thyroid carcinoma-CSCs through a small interfering RNA library targeting 719 kinases [29]. The heparan sulfate proteoglycan Syndecan-1 is positively correlated with the stemness of inflammatory breast cancer cells, which are characterized by CSC phenotype, via the IL-6/Stat3 pathway [30]. Notably, the CSC markers CD44 and CD24 collaboratively drive the reprogramming of NPC cells through Stat3-mediated stemness and EMT activation [31]. In the current study, we found that Stat3 signaling is the mostly-enriched pathway regulated by limonin in NPC SP cells and further rescue experiments confirmed that reactivating Stat3 partially reversed the inhibitory effects of limonin on the stemness of NPC SP cells using colivelin and IL-6. However, it must be noted that other pathways, such as Hippo and PI3K/Akt pathways, are also enriched by limonin treatment. Additionally, Hippo and PI3K/Akt pathways are also critical for CSCs progression [18,32]. Furthermore, our work showed that limonin facilitates the NPC SP cells entry into cell cycle and the PI3K/Akt pathway has been found to be responsible for regulating CSCs cell cycle [32]. Thus, limonin attenuates the stemness of NPC cells maybe through regulating other signaling, such as Hippo and PI3K/Akt pathways.

All in all, this work establishes that limonin could target NPC CSCs and thus enhances the radiosensitivity through inhibiting Stat3 signaling.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109366.

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