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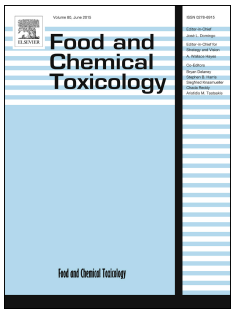

Limonin attenuates the stemness of cervical carcinoma cells by promoting YAP nuclear-cytoplasmic translocation

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Limonin attenuates the stemness of cervical carcinoma cells by promoting YAP nuclear-cytoplasmic translocation

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Abstract

This work aimed to investigate the role and related mechanism of limonin in regulating the stemness of cervical carcinoma (CC) cells. In the present study, we constructed adriamycin-resistant CC cells and found that they exhibited greater stemness than parental cells. Additionally, limonin attenuated the stemness of CC cells that were resistant or sensitive to adriamycin, as evidenced by the decreases in spheroid formation capacity, stemness markers expression and ALDH1 activity, whereas limonin did not affect the viability of normal cervical epithelial cells. Furthermore, limonin enhanced adriamycin sensitivity and attenuated adriamycin resistance in CC cells. Mechanistically, the nuclear-cytoplasmic translocation of YAP, not TAZ, was promoted by limonin in CC cells. Additionally, YAP overexpression attenuated the inhibitory effects of limonin on CC cell stemness. Therefore, limonin can attenuate the stemness, and thus the chemoresistance, of CC cells by promoting the nuclear-cytoplasmic translocation of YAP.

Keywords Limonin, YAP, Cervical carcinoma, Stemness, Nuclear-cytoplasmic translocation

1 Introduction

Cervical carcinoma (CC) is a common malignant tumour in women and the leading cause of morbidity and mortality in women in less-developed countries and regions [1]. Although surgery combined with radiotherapy and chemotherapy can improve the prognosis of early CC, recurrent and metastatic CC is difficult to cure, which is one of the main factors leading to a higher mortality rate for CC [2]. Thus, there is still an urgent need to find novel methods to treat CC.

Limonin is a furanolactone enriched in citrus fruits that has been shown to exhibit antibacterial, anti-inflammatory and anti-viral activity. Hassan et al. showed that limonin alleviates macro- and micro-vascular complications of metabolic syndrome in rats [3]. A novel limonin derivative was reported to modulate the inflammatory response by suppressing the TLR4/NF-κB signalling pathway [4]. Moreover, limonin inhibits eugenol-induced calcium and cAMP levels and the PKA/CREB signalling pathway in non-neuronal 3T3-L1 cells [5] and has the therapeutic efficacy against nonbacterial prostatitis [6]. Recently, Yao et al. reported that limonin could suppress tumour glycolysis and induce apoptosis in hepatocellular carcinoma by blocking hexokinase-2 phosphorylation [7]. However, the roles and related mechanism of limonin in CC progression have never been evaluated.

Cancer stem cells (CSCs) are a small proportion of cells in tumour tissue with stemness properties; they have self-renewal potential and uncertain differentiation and

can lead to tumourigenesis [8]. CSCs have been considered the root of tumour progression, metastasis and chemoresistance [9], and their roles and related mechanisms in blood tumours, breast cancer and brain tumours have been reported in detail. For example, Zheng et al. the inhibition of YAP/TAZ activity by a STARD13-related ceRNA network suppresses the stemness of breast cancer cells by co-regulating Hippo and Rho-GTPase/F-actin signalling [10]. PP2A inhibition sensitizes CSCs to ABL tyrosine kinase inhibitors in BCR-ABL(+) human leukaemia [11], and the EZH2 inhibitor GSK343 suppresses cancer stem-like phenotypes and reverses mesenchymal transition in glioma cells [12]. However, the roles and related mechanisms of CSCs in CC progression are still not clear.

The transcription factor YAP is a critical downstream effector of Hippo signalling [13] that has been shown to promote tumour proliferation and metastasis [14]. Importantly, YAP has been considered the root of cancer [15], and increased YAP transcriptional activity contributes to CSC progression [16]. Therefore, YAP has gradually become a potential target for tumour treatment. In the present study, we found that limonin could attenuate the stemness, and thus the chemoresistance, of CC cells by reducing YAP transcriptional activity.

2 Materials and methods

2.1 Cell culture and reagents

The healthy primary normal cervical epithelial cell line HcerEpic and the CC cell lines HeLa and C33A were purchased from ATCC. All of the cell lines were cultured in Dulbecco's Minimum Essential Medium (DMEM) (Gibco, USA) with 10% FBS

(foetal bovine serum, BI), 80 U/mL penicillin and 0.08 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Adriamycin-resistant HeLa cells were established by culture with 1 μM adriamycin, and cell clones were collected and screened. Then, the cells were cultured long-term with 5 nM adriamycin, and those cells with confirmed adriamycin resistance before experimental use were denoted as HeLa-ADM cells. Limonin was purchased from Selleck.cn (Houston, Texas, USA).

2.2 Plasmid construction and transfection

The YAP coding sequences were inserted into the pcDNA3.1 (+) vector, and the resulting plasmid was named pc-YAP. The following primers were used for constructing pc-YAP: Forward，5’ –ATGGATCCCGGGCAGCAGCCGCCGC- 3’; Reverse, 5’- CTATAACCATGTAAGAAAGCTTTCT -3’. CC cells were transfected with pc-YAP using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

2.3 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to detect mRNA levels. Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations. Then complementary DNA (cDNA) was reverse transcribed with the Hifair™ II 1st Strand cDNA Synthesis Kit (YEASEN) following the standard procedure. mRNA levels were examined with Hieff Unicon aqMan Multiplex qPCR Master Mix (YEASEN) on an ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). GAPDH served as an internal reference. The $2^{-\Delta\Delta^{ct}}$ method was used to calculate the relative expression of mRNAs.

2.4 Western blot

The detailed procedure was described previously [17]. The antibodies against YAP (ab52771), TAZ (ab84927), CTGF (ab6992), Nanog (ab21624), ALDH1 (ab23375), E-cadherin (ab1416), vimentin (ab92547) and GAPDH (ab8245) were purchased from Abcam (Cambridge, MA, USA). Blots were washed and incubated with a peroxidase-conjugated antibody (Beyotime, Beijing, China), and chemiluminescence was detected using an enhanced chemiluminescence kit (Beyotime) followed by exposure with the Tanon 5200 imaging system.

2.5 Cell spheroid formation assay

CC cell spheroid formation was analysed under anchorage-independent conditions in methylcellulose (Sigma). Briefly, CC cells exposed to different treatments were digested with trypsin-EDTA (Sigma) and then cultured in ultra-low attachment 24-well plates (Corning, Union City, CA) at 500 cells/well in serum-free medium: 200 mL of DMEM/F12 or 200 mL of RPMI 1640 basal medium supplemented with 20 ng/mL EGF, 10 ng/mL bFGF, and 2% B27 (all from Sigma and Gibco). After 8 days, the number of spheroids containing at least five cells and at least 50 μm in diameter was determined by microscopy. This experiment was performed in triplicate and repeated at least three times independently.

2.6 ALDH1 activity assay

ALDH1 activity was assayed by the ALDEFLUOR™ Kit (Cat # KA3742, Stemcell Technologies) following the standard procedure.

2.7 Immunofluorescence (IF) assays

YAP localization was analysed by IF assays. Coverslips with cells were washed gently with PBS and then fixed with 4% fresh para-formaldehyde for 15 min. The coverslips were washed with PBS, and the cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature, blocked with 10% goat serum for 30 min at 20 °C, and incubated with primary antibody against YAP (diluted 1:100; Abcam) overnight at 4 °C. The coverslips were washed extensively with PBS and incubated with species-specific FITC-labelled secondary antibody (Proteintech, Chicago, USA) at a dilution of 1:200 for 60 min at room temperature. The coverslips were treated with 4,6-diamidino-2-phenylindole (DAPI) for 5 min for nuclear staining and then mounted on glass slides. Images were acquired using a fluorescence microscope (Olympus).

2.8 Luciferase reporter assay

A luciferase reporter assay was performed to detect YAP transcriptional activity in CC cells exposed to different treatments. Briefly, the 8xGTIIC-luciferase plasmid, which contains a YAP-responsive synthetic promoter driving luciferase expression, was co-transfected into CC cells with a β-gal plasmid (Ambion, USA) using Lipofectamine^{TM2000}. After 72 h, luciferase activity was examined. β-Gal activity was used as a normalization control for luciferase activity.

2.9 Cell viability assay

Cells were digested and 3×10^3 cells were seeded into 96-well plates and treated with limonin (5 μM, 10 μM, or 20 μM), adriamycin (10 nM) plus limonin (20 μM) or control (no treatment). After 24 h, 48 h and 72 h, cell viability was examined by using

a CCK8 assay kit (Cat# HY-K0301, MedChemExpress, USA) following the manufacturer's protocols.

2.10 Cell Apoptosis

After CC cells were exposed to different treatments, apoptosis was measured using flow cytometry analysis with Annexin V-FITC and propidium iodide (PI) (Cat# C1062, Beyotime, China). Cells were harvested, washed with ice-cold PBS, and stained with Annexin V-FITC and PI (Beyotime) following the manufacturer's recommendations. Flow cytometry analysis was then performed (BD Biosciences).

2.11 Cell migration assay

The detailed assay procedure was reported in a previous study [18]. Briefly, CC cells were digested and resuspended, and 8×10^4 cells were added to the upper chambers in a 24-well plate containing MILLIcell PET Hanging Cell Culture Inserts (pore size, 8 μm; PET; MILLIPORE), and then treated with or without limonin (5 μM, 10 μMor 20 μM), Eight hundred microlitres of medium containing 20% FBS was used as a chemo-attractant in the bottom chamber. After 24 h, cells that migrated into the bottom chamber were fixed in methanol for 15 min and stained with 0.1% crystal violet for 15 min. Migrated cells in five random fields from each well were counted in triplicate by using phase contrast microscopy. Cells were also quantified with a microplate reader (OD 570 nm) after destaining with 30% glacial acetic acid.

2.12 Statistical analysis

All data were obtained from at least three independent experiments ($n \geq 3$), and the results are presented as the mean ± SD (standard deviation). Differences were

analysed using one-way ANOVA with the Tukey–Kramer post-test, and $P < 0.05$ was considered significant.

3 Results

3.1 Limonin attenuates the stemness of CC cells in a concentration dependent manner

Whether limonin inhibits the stemness of CC cells has not been evaluated previously. We found that limonin suppressed the expression of stemness markers (ALDH1 and Nanog) in a dose-dependent manner via qRT-PCR and western blot analysis (**Figure S1A - C**). We also examined whether limonin affects the viability of normal cervical epithelial cells. Cell viability and apoptosis assays indicated that limonin had no effect on the viability or apoptosis of HcerEpic cells (**Figure 1A** and **1B**). Furthermore, cell spheroid formation assays showed that limonin significantly decreased the capacity for spheroid formation, characterized as decreases in spheroid size and number (**Figure 1C** and **1D**). Additionally, ALDH1 activity in CC cells was suppressed by limonin treatment (**Figure 1E**). Therefore, these results suggest that limonin attenuated the stemness of CC cells.

3.2 Limonin attenuates the migration and EMT (epithelial-mesenchymal transition) of adherent CC cells and non-adherent spheroid cells

Because CSCs contribute to cell migration [8], we further determined whether limonin affects CC cell migration. Transwell migration analysis showed that limonin markedly inhibited the migration ability of CC cells (**Figure 2A** and **2B**). Additionally, limonin suppressed EMT process, as evidenced by the increased expression of the

epithelial marker E-cadherin and the decreased expression of mesenchymal marker vimentin (**Figure S1D - F**). As cell spheroids are enriched for CSCs, we explored whether limonin directly affects the characteristics of cells in CC cell spheroids. As expected, CC cell spheroids displayed higher expression of stemness markers (ALDH1 and Nanog) (**Figure 2C** and **2D**) and higher ALDH1 activity thant adherent CC cells (**Figure 2E**). Then, CC cell spheroids were digested, resuspended, seeded into 96-well plates, and treated with limonin. As shown in **Figure 3A**, limonin significantly decreased cell spheroid viability. Moreover, consistent results were obtained from Transwell migration analysis (**Figure 3B** and **3C**). Notably, EMT was attenuated in CC cell spheroids (**Figure 3D** and **3E**).

3.3 Limonin suppresses the transcriptional activity of YAP by promoting its nuclear-cytoplasmic translocation

Because YAP/TAZ have been shown to contribute to CSC progression, we wondered whether limonin affects YAP/TAZ activity. As shown in **Figure 4A**, the nuclear-cytoplasmic translocation of YAP, not TAZ, was promoted by limonin treatment. Consistently, limonin treatment decreased nuclear YAP expression and increased cytoplasmic YAP expression, whereas the nuclear/cytoplasmic ratio of TAZ was unaffected (**Figure S2A**). Furthermore, a luciferase reporter assay showed that limonin decreased YAP transcriptional activity, characterized by decreased activity of 8xGTIIC-luciferase, a YAP-responsive synthetic promoter driving luciferase expression (**Figure 4B**). Additionally, the expression of CTGF, a target of YAP, was suppressed by limonin treatment (**Figure 4C** and **4D**). Limonin treatment did not alter

the mRNA levels of YAP/TAZ but did, decrease the protein levels of YAPbut not TAZ (**Figure S2B** and **S2C**).

3.4 Limonin attenuates the stemness of CC cells in a YAP-dependent manner

Next, we further investigated whether limonin attenuates the stemness of CC cells in a YAP-dependent manner. CC cells were transfected with pc-YAP after limonin treatment, and as expected, pc-YAP transfection rescued the limonin-induced decrease in YAP expression (**Figure S3A**). Additionally, YAP overexpression rescued the limonin-mediated attenuation of CC cell stemness, as evidenced by the recovery of stemness marker expression (**Figure S3B - D**), cell spheroid formation ability (**Figure 5A** and **5B**) and ALDH1 activity (**Figure 5C**). Consistently, YAP overexpression attenuated the inhibitory effects of limonin on CC cell migration and EMT (**Figure 5D** and **5E, Figure S3E - G**). Taken together, our results demonstrate that limonin attenuates the stemness of CC cells by promoting YAP nuclear-cytoplasmic translocation.

3.5 Adriamycin-resistant CC cells display greater stemness than parental cells

Because CSCs can promote chemoresistance, we constructed adriamycin-resistant CC cells, named HeLa-ADM cells. As expected, HeLa-ADM cells displayed a greater stemness than the parental cells, characterized by higher expression of stemness markers (**Figure 6A** and **6B**), cell spheroid formation capacity (**Figure 6C**) and ALDH1 activity (**Figure 6D**). Additionally, HeLa-ADM cells exhibited greater migration ability (**Figure 6E** and **6F**). Notably, the nuclear/cytoplasmic ratio of YAP was higher in HeLa-ADM cells than in parental

cells (**Figure 6G**).

3.6 Limonin attenuates adriamycin resistance and enhances adriamycin sensitivity in CC cells through YAP

Finally, we evaluated whether limonin attenuates the adriamycin resistance of CC cells. Cell viability and apoptosis assays indicated that limonin attenuated adriamycin resistance in HeLa-ADM cells, and enhanced adriamycin sensitivity in HeLa cells (**Figure 7A** and **7B**); this effect was rescued by YAP overexpression. Notably, limonin attenuated the stemness of HeLa-ADM cells, as evidenced by the decreases in stemness marker expression (**Figure 7C** and **7D**), cell spheroid formation (**Figure 7E** and **7F**) and ALDH1 activity (**Figure 7G**); these effects were rescued by YAP overexpression. Therefore, we concluded that limonin can enhance adriamycin sensitivity and attenuate adriamycin resistance in CC cells by regulating CC cell stemness through YAP.

4 Discussion

Although the incidence of invasive CC has decreased, the incidence of early CC has increased, especially among younger women [19]. CSCs are the basis of cancers, and a large number of studies have shown that CC development and progression are significantly related to CSCs [20]. To find the root cause of CC, research on cervical CSCs has become a popular topic in terms of diagnosis and treatment. Here, we show that limonin can attenuate the stemness of CC cells that are adriamycin resistant or sensitive by promoting YAP nuclear-cytoplasmic translocation. To the best of our knowledge, this is the first study revealing the roles of limonin in CC cell stemness

and chemoresistance.

Because cell spheroids are enriched for CSCs [21], we explored the effects of limonin on CC cell spheroid progression and found that limonin inhibited spheroid-derived cell viability, migration and stemness; these effects indicate that limonin might directly kill CSCs among the CC cell population. Consistent results were obtained in another study demonstrating that Tanshinone IIA inhibits CC stem cell migration and invasion [22]. Notably, we found that limonin exerted modest but not significant cytotoxicity in HcerEpic cells (**Figure 1E**). This could be due to the increased amount of DMSO in high concentrations of limonin. Moreover, we admit that other techniques, such as flow cytometry, could be used to sort cervical CSCs for testing. Importantly, we found that the migration ability of HeLa-ADM cells was greater than that of parental HeLa cells (**Figures 2** and **3**), which supports the notion that cell stemness contributes to cell migration and invasion [23]. Importantly, limonin is one of many furanolactones, this chemical family and their crosstalk in in CC cell signalling need to be studied. Moreover, this work was performed *in vitro*, and further *in vivo* experiments should be conducted to confirm effects observed *in vitro*.

Both YAP and TAZ are key downstream effectors of the Hippo pathway, which is involved in organ development [24]. Importantly, YAP/TAZ are regarded as the root of cancer [15] and are thus therapeutic targets [25]. Increased YAP/TAZ transcriptional activity has been shown to contribute to CSC-related cancer progression [14, 26]. Here, we show that limonin promotes the nuclear-cytoplasmic

translocation of YAP, decreases its nuclear/cytoplasmic ratio and thus reduces its transcriptional activity, whereas limonin had no effect on TAZ, perhaps due to the different structures of YAP and TAZ. Likewise, a previous study showed that glucocorticoid receptor signalling could activate YAP but not TAZ in breast cancer [27]. Additionally, verteporfin exhibits YAP-independent anti-proliferative and cytotoxic effects in endometrial cancer cells [28]. However, the mechanisms contributing to the limonin-mediated inhibition of YAP activity remain unclear and should be explored in the future.

In conclusion, this work reveals that limonin can attenuate the stemness of CC cells and enhance the adriamycin sensitivity of CC cells that are resistant or sensitive to adriamycin. Thus, limonin provides new clues for the treatment of CC patients, especially those with chemoresistance.

5 Acknowledgements

Not applicable.

6 Author contributions

Conceptualization: Wenxia Du; Methodology, Lijuan Cui; Software, Lijuan Cui; Validation: Wei Zhao and Mingyuan Wu; Formal Analysis: Wei Zhao and Mingyuan Wu; Investigation: Wei Zhao and Mingyuan Wu; Resources: Wenxia Du; Data Curation: Wei Zhao, Mingyuan Wu and Wenxia Du; Writing – Original Draft Preparation: Wei Zhao and Mingyuan Wu; Writing – Review & Editing: Wei Zhao, Mingyuan Wu, Lijuan Cui and Wenxia Du; Visualization: Mingyuan Wu and Wenxia Du; Supervision: Wenxia Du; Project Administration: Wenxia Du.

7 Conflicts of interest

The authors declare no conflict of interest.

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9 Figure legends

Figure 1. Limonin attenuates the stemness of CC cells in a concentration-dependent manner. (**A**) HcerEpic cells were treated with the indicated concentrations of limonin for 24 h, 48 h and 72 h, and viability was then detected via a CCK8 assay. (**B**) HcerEpic cells were treated with different concentrations of limonin for 48 h and 72 h, and apoptosis was then detected. (**C** and **D**) The potential for CC cell spheroid formation was evaluated following treatment with of the indicated concentrations of limonin for 10 days by measuring spheroid size (**C**) and numbers (**D**). (**E**)

ALDH1 activity was evaluated in cells treated with different concentrations of limonin for 48 h using an ALDEFLUOR™ Kit. Data are presented as the mean ± s.d. *P < 0.05, **P < 0.01 vs. control.

Figure 2. Limonin attenuates the migration and EMT of adherent CC cells and non-adherent spheroid cells. (**A** and **B**) The cell migration ability of CC cells was determined following treatment with different concentrations of limonin, as indicated. (**C** and **D**) E ALDH1 and Nanog expression in parental HeLa cells and HeLa spheroids was examined. (**E**) ALDH1 activity was measured in parental HeLa cells and HeLa spheroids using an ALDEFLUOR™ Kit. Data were presented as mean ± s.d. *P < 0.05, **P < 0.01 vs. control or HeLa cells.

Figure 3. Limonin suppresses HeLa spheroid cell viability, migration and t EMT. (**A**) HeLa spheroid cells were treated with different concentrations of limonin for 24 h, 48 h and 72 h, and viability was then detected via a CCK8 assay. (**B** and **C**) The migratory ability of HeLa spheroid cells was determined following treatment with different concentrations of limonin, as indicated. (**D** and **E**) The expression of E-cadherin and vimentin was detected in the cells described in (**A**). Data are presented as the mean ± s.d. *P < 0.05, **P < 0.01 vs. control.

Figure 4. Limonin suppresses the transcriptional activity of YAP by promoting its nuclear-cytoplasmic translocation. (**A**) The nuclear-cytoplasmic translocation of YAP/TAZ in HeLa cells treated with or without limonin was examined by immunofluorescence assay. (**B**) The luciferase activity of 8xGTIIC-luciferase, a YAP-responsive synthetic promoter driving luciferase expression plasmid, was evaluated in CC cells following limonin treatment or no treatment. (**C** and **D**) CTGF mRNA and protein levels were determined in the cells described in (**B**).

Figure 5. Limonin attenuates the stemness of CC cells in a YAP-dependent manner. (**A** and **B**)

CC cell spheroid size (**A**) and number (**B**) were detected in CC cells following limonin (20 μM) treatment with or without pc-YAP transfection. (**C**) ALDH1 activity was measured in the cells depicted in (**A**). (**D** and **E**) Cell migration ability was detected in the cells depicted in (**A**). Data are presented as the mean ± s.d. **P < 0.01 vs. control.

Figure 6. Adriamycin-resistant CC (HeLa-ADM) cells display greater stemness than parental cells. (**A** and **B**) The expression of stemness markers (ALDH1 and Nanog) was detected in HeLa and HeLa-ADM cells. (**C**) HeLa and HeLa-ADM cell spheroid size and numbers were detected. (**D**) ALDH1 activity was measured in HeLa and HeLa-ADM cells. (**E** and **F**) The migration of HeLa and HeLa-ADM cells was determined. (**G**) Nuclear and cytoplasmic levels of YAP were detected in HeLa and HeLa-ADM cells. Data are presented as the mean ± s.d. *P < 0.05, **P < 0.01 vs. HeLa.

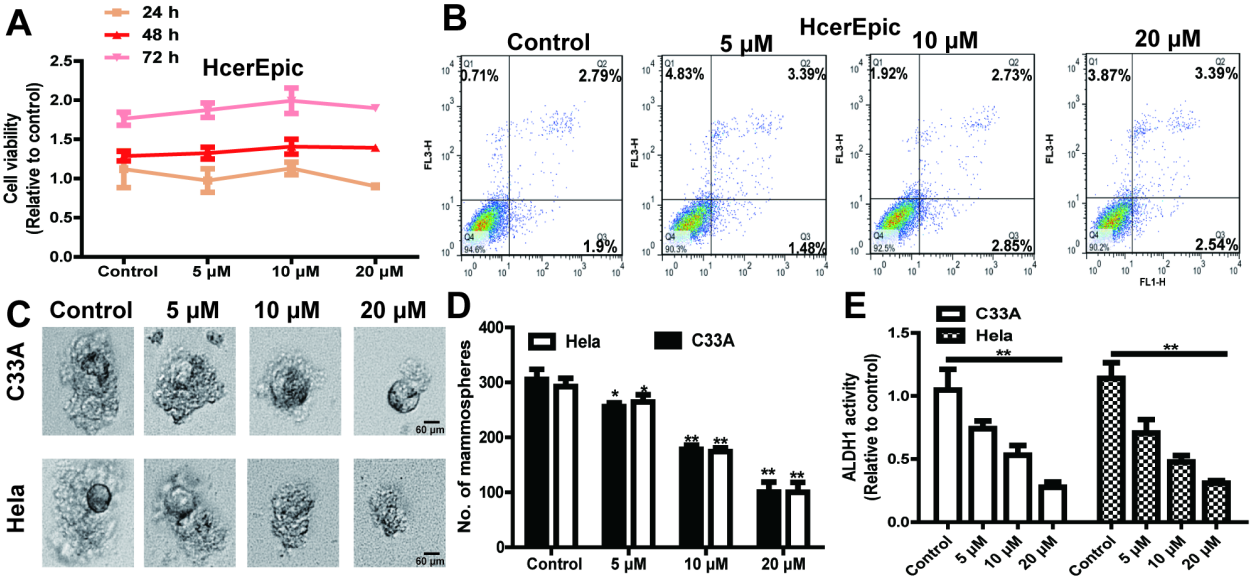
Figure 7. Limonin enhances adriamycin sensitivity in CC cells. (**A** and **B**) The viability of HeLa and HeLa-ADM cells treated with limonin and transfected or not with pc-YAP was determined by CCK8 assay. (**C** and **D**) The expression of stemness markers (ALDH1 and Nanog) was detected in HeLa-ADM cells treated with limonin and transfected or not with pc-YAP. (**E** and **F**) Spheroid size and number were detected in the cells described in (**C**). (**G**) ALDH1 activity was measured in the cells described in (**C**). Data are presented as mean ± s.d. *P < 0.05, **P < 0.01 vs. control.

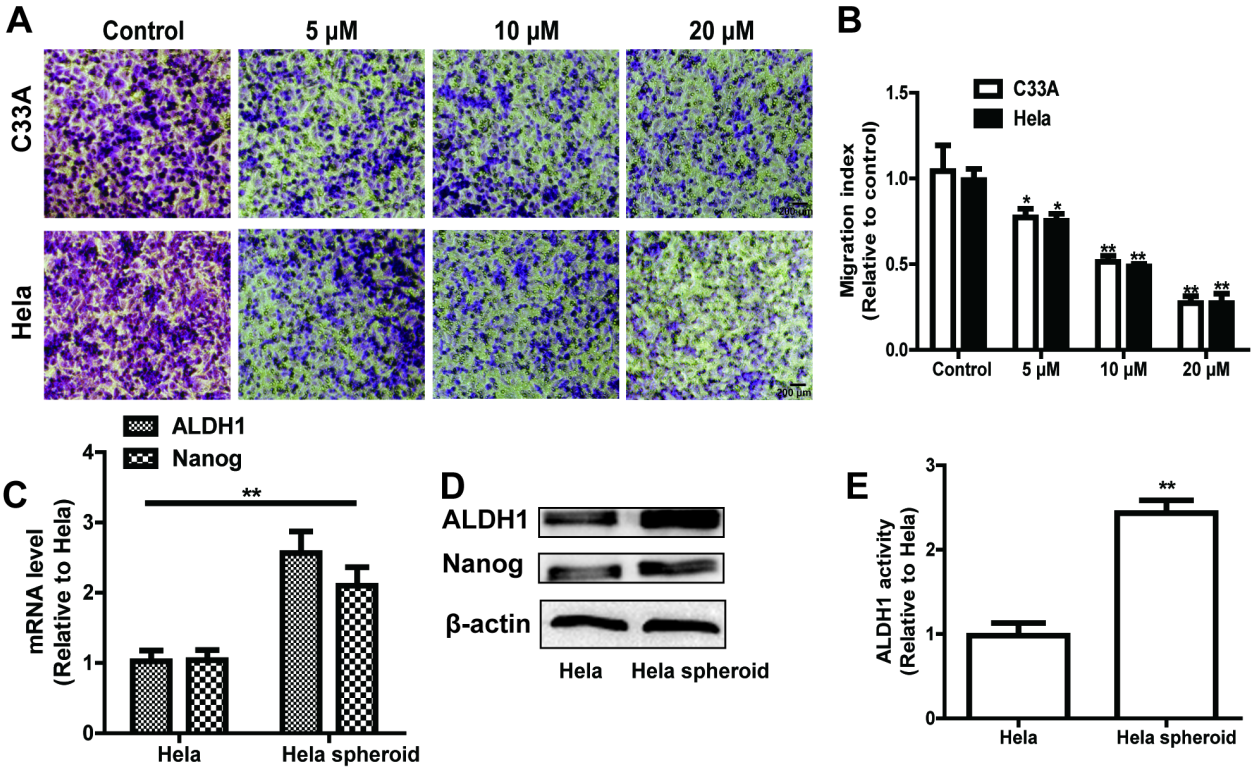
Figure S1. Limonin negatively regulates the expression of stemness markers and EMT in CC cells. (**A** and **B**) mRNA levels of ALDH1 (**A**) and Nanog (**B**) were detected in C33A and HeLa cells following treatment with different concentrations of limonin for 48 h. (**C**) Protein levels of ALDH1 and Nanog were determined in the cells described in (**A**). (**D** - **F**) mRNA

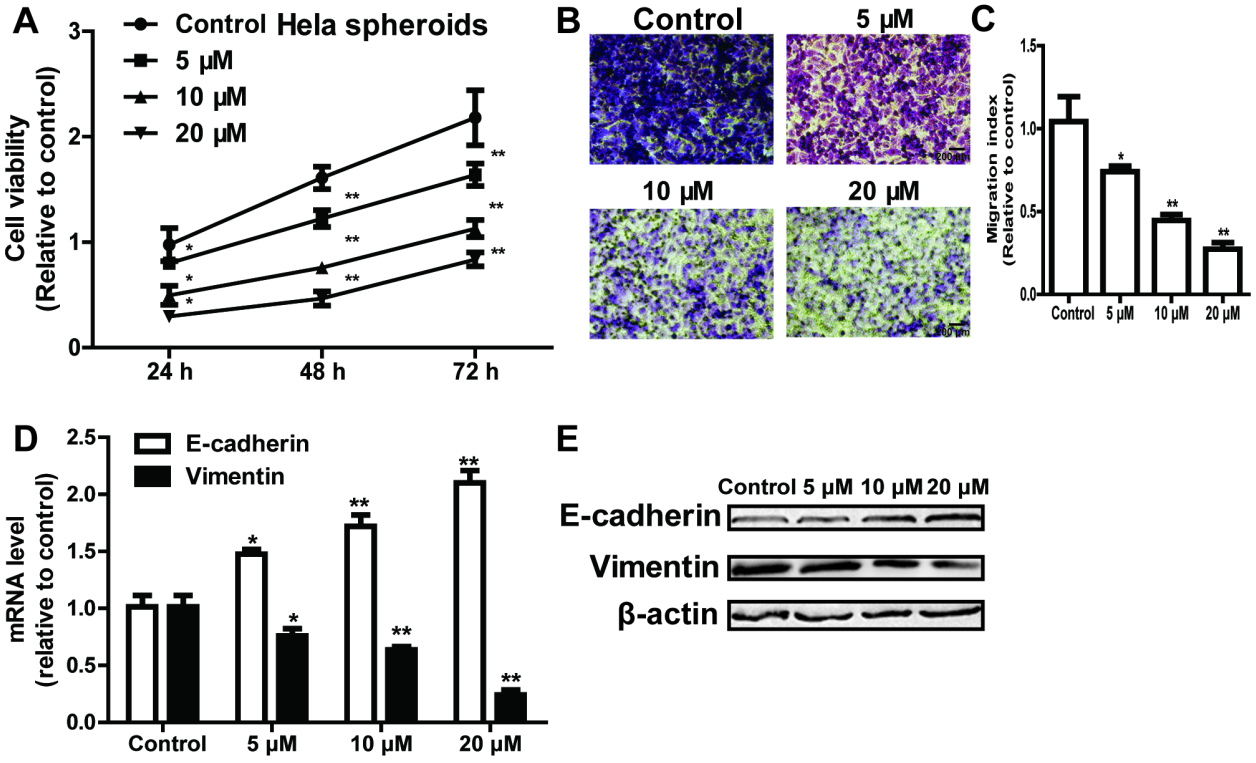
levels of E-cadherin (**D**) and vimentin (**E**) were detected in C33A and HeLa cells following treatment with different concentrations of limonin for 48 h. (**C**) Protein levels of E-cadherin and vimentin were examined in the cells described in (**D**). Data are presented as the mean ± s.d. *P < 0.05, **P < 0.01 vs. control.

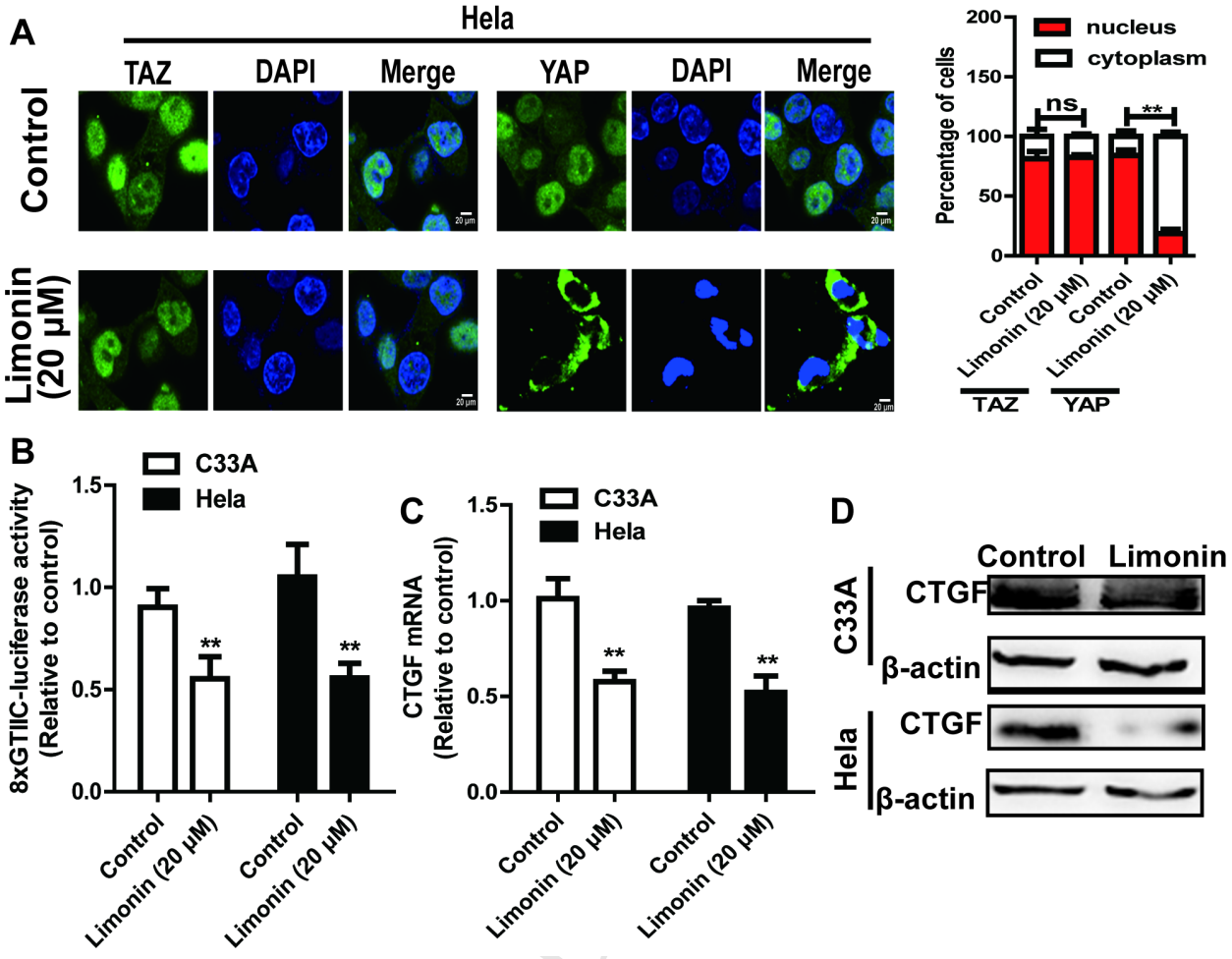
Figure S2. Limonin suppresses YAP nuclear-cytoplasmic translocation. (**A**) YAP/TAZ protein levels in the nucleus and cytoplasm were measured in HeLa cells following limonin treatment or no treatment. (**B** and **C**) YAP/TAZ mRNA (**B**) and protein (**C**) levels were detected in CC cells following limonin treatment or no treatment. Data are presented as the mean ± s.d. **P < 0.01 vs. control.

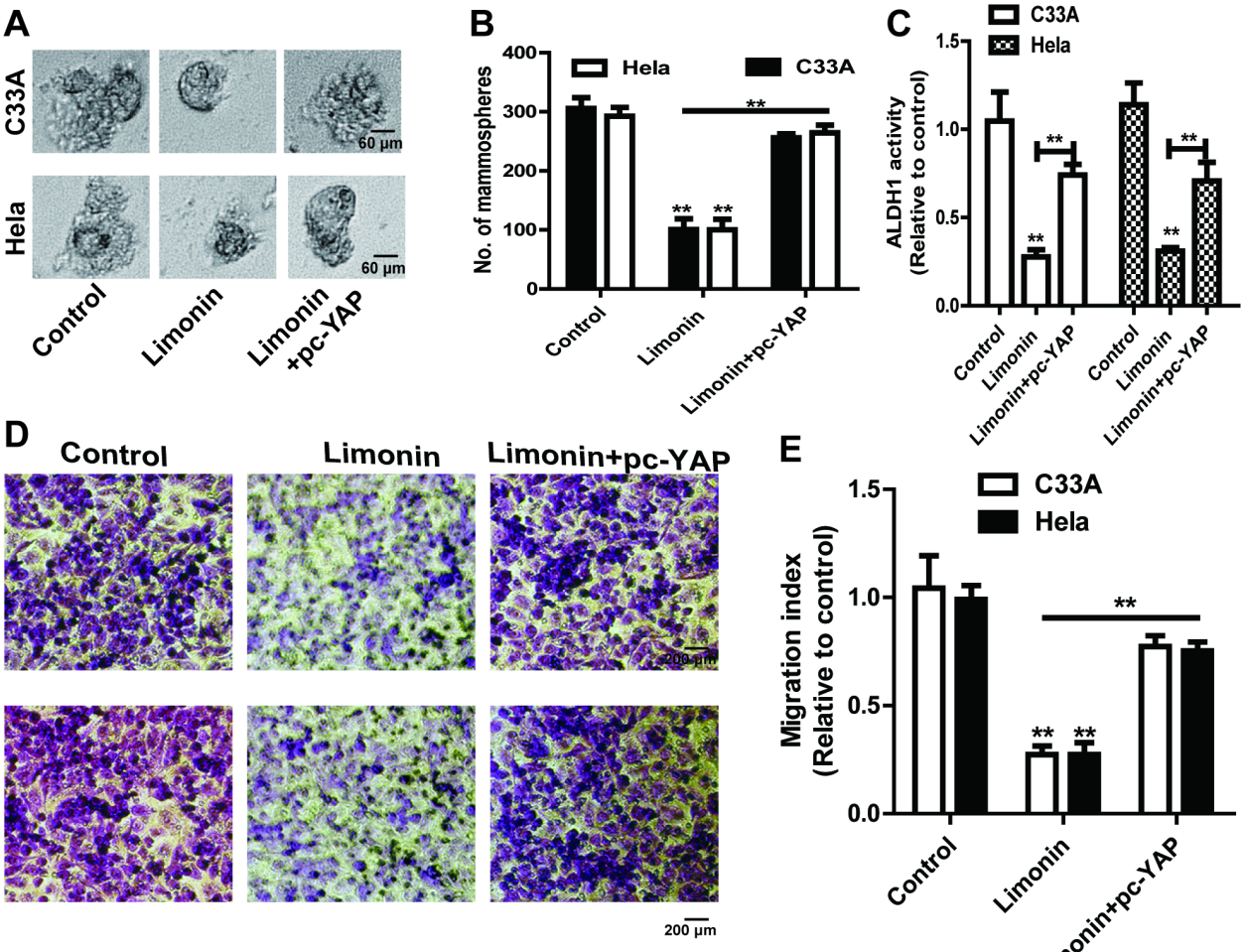
Figure S3. Limonin attenuates the expression of stemness markers and EMT in CC cells in a YAP-dependent manner. (**A**) YAP protein levels were examined in CC cells following limonin (20 μM) treatment with or without pc-YAP transfection. (**B - D**) ALDH1 and Nanog mRNA and protein levels were measured in the CC cells depicted in (**A**). (**E - G**) E-cadherin and vimentin expression was evaluated in the cells depicted in (**A**). Data are presented as the mean ± s.d; **P < 0.01 vs. control.

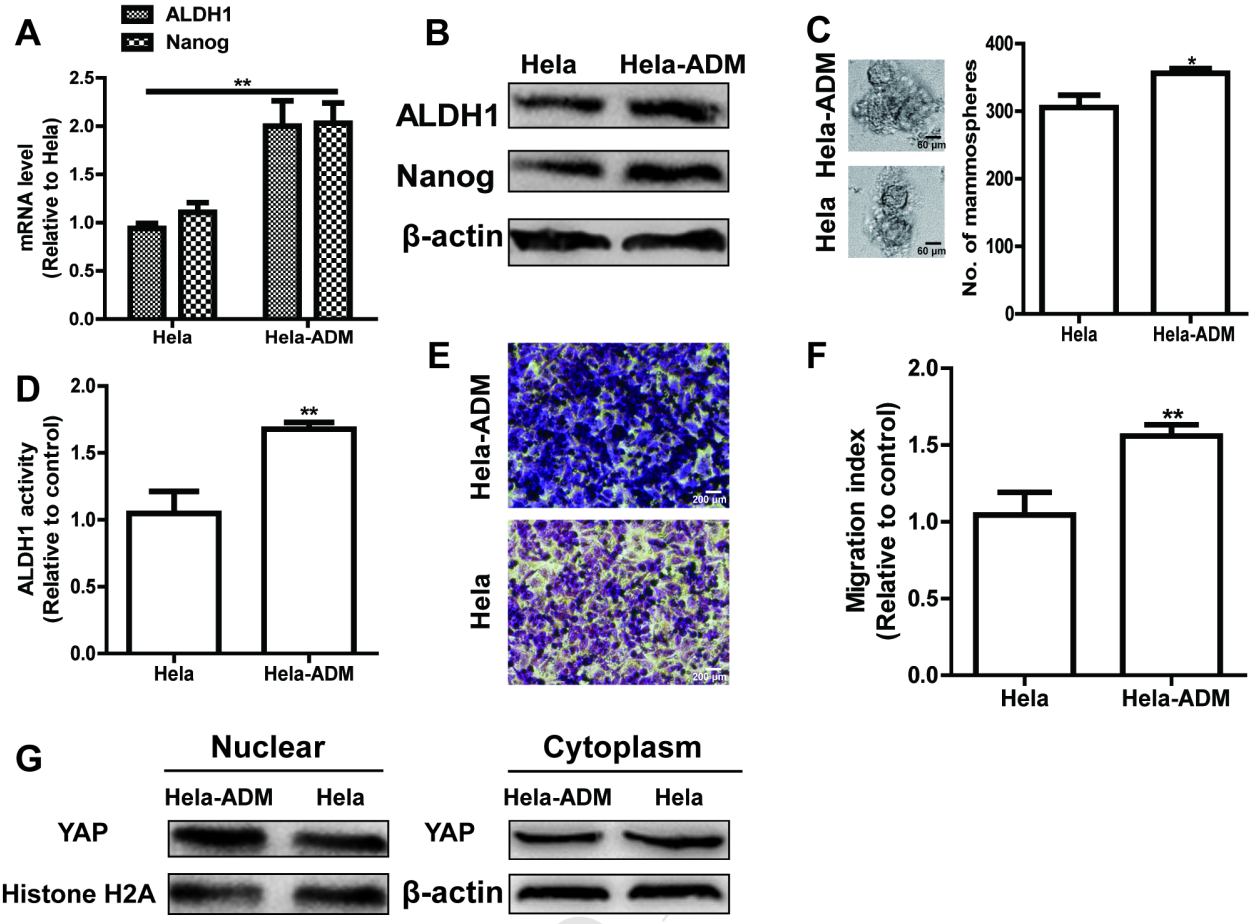


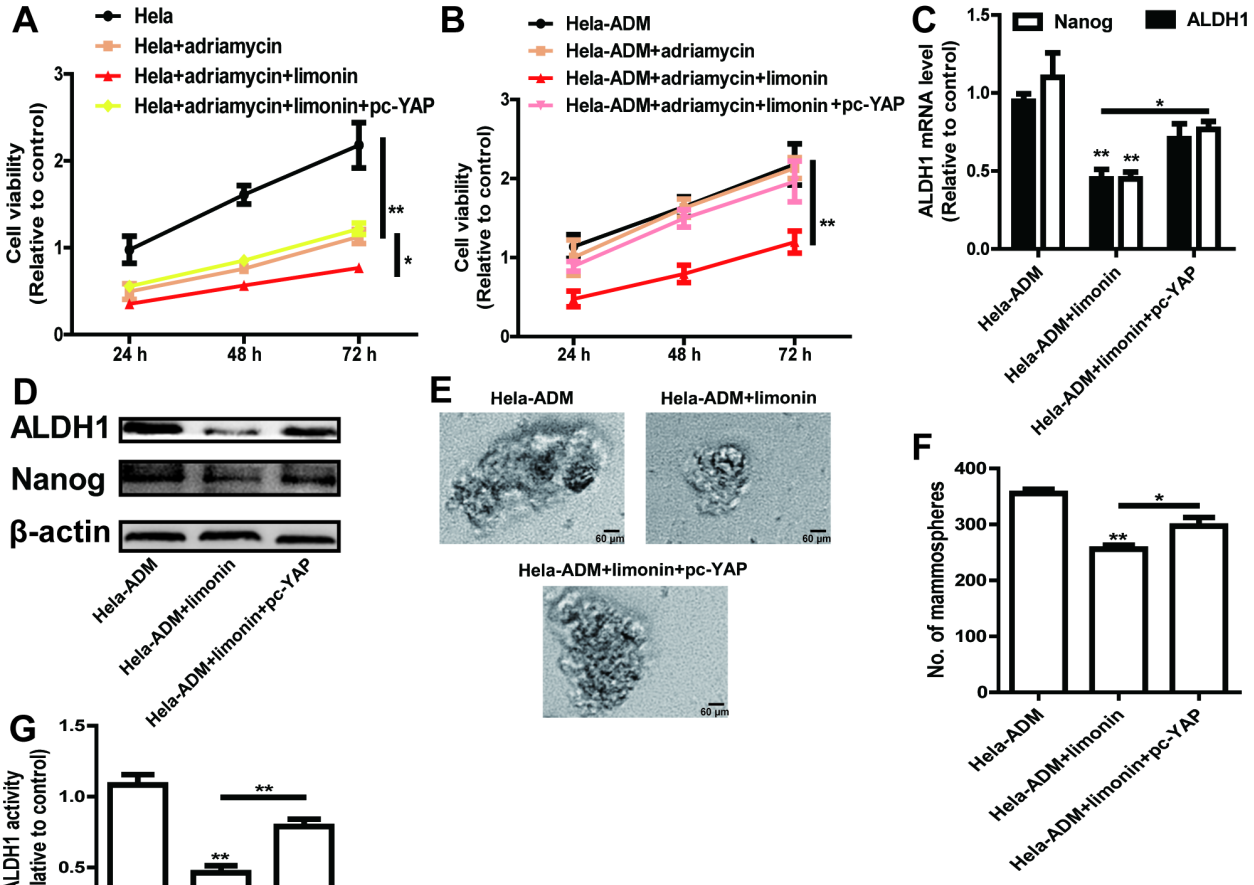





A
Hela
TAZ
DAPI
Merge
YAP
DAPI
Merge
Control
Limonin (20 μM)
20 μm
nucleus
cytoplasm
Percentage of cells
200
150
100
50
0
ns
**
Control
Limonin (20 μM)
Control
Limonin (20 μM)
TAZ
YAP
B
C33A
Hela
8xGTIIC-luciferase activity (Relative to control)
1.5
1.0
0.5
0.0
**
**
Control
Limonin (20 μM)
Control
Limonin (20 μM)
C
C33A
Hela
CTGF mRNA (Relative to control)
1.5
1.0
0.5
0.0
**
**
Control
Limonin (20 μM)
Control
Limonin (20 μM)
D
Control
Limonin
C33A
CTGF
β-actin
Hela
CTGF
β-actin


A
C33A
Hela
60 μm
60 μm
Control
Limonin
Limonin +pc-YAP
B
No. of mammospheres
Hela
C33A
**
Control
Limonin
Limonin+pc-YAP
C
C33A
Hela
ALDH1 activity (Relative to control)
**
Control
Limonin
Limonin+pc-YAP
D
Control
Limonin
Limonin+pc-YAP
200 μm
E
C33A
Hela
Migration index (Relative to control)
**
Control
Limonin
Limonin+pc-YAP




A
Hela
Hela+adriamycin
Hela+adriamycin+limonin
Hela+adriamycin+limonin+pc-YAP
Cell viability (Relative to control)
24 h
48 h
72 h
B
Hela-ADM
Hela-ADM+adriamycin
Hela-ADM+adriamycin+limonin
Hela-ADM+adriamycin+limonin +pc-YAP
C
Nanog
ALDH1
ALDH1 mRNA level (Relative to control)
Hela-ADM
Hela-ADM+limonin
Hela-ADM+limonin+pc-YAP
D
ALDH1
Nanog
β-actin
E
Hela-ADM
Hela-ADM+limonin
Hela-ADM+limonin+pc-YAP
F
No. of mammospheres
G
ALDH1 activity (Relative to control)

Highlights:

A new critical role of limonin in CC cells stemness is proposed;

The mechanisms of limonin in CC cells stemness are further studied;

A novel mode of regulation of YAP activity by limonin in CC cells.

The novel mechanism provides a new potential drug for CC.