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Anticancer Potential of Myricetin against Huh7- and Hep3B-Derived Liver Cancer Stem Cells through the Regulation of Apoptosis, Autophagy, and Stemness

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Abstract

Liver cancer stem cells (LCSCs) play a significant role in the development, metastasis, treatment resistance, and recurrence of hepatocellular carcinoma (HCC). Targeting LCSCs offers a novel strategy to overcome treatment resistance in HCC. Myricetin, a flavonol from the flavonoid family, is known for its diverse biological activities, including anticancer effects. However, its potential for eradicating LCSCs had not been thoroughly investigated prior to this study. This study evaluated the effects of myricetin on LCSCs derived from Huh7 and Hep3B cell lines both *in vitro* and *in vivo*. LCSCs were treated with myricetin to assess cell proliferation, cell cycle arrest, apoptosis induction, autophagy regulation, stemness and EMT marker expression, and tumor growth suppression using a chicken embryo CAM model. Additionally, the combination therapy of myricetin with chloroquine, an autophagy inhibitor, was explored. Myricetin significantly inhibited the proliferation of Huh7- and Hep3B-derived LCSCs and suppressed tumor growth in the CAM model. It induced cell cycle arrest at the G0/G1 phase and triggered apoptosis through intrinsic and extrinsic pathways. Myricetin also stimulated autophagy by inhibiting the PI3K/AKT/mTOR pathway, reduced the expression of stemness markers, including Sox2, Oct4, Nanog, and ALDH1A1, and suppressed EMT. Combining myricetin with chloroquine enhanced apoptotic effects and further downregulated stemness markers by inhibiting STAT3 activation, demonstrating greater efficacy than myricetin alone. The findings establish myricetin, either as a standalone treatment or in combination with chloroquine, as a promising therapeutic candidate for targeting LCSC growth and overcoming chemotherapy resistance in HCC.

Key Words: Liver cancer stem cell, Myricetin, Chloroquine, Apoptosis, Autophagy

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 90% of all liver cancer cases, and is considered one of the most frequently diagnosed malignancies worldwide (Dopazo *et al*., 2024). Over the past two decades, the global prevalence of HCC has risen by nearly 75%, with projections indicating that liver cancer may result in over 1 million deaths annually by 2030. Chronic liver diseases caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are the primary risk factors for HCC development (Howell *et al*., 2021). Early-stage HCC is treated with curative approaches such as hepatectomy and liver transplantation. For intermediate-stage cases, therapies such as transarterial chemoembolization (TACE), targeted drugs like sorafenib, and immunotherapy are commonly employed (Yang *et al*., 2024). Despite advancements in treatment, the overall prognosis remains poor due to frequent late-stage diagnosis and high rates of recurrence.

A significant factor contributing to HCC progression and recurrence is the presence of liver cancer stem cells (LCSCs), a subpopulation of tumor cells with unique properties, including self-renewal, differentiation, tumor initiation, and resistance to conventional therapies (Lee *et al*., 2022). LCSCs also play a critical role in metastasis and therapeutic resistance, primarily due to their ability to evade programmed cell death and maintain a stem-like phenotype within the tumor microenvironment (Castelli *et al*., 2021). These cells express elevated levels of stemness markers, such as Sox2, Oct4, Nanog, and aldehyde dehydrogenase 1A1 (ALDH1A1), which further promote their

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survival and proliferation (Lee *et al*., 2022). Additionally, LCSCs facilitate epithelial-mesenchymal transition (EMT), a process that plays a key role in tumor metastasis, as well as in CSC formation and maintenance (Pradella *et al*., 2017; Babaei *et al*., 2021). Notably, stemness markers and EMT in LCSCs are regulated by upstream signaling pathways, such as Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) (Jia *et al*., 2021). Given their pivotal role in HCC progression, targeting the stem-like characteristics of LCSCs and the signaling pathways regulating their activity is vital for developing cancer stem cell (CSC)-focused therapies. Such strategies hold promise for achieving improved and sustained treatment outcomes in HCC patients.

CSCs evade cell death through diverse survival mechanisms (Safa, 2020). Cell death is broadly categorized into accidental cell death (ACD) and programmed cell death (PCD), with PCD further classified into apoptosis (type I), autophagy (type II), and necroptosis (type III) based on their distinct morphological and functional characteristics (Shen *et al*., 2023). Among these, apoptosis is the predominant form of PCD and plays a pivotal role in suppressing cancer cell proliferation (Moyer *et al*., 2025). This process is marked by cell shrinkage, membrane vacuolization, and chromatin condensation, and is regulated by the caspase protein family via the intrinsic (mitochondrial) and extrinsic (death receptor) pathways (Kashyap *et al*., 2021). Therefore, inducing apoptosis is a key strategy for limiting the survival and proliferation of LCSCs.

Autophagy, a cellular process essential for maintaining energy homeostasis during nutrient deprivation and stress, also plays a dual role in cancer progression (Liu *et al*., 2023). It involves the formation of autophagosomes that degrade and recycle cellular components through lysosomal fusion. In liver cancer, autophagy acts as both a tumor suppressor and a pro-survival mechanism, depending on the context. For instance, the anticancer drug sorafenib induces autophagy, which enhances tumor suppression but also promotes cancer cell survival. Sorafenib increases autophagy markers such as LC3-II and Beclin-1, but combining it with the autophagy inhibitor chloroquine significantly improves its anticancer efficacy, underscoring autophagy's protective role in cancer cell survival (Prieto-Domínguez *et al*., 2016; Sun *et al*., 2017).

Emerging evidence suggests that autophagy is intricately involved in CSC function, supporting their survival, stemness, and tumorigenic capacity (Wang *et al*., 2022). For example, autophagy facilitates CSC survival by removing cytoplasmic ubiquitinated p53 in lung CSCs, thereby regulating post-transcriptional p53 levels and promoting tumor formation (Wang *et al*., 2021). In contrast, curcumin has been shown to induce autophagy in glioblastoma stem cells, which subsequently reduces their self-renewal and differentiation potential in mouse models (Zhuang *et al*., 2012). These findings highlight the context-dependent role of autophagy in CSCs and suggest that modulating autophagy may enhance anticancer therapies by targeting CSC characteristics, ultimately reducing recurrence and resistance.

Flavonoids, a class of polyphenolic compounds found in plant-based foods such as fruits, vegetables, nuts, and teas, are widely recognized for their potential anticancer properties (Meerson *et al*., 2021). Myricetin, a specific type of flavonoid classified as a flavonol, exhibits anticancer, antioxidant, and anti-inflammatory effects, as well as the ability to enhance the sensitivity of cancer cells to chemotherapy (Agraharam *et al*., 2022). It has demonstrated anticancer activity against multiple cancers, including thyroid, colon, breast, prostate, bladder, liver, gastric, and pancreatic cancers (Kumar *et al*., 2023). In human thyroid cancer cells, myricetin induces sub-G1 phase arrest and mitochondrial dysfunction-mediated apoptosis (Ha *et al*., 2017). In colon and gastric cancer cells, it promotes apoptosis and cytoprotective autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway (Zhu *et al*., 2020; Han *et al*., 2022). In HCC cells, myricetin induces G2/M phase cell cycle arrest and cytoprotective autophagy by inhibiting STAT3 and p38 MAPK signaling pathways (Yang *et al*., 2021). Additionally, it promotes apoptosis through AKT/p70S6K/Bad signaling and the mitochondrial apoptotic pathway (Zhang *et al*., 2013).

However, the potential effects of myricetin on CSCs remain largely unexplored. In this study, we investigate the selective inhibitory effects of myricetin on LCSCs derived from Huh7 and Hep3B cell lines, focusing on its ability to regulate apoptosis, autophagy, and stemness-related signaling pathways. Furthermore, we evaluate the combinatorial potential of myricetin with chloroquine, an autophagy inhibitor, and demonstrate that co-treatment markedly enhances the suppression of LCSC viability and stem-like features. These findings provide new insights into a promising therapeutic strategy that targets both stemness maintenance and autophagy in CSCs. Given the central role of CSCs in therapeutic resistance and tumor relapse, this combinatorial approach may offer a novel direction for improving the efficacy of HCC treatment.

MATERIALS AND METHODS

Materials

Myricetin, ampelopsin, and chloroquine were sourced from MedChemExpress (South Brunswick, NJ, USA). Heparin, accutase, extracellular matrix gel (derived from Engelbreth-Holm-Swarm murine sarcoma), and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylrhodamine ethyl ester (TMRE) was acquired from Invitrogen (Carlsbad, CA, USA). DME/F-12 (1:1) and B-27 supplement were purchased from HyClone (Marlborough, MA, USA) and Gibco (Grand Island, NY, USA), respectively. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were supplied by Prospecbio (East Brunswick, NJ, USA). Antibiotics (penicillin/streptomycin/amphotericin B) were provided by Lonza (Walkersville, MD, USA). All primary and secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

LCSC culture

The Huh7 and Hep3B HCC cell lines were provided by the Korean Cell Line Bank (Seoul, Korea). Following the protocols established in our previous studies, LCSCs were selectively propagated from the HCC cell lines using tumorsphere culture in serum-free DME/F-12 medium supplemented with B-27, heparin, antibiotics, bFGF, and EGF (Kim *et al*., 2021; Kwon and Jung, 2023). The stem-like characteristics of the tumorsphere cells were confirmed. Serum-free cultured tumorsphere cells showed significantly higher levels of key liver stemness markers, including integrin α6, Oct4, and Sox2, than adherent cells in serum-containing media (Kim *et al*., 2021). Extreme limiting dilution analysis (ELDA) further confirmed their higher stem cell frequency and enhanced self-renewal

capacity (Kwon and Jung, 2023). The resulting tumorspheres were subcultured by dissociation with accutase and maintained in a 5% CO_2 incubator.

Luciferase-based ATP luminescence assay

Huh7- and Hep3B-derived LCSCs were plated in 96-well white plates and exposed to myricetin, ampelopsin, or chloroquine at specified concentrations and time intervals. LCSC proliferation and viability were assessed using a luciferase-based ATP luminescence assay (Promega, Madison, WI, USA). The IC_{50} values were calculated using curve-fitting software in GraphPad Prism 6 (La Jolla, CA, USA) (Kwon and Jung, 2023; Cho and Jung, 2025).

Chicken embryo chorioallantoic membrane (CAM) assay

The doses of myricetin used in the CAM assay were determined based on the effective concentration range identified in *in vitro* experiments. Fertilized chicken eggs were incubated at 37°C for one week, and a small window, measuring less than 1 cm, was carefully created. Huh7-derived LCSCs were mixed with myricetin and ECM gel, allowed to solidify in a cell incubator for 1 h, and subsequently injected onto the surface of the CAM. After an additional 7 days of incubation, the tumors were excised, and their formation rate, weight, and diameter were measured (Palumbo *et al*., 2023; Cho and Jung, 2025).

Cell cycle and apoptosis measurement

Huh7- and Hep3B-derived LCSCs were plated in 60-mm dishes and exposed to myricetin, ampelopsin, or chloroquine at specified concentrations and durations. After treatment, the cells were harvested and stained using either the Luminex Muse® Cell Cycle reagent or the Annexin V & Dead Cell reagent (Austin, TX, USA), following the manufacturer's protocols. Flow cytometry analysis of the cell cycle and apoptosis was performed using the Guava® Muse® Cell Analyzer with MuseSoft_V1.8.0.3 software (Kwon and Jung, 2023; Cho and Jung, 2025).

Nuclear morphology and mitochondrial membrane potential (MMP) measurement

Huh7- and Hep3B-derived LCSCs were plated in 24-well plates and exposed to myricetin at specified concentrations for 24 h. Following treatment, the cells were stained with 20 μg/mL of DAPI to assess nuclear morphology or 100 nM of TMRE to measure MMP. Fluorescence images were captured using a fluorescence microscope at 40× and 20× magnification, respectively, and fluorescence intensity was quantified with NIH ImageJ 1.5 software (Yuk and Jung, 2024).

Immunoblotting analysis

Cell lysates were resolved using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with skim milk in TBST, incubated with primary antibodies (1:2000-1:10,000) overnight at 4°C, and then with HRP-conjugated secondary antibodies (1:3000) for 1 h. Signals were detected using a Bio-Rad enhanced chemiluminescence kit (Hercules, CA, USA), and protein expression was quantified using NIH ImageJ 1.5 software by normalizing target protein intensity to β-actin (Kwon and Jung, 2023; Cho and Jung, 2025).

Statistical analysis

Data are presented as mean ± SD from at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test in SPSS v9.0, with $p<0.05$ considered significant (Kwon and Jung, 2023; Cho and Jung, 2025).

RESULTS

Myricetin inhibits the proliferation of Huh7- and Hep3B-derived LCSCs *in vitro*

We utilized the tumorsphere cultures established in our previous study to expand Huh7- and Hep3B-derived LCSCs, which were subsequently used in all experiments conducted in this study (Kim *et al*., 2021; Kwon and Jung, 2023). A luciferase-based ATP luminescence assay was performed to compare the inhibitory effects of myricetin and another flavonoid, ampelopsin (dihydromyricetin), on the proliferation of LCSCs. Huh7- and Hep3B-derived LCSCs were treated with various concentrations of ampelopsin and myricetin (0-300 μM) for 0, 24, 48, and 72 h. The results revealed that myricetin effectively inhibited cell proliferation in a time- and concentration-dependent manner compared to ampelopsin, while also reducing the size of tumorspheres. For Huh7-derived LCSCs, the IC_{50} value of ampelopsin exceeded 300 μM, whereas the IC_{50} values of myricetin were 185.7, 144.7, and 139.8 μM at 24, 48, and 72 h, respectively (Fig. 1A). Similarly, for Hep3B-derived LCSCs, the IC_{50} values of ampelopsin were 229.3, 213.5, and 199.8 μM, while those of myricetin were 202.2, 165.2, and 153.8 μM, indicating superior efficacy of myricetin in a time-dependent manner (Fig. 1B). Notably, myricetin demonstrated stronger inhibitory effects on LCSC viability compared to ampelopsin. This difference may be partially due to structural variations between the two compounds, as myricetin contains a planar C2=C3 double bond absent in ampelopsin. This planar structure may facilitate better cellular uptake or target interaction, potentially enhancing its anticancer activity. Further studies are needed to validate this structure-function relationship.

Myricetin inhibits tumor growth of Huh7-derived LCSCs *in vivo*

To evaluate the tumorigenic potential and growth-inhibitory effects of myricetin on LCSCs *in vivo*, a chick embryo chorioallantoic membrane (CAM) tumor model was used. Huh7-derived LCSCs were transplanted into the CAM model and treated with 100 or 200 μg of myricetin, followed by a 7-day incubation period. As shown in Fig. 2, the tumor formation rate in the untreated control group was 92% (13 tumors out of 14 eggs), while the rates were reduced to 35% (5 tumors out of 14 eggs) and 21% (3 tumors out of 14 eggs) in the 100 μg and 200 μg myricetin-treated groups, respectively. Furthermore, the tumor weight in the control group was 110.02 ± 72.85 mg, whereas the weights were significantly reduced to 8.74 ± 7.05 mg and 5.87 ± 3.14 mg in the 100 μg and 200 μg myricetin-treated groups, respectively. Similarly, the tumor diameter in the control group was 5.44 ± 1.56 mm, compared to 2.3 ± 0.85 mm and 2.17 ± 0.29 mm in the 100 μg and 200 μg myricetin-treated groups, respectively. These findings indicate that myricetin significantly inhibits tumor growth of Huh7-derived LCSCs in a dose-dependent manner *in vivo*. Thus, myricetin demonstrates strong antitumor activity targeting LCSCs *in vivo*.

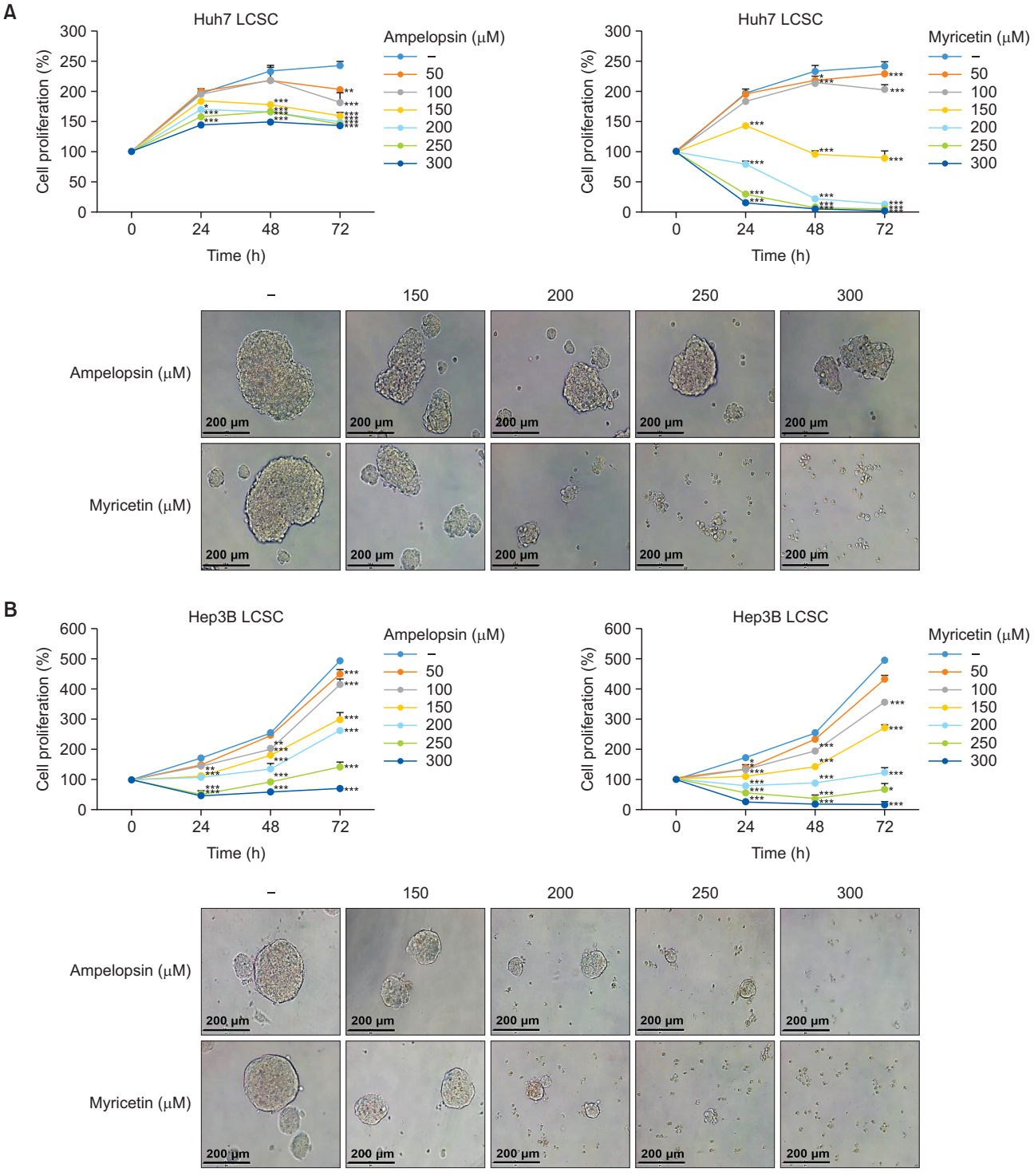

Fig. 1. Effects of myricetin on the proliferation of Huh7- and Hep3B-derived LCSCs. (A) Huh7- and (B) Hep3B-derived LCSCs were exposed to the indicated concentrations of ampelopsin and myricetin for 0, 24, 48, and 72 h, and cell proliferation was assessed using a luciferase-based ATP luminescence assay. Tumorspheres were observed under an optical microscope after 72 h of ampelopsin and myricetin treatment, and cell images were captured at 20× magnification. Data are presented as mean ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to the vehicle control. LCSC, liver cancer stem cell.

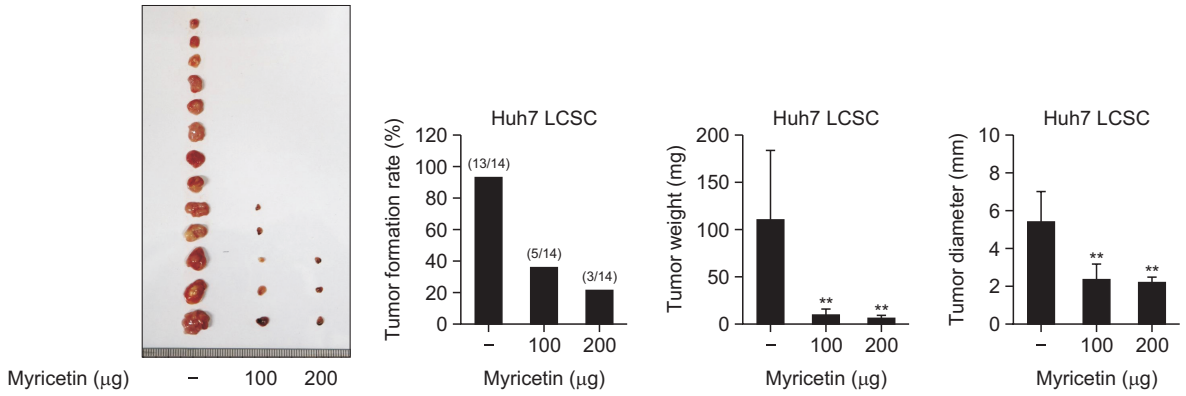

Fig. 2. Effects of myricetin on *in vivo* tumor growth of Huh7-derived LCSCs in a CAM model. Huh7-derived LCSCs were combined with ECM gel, with or without myricetin (100, 200 μg/egg), and applied to the CAM surface of fertilized chick eggs. After 7 days of incubation, tumors were excised, and their formation rate, weight, and diameter were measured. Data are presented as mean ± SD (n=14). $^{**}p<0.01$ compared to the vehicle control. LCSC, liver cancer stem cell; CAM, chicken embryo chorioallantoic membrane.

Myricetin induces cell cycle arrest and apoptosis in Huh7- and Hep3B-derived LCSCs

The cell cycle plays a critical role in the self-renewal of CSCs and the maintenance of cellular heterogeneity within tumors (Sun *et al*., 2021). To determine whether myricetin inhibits LCSC proliferation by regulating cell cycle progression, Huh7- and Hep3B-derived LCSCs were treated with ampelopsin or myricetin at 200 and 300 μM for 24 h, followed by cell cycle analysis. As shown in Fig. 3A, myricetin treatment led to a significant accumulation of cells in the G0/G1 phase in a concentration-dependent manner compared to both the control and ampelopsin-treated groups. These results indicate that myricetin induces G0/G1 phase arrest, thereby contributing to the inhibition of LCSC proliferation prior to the onset of cell death.

Inducing apoptosis is also considered a promising strategy for targeting CSCs, which are known for their resistance to programmed cell death (Safa, 2020). To assess the pro-apoptotic effects of myricetin, Huh7- and Hep3B-derived LCSCs were treated with ampelopsin or myricetin at 200 and 400 μM for 72 h. As demonstrated in Fig. 3B, myricetin significantly increased the proportion of apoptotic cells in a concentration-dependent manner compared to ampelopsin. These findings suggest that myricetin inhibits LCSC growth by inducing both cell cycle arrest at the G0/G1 phase and subsequent apoptosis.

Myricetin promotes apoptotic characteristics in Huh7- and Hep3B-derived LCSCs

We further investigated the apoptotic effects of myricetin on Huh7- and Hep3B-derived LCSCs. Apoptosis is characterized by distinct morphological changes such as nuclear condensation and fragmentation (Moyer *et al*., 2025). To evaluate whether myricetin induces apoptosis in a concentration-dependent manner, Huh7- and Hep3B-derived LCSCs were treated with 100, 200, and 300 μM of myricetin for 24 h, and nuclear morphology was analyzed using DAPI staining. As shown in Fig. 4A, myricetin treatment induced nuclear condensation and fragmentation in both Huh7- and Hep3B-derived LCSCs in a concentration-dependent manner, suggesting that myricetin effectively promotes apoptosis.

Mitochondrial membrane potential (MMP) is essential for ATP synthesis and is often maintained at high levels in CSCs to enhance their resistance to apoptosis (Zhang *et al*., 2015). Therefore, MMP plays a crucial role in the survival and proliferation of CSCs. To investigate the impact of myricetin on MMP levels in LCSCs, Huh7- and Hep3B-derived LCSCs were treated with 100, 200, and 300 μM of myricetin for 24 h, followed by staining with TMRE, a dye that accumulates in functional mitochondria. As shown in Fig. 4B, myricetin caused a significant loss of MMP in both Huh7- and Hep3B-derived LCSCs in a concentration-dependent manner. These results collectively indicate that myricetin exhibits antiproliferative effects on LCSCs, potentially through mitochondria-mediated apoptosis.

Myricetin induces extrinsic and intrinsic apoptosis and autophagy pathways in Huh7- and Hep3B-derived LCSCs

We further examined the effects of myricetin on molecular markers involved in the activation of autophagy as well as extrinsic and intrinsic cell death pathways in LCSCs. Both autophagy and apoptosis play critical roles in CSCs, often interacting in a complex manner through mutual regulation and cross-signaling under specific conditions, despite being independently regulated processes. As shown in Fig. 5, myricetin treatment in Huh7- and Hep3B-derived LCSCs increased the protein levels of DR5, a death receptor on the cell surface, and cleaved caspase-8, a key effector of the extrinsic apoptosis pathway. Concurrently, survivin and Bcl-2, which inhibit caspase activation, were downregulated, while Bad, Bax, cleaved caspase-9, and cleaved caspase-3, central mediators of intrinsic apoptosis, were upregulated. Additionally, Beclin-1, a key regulator of autophagosome formation, showed mild upregulation following myricetin treatment. Myricetin also increased the protein levels of autophagy-related gene 5 (Atg5) and promoted the conversion of LC3-I to LC3-II, indicating enhanced autophagosome formation and maturation. Importantly, p62 levels were markedly decreased after myricetin treatment, further supporting the activation of autophagic flux rather than a simple accumulation of autophagosomes. These findings suggest that myricetin induces both extrinsic and intrinsic apoptosis while activating autophagy pathways in LCSCs, highlighting its potential as a multifaceted anticancer agent.

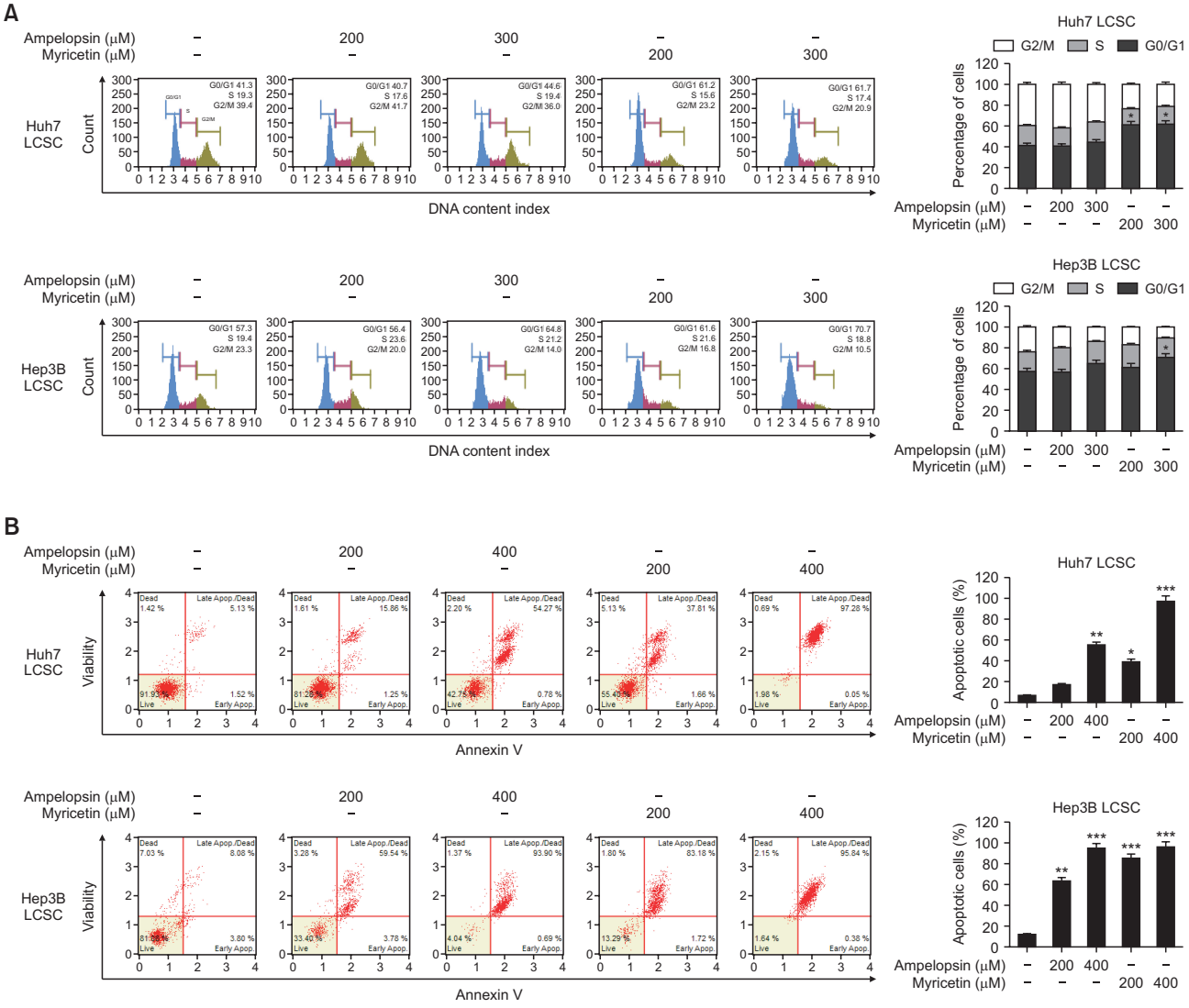

Fig. 3. Effects of myricetin on cell cycle progression and apoptosis in Huh7- and Hep3B-derived LCSCs. Huh7- and Hep3B-derived LCSCs were treated with ampelopsin or myricetin at the indicated concentrations. (A) Cell cycle distribution was analyzed after 24 h of treatment using the Muse® Cell Cycle Kit. (B) Apoptosis was assessed after 72 h of treatment using the Muse® Annexin V & Dead Cell Kit. Flow cytometry analysis was performed using the Guava® Muse® Cell Analyzer. Data are presented as mean ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to the vehicle control. LCSC, liver cancer stem cell.

Myricetin induces apoptosis and autophagy by inhibiting the PI3K/AKT/mTOR pathway in Huh7- and Hep3B-derived LCSCs

The inhibition of the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is critical in regulating the survival and resistance of CSCs, and it can simultaneously trigger autophagy and apoptosis (Zhu *et al*., 2020; Han *et al*., 2022). To evaluate whether myricetin affects this pathway, we examined the expression levels of key molecular markers of the PI3K/AKT/mTOR signaling cascade in Huh7- and Hep3B-derived LCSCs. As depicted in Fig. 6, myricetin treatment significantly reduced the levels of both phosphorylated and non-phosphorylated forms of PI3K, AKT, and mTOR in Huh7- and Hep3B-derived LCSCs. These findings indicate that myricetin inhibits the growth of LCSCs by inactivating the PI3K/AKT/mTOR pathway, thereby inducing apoptosis and autophagy.

Myricetin downregulates stemness markers and EMT in Huh7- and Hep3B-derived LCSCs

Targeting specific stem cell-related markers to inhibit the survival and proliferation of CSCs is a promising strategy for eradicating LCSCs (Lee *et al*., 2022). To evaluate the effect of myricetin on stemness, we examined the expression of essential stemness-related markers in Huh7- and Hep3B-derived LCSCs. As illustrated in Fig. 7A, myricetin treatment led to a significant reduction in the levels of Sox2, Oct4, and Nanog, essential transcription factors that regulate CSC stemness and self-renewal. Additionally, it downregulated ALDH1A1, a protein involved in chemoresistance by detoxifying harmful aldehydes in CSCs.

We further investigated whether myricetin influences EMT,

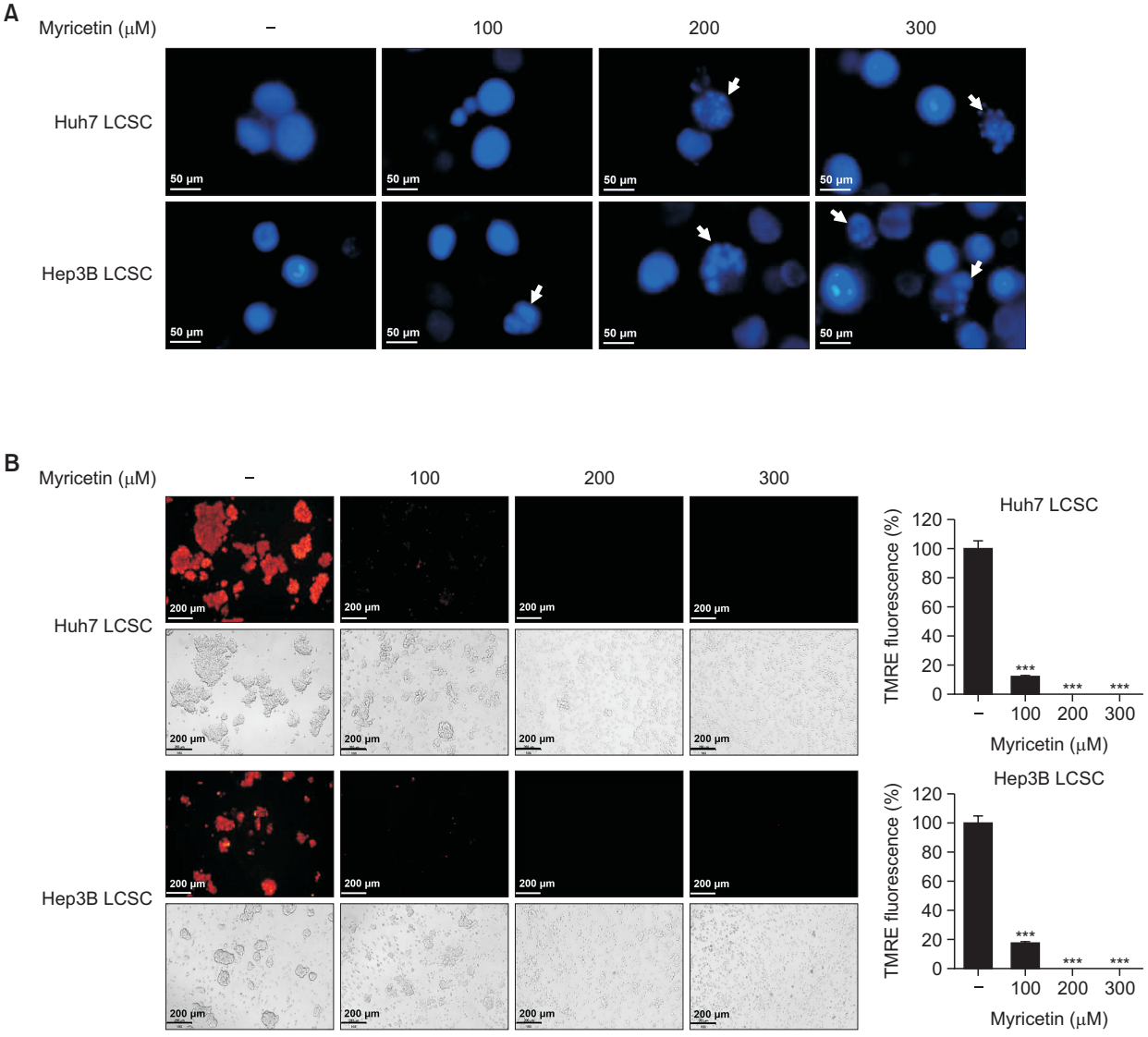

Fig. 4. Effects of myricetin on apoptotic characteristics in Huh7- and Hep3B-derived LCSCs. (A, B) Huh7- and Hep3B-derived LCSCs were treated with myricetin at the indicated concentrations for 24 h. (A) DAPI staining was used to observe nuclei under a fluorescence microscope, with nuclear condensation and fragmentation marked by white arrows. (B) Mitochondrial membrane potential was measured using TMRE, a fluorescent probe. Data are presented as mean ± SD (n=3). ***p<0.001 compared to the vehicle control. LCSC, liver cancer stem cell; DAPI, 4′,6-diamidino-2-phenylindole; TMRE, tetramethylrhodamine ethyl ester perchlorate.

a key process that enhances metastasis, chemoresistance, and tumor stemness. The hallmark of EMT is the upregulation of N-cadherin alongside the downregulation of E-cadherin, a process regulated by a complex network of signaling pathways and transcription factors (Pradella *et al*., 2017; Babaei *et al*., 2021). As depicted in Fig. 7B, myricetin treatment significantly reduced the expression levels of EMT-related transcription factors, including SLUG and TWIST1/2, in Huh7- and Hep3B-derived LCSCs. Moreover, myricetin treatment increased E-cadherin expression while decreasing N-cadherin expression. These findings suggest that myricetin effectively suppresses stemness marker expression and EMT in LCSCs, potentially attenuating their CSC-like properties and offering therapeutic implications for cancer treatment.

Chloroquine inhibits myricetin-induced autophagy and enhances apoptosis in Huh7- and Hep3B-derived LCSCs

Autophagy in cancer cells can have cytotoxic or cytoprotective effects depending on the type of stress or drug treatment (Shin, 2020). To investigate the role of autophagy in myricetin-induced apoptosis in LCSCs, autophagy was inhibited by pretreating Huh7- and Hep3B-derived LCSCs with chloroquine (CQ), an autophagy inhibitor, for 1 h. As shown in Fig. 8A, co-treatment with myricetin and CQ significantly reduced the viability of LCSCs compared to treatment with myricetin alone. Additionally, as shown in Fig. 8B and 8C, the combination treatment induced a higher apoptotic rate, decreased survivin protein levels, and increased levels of cleaved caspase-3 and cleaved caspase-9 compared to myricetin alone. Interestingly, the combination treatment significantly increased the

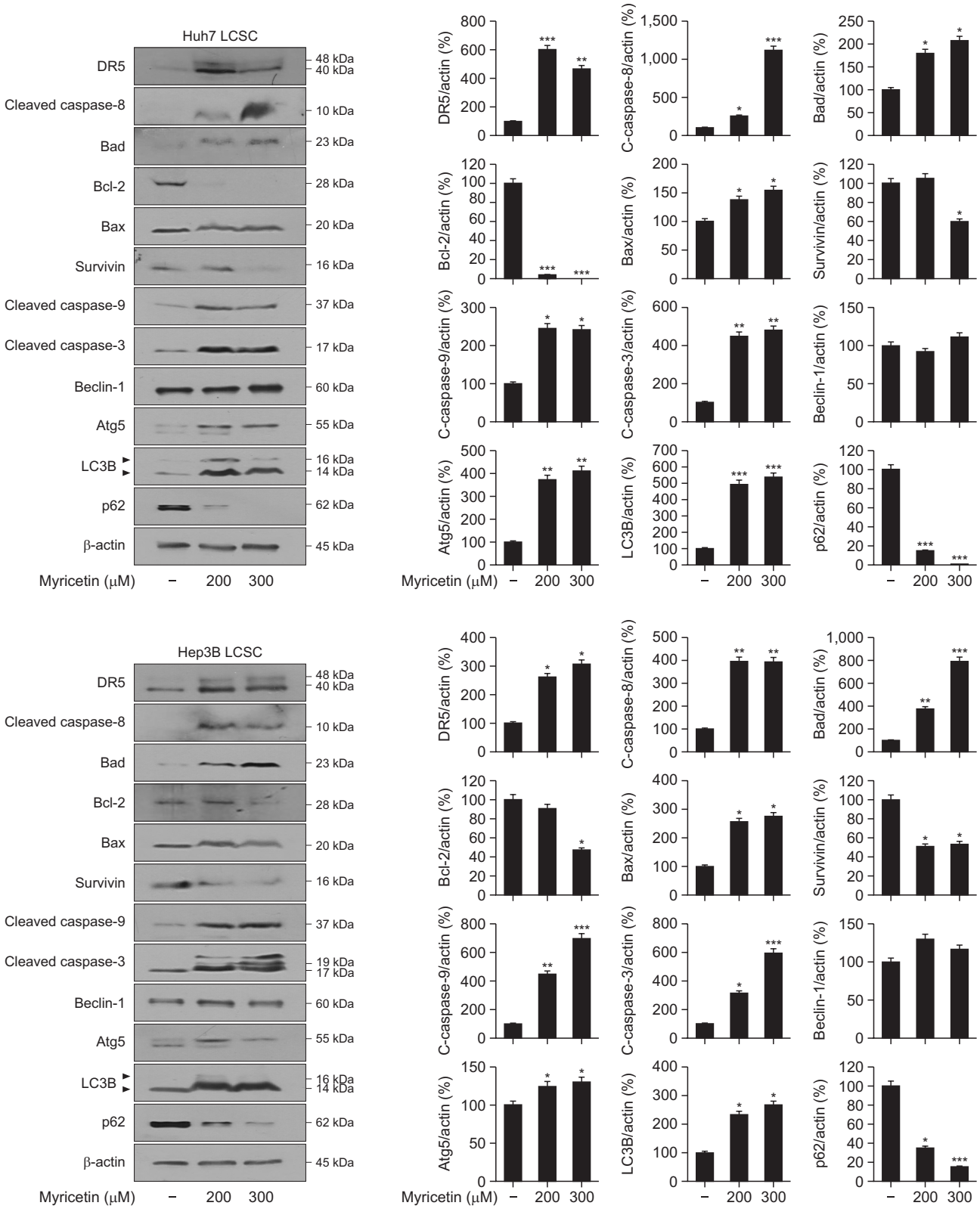

Fig. 5. Effects of myricetin on apoptosis and autophagy pathways in Huh7- and Hep3B-derived LCSCs. Huh7- and Hep3B-derived LCSCs were exposed to myricetin at the indicated concentrations for 24 h. The expression of apoptosis and autophagy markers was analyzed by western blotting and quantified by normalizing protein intensity to β-actin. Data are presented as mean ± SD (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 compared to the vehicle control. LCSC, liver cancer stem cell; DR5, death receptor 5; Bad, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Atg5, autophagy-related gene 5; LC3B, microtubule-associated protein 1 light chain 3 beta; p62, sequestosome 1.

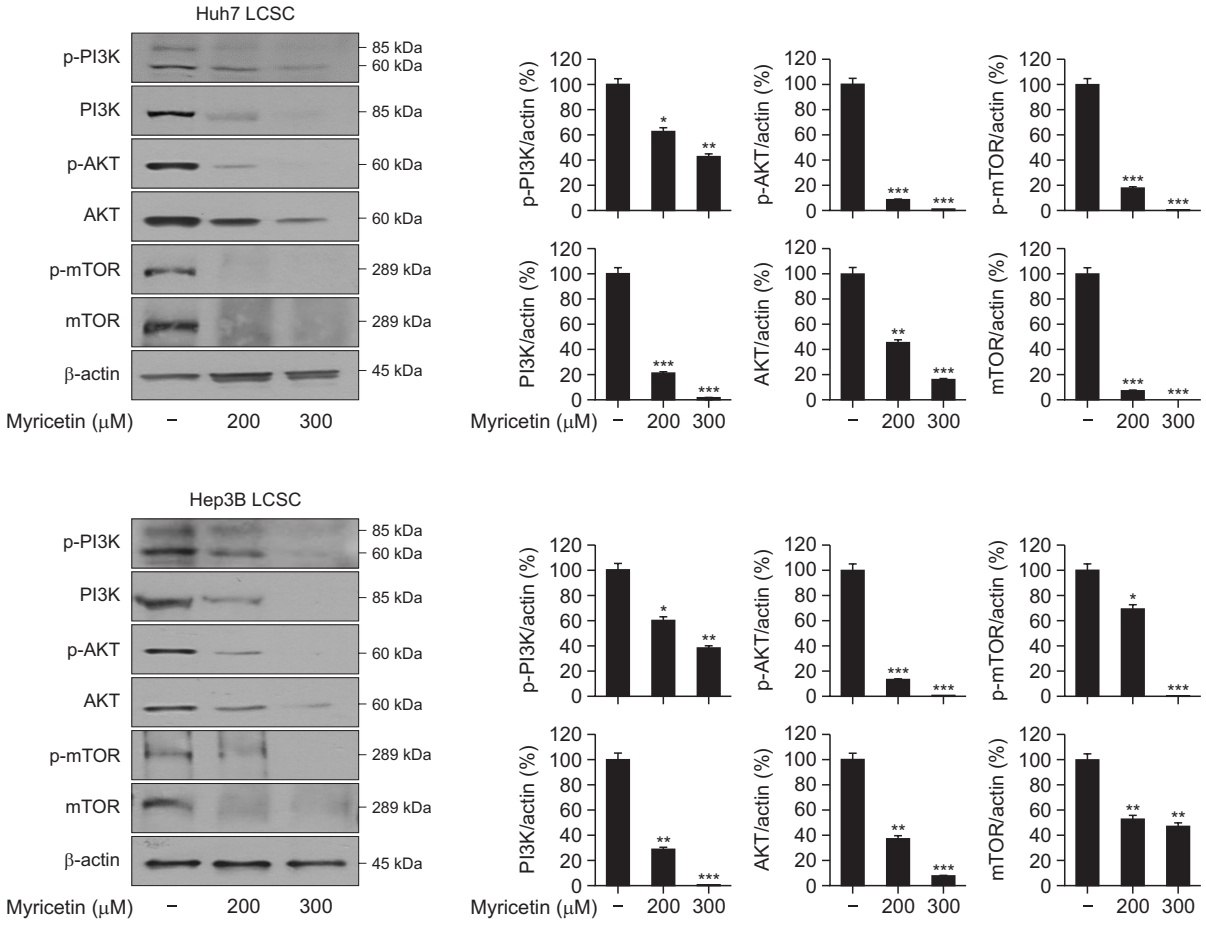

Fig. 6. Effects of myricetin on the PI3K/AKT/mTOR pathway in Huh7- and Hep3B-derived LCSCs. Huh7- and Hep3B-derived LCSCs were treated with myricetin at the indicated concentrations for 24 h. Protein expression levels were analyzed by western blotting and normalized to β-actin. Data are presented as mean ± SD (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 compared to the vehicle control. LCSC, liver cancer stem cell; p-, phosphorylated; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin.

expression of the cleaved p19 form of caspase-3, indicating that it accelerates p19 generation, which then undergoes further cleavage, ultimately enhancing apoptosis. These results collectively suggest that CQ-mediated inhibition of autophagy enhances the apoptotic effects of myricetin and reduces LCSC viability, highlighting the cytoprotective role of myricetin-induced autophagy in cell survival.

Co-treatment with myricetin and chloroquine enhances the suppression of LCSC stemness by inhibiting STAT3 activation

Chloroquine (CQ) can suppress the stemness and survival mechanisms of CSCs by inhibiting the JAK2/STAT3 pathway, in addition to its autophagy-inhibiting properties (Choi *et al*., 2014). To investigate whether the enhanced effect of the combination treatment was mediated through this pathway, we analyzed the Western blot data using total JAK2 and STAT3 protein levels as normalization controls for their phosphorylated forms. As shown in Fig. 9, the p-JAK2/JAK2 ratio under combination treatment was comparable to that of myricetin alone, suggesting that JAK2 phosphorylation was not significantly altered. In contrast, the p-STAT3/STAT3 ratio was markedly decreased in both Huh7 and Hep3B LCSCs following combination treatment, indicating that STAT3 deactivation is likely a key mediator of the observed anti-stemness effect.

Consistent with this, the combination treatment further reduced the expression of key stemness-related markers, including Sox2, Oct4, Nanog, and ALDH1A1, compared to single-agent treatment. These findings suggest that the enhanced suppression of stemness by combining myricetin with CQ may be primarily attributed to the inhibition of STAT3 activation, supporting the potential of this combination as a more effective therapeutic strategy for targeting LCSCs.

DISCUSSION

HCC significantly contributes to cancer mortality due to its resistance to chemotherapy and frequent post-treatment recurrence, which result in poor therapeutic outcomes (Dopazo *et al*., 2024). LCSCs play a pivotal role in HCC progression by driving self-renewal, differentiation, tumor initiation, chemotherapy resistance, metastasis, and recurrence (Lee *et al*., 2022). Therefore, advancing therapies that specifically target LCSCs holds promise for improving HCC treatment efficacy.

Over 6,000 flavonoids have been identified, isolated, and

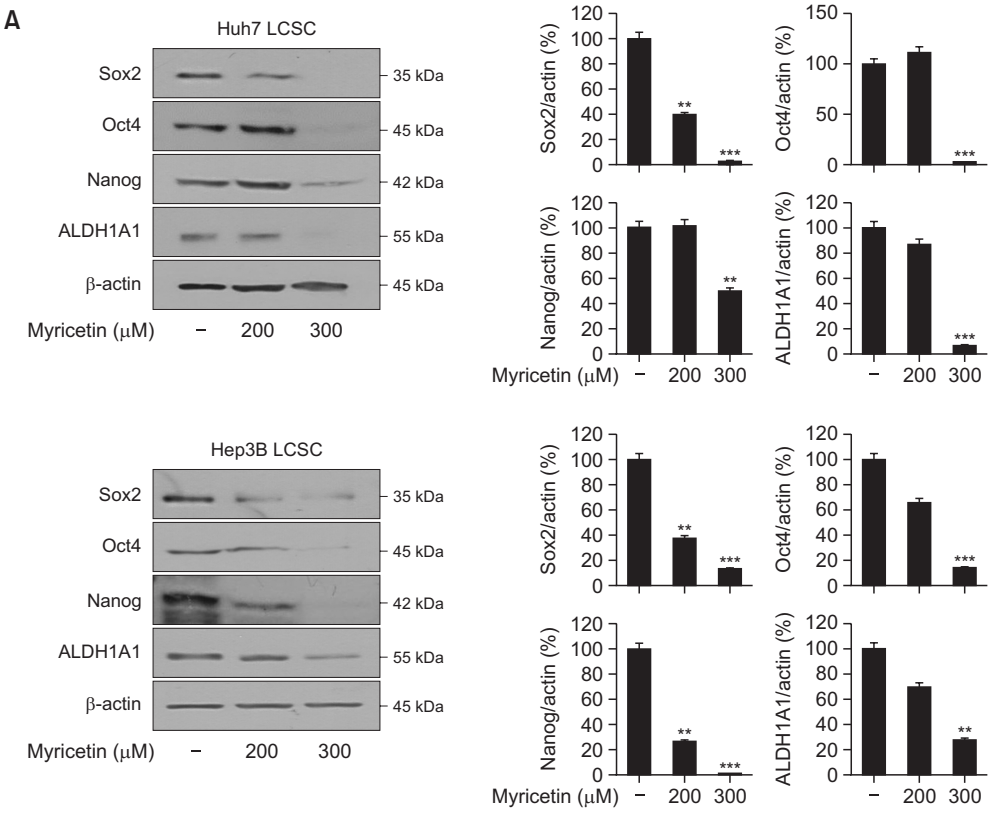

Fig. 7. Effects of myricetin on stemness and EMT markers in Huh7- and Hep3B-derived LCSCs. (A, B) Huh7- and Hep3B-derived LCSCs were treated with myricetin at the indicated concentrations for 24 h. (A) Stemness and (B) EMT marker expression levels were evaluated by western blotting and normalized to β-actin or α-tubulin. Data are presented as mean ± SD (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 compared to the vehicle control. LCSC, liver cancer stem cell; Sox2, SRY-box transcription factor 2; Oct4, octamer-binding transcription factor 4; Nanog, Nanog homeobox; ALDH1A1, aldehyde dehydrogenase 1 family member A1; EMT, epithelial-mesenchymal transition; SLUG, Zinc finger protein SNAI2; TWIST1/2, twist-related protein 1/2; E-Cadherin, epithelial cadherin; N-cadherin, neural cadherin.

categorized into subclasses such as flavones, flavonols, flavanols, isoflavones, and isoflavans (Meerson *et al*., 2021). Studies have demonstrated that flavonoids exhibit inhibitory effects on CSC self-renewal, stemness traits, epithelial-mesenchymal transition (EMT) processes, and survival across various tumor types. For example, quercetin has been shown to suppress CD24 and CD133 expression in pancreatic CSCs, promoting differentiation through β-catenin modulation (Hoca *et al*., 2020). Similarly, apigenin has been reported to inhibit the self-renewal, proliferation, colony formation, and invasiveness of glioblastoma stem cells (Kim *et al*., 2016). Consequently, several dietary polyphenolic compounds, including flavonoids, are being actively studied for their anti-CSC properties and potential applications in cancer chemoprevention and therapy.

This study demonstrated that myricetin, a natural flavonol, plays a significant role in inhibiting the growth of LCSCs. While the anticancer properties of myricetin have been investigated in various conventional cancer cell lines, its selective activity against CSCs remains poorly characterized. In this study, we specifically focused on the effects of myricetin on LCSCs and demonstrated that its combination with chloroquine further enhanced the suppression of stemness-associated traits. These findings highlight a novel therapeutic approach that targets both autophagy regulation and stemness maintenance in CSCs. The findings showed that myricetin effectively suppressed the growth of Huh7- and Hep3B-derived LCSCs both *in vitro* and *in vivo* by inducing cell cycle arrest in the G0/G1 phase and promoting apoptosis. The suppression of Huh7- and Hep3B-derived LCSCs by myricetin was linked to the activation of intrinsic apoptotic pathways, characterized by nuclear condensation, fragmentation, MMP depletion, and modulation of the Bad/Bcl-2/Bax/survivin/caspase-9/caspase-3 signaling cascade. Myricetin also induced extrinsic apoptosis by increasing the expression levels of DR5 and cleaved caspase-8. It is well known that when TRAIL, a representative ligand of DR5, binds to DR5, it activates the caspase cascade, leading to apoptosis. However, several chemotherapeutic agents (e.g., doxorubicin, cisplatin, paclitaxel) and natural compounds (e.g., curcumin, resveratrol, genistein) have been reported to activate DR5 and promote apoptosis by either increasing DR5 expression or enhancing its sensitivity, even in the absence of ligand binding (Artykov *et al*., 2020; Gan *et al*., 2022). Therefore, myricetin may also induce extrinsic apoptosis by upregulating DR5 expression in a ligand-independent manner. Additionally, myricetin stimulated autophagy, as evidenced by the upregulation of Beclin-1 and Atg5, the conversion of LC3-I to LC3-II, and the concomitant decrease in p62 levels, indicating activation of autophagic flux rather than simple autophagosome accumulation. The apoptosis- and autophagy-inducing effects of myricetin were closely linked to the inactivation of

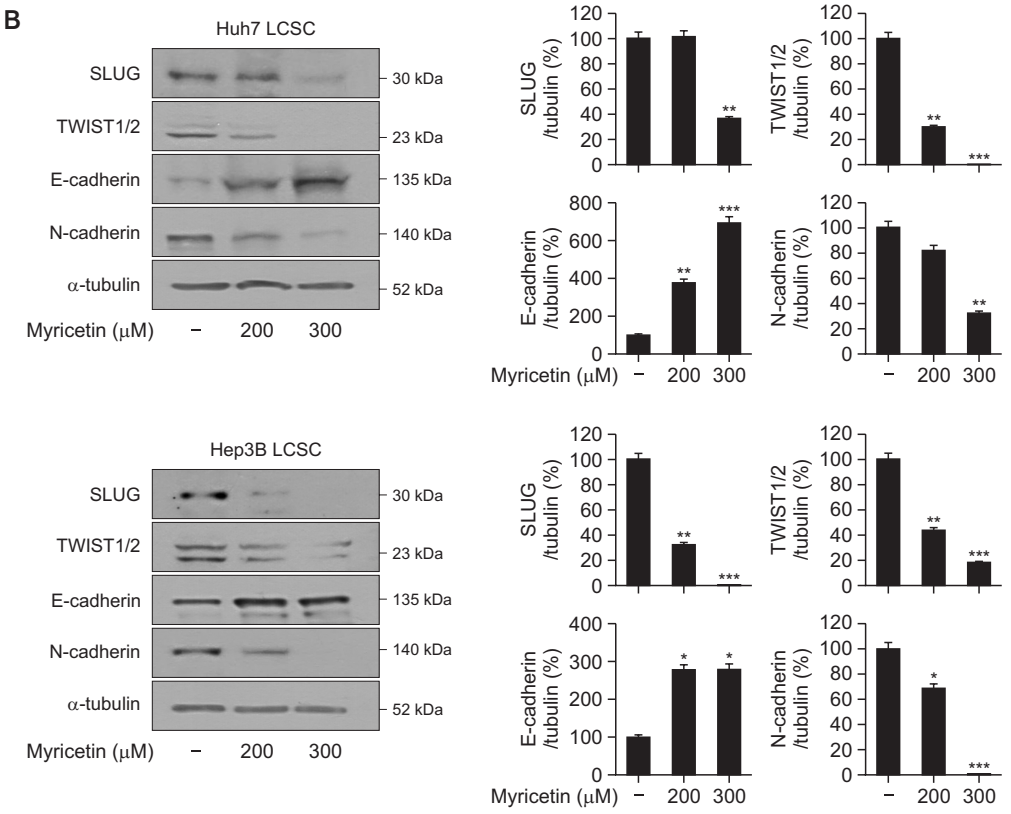

Fig. 7. Continued.

the PI3K/AKT/mTOR signaling pathway. Furthermore, myricetin downregulated the expression of key stemness markers, including Sox2, Oct4, Nanog, and ALDH1A1, and suppressed EMT by modulating the expression of SLUG, TWIST1/2, E-cadherin, and N-cadherin. Notably, the combination treatment of myricetin and chloroquine, a late-stage autophagy inhibitor, enhanced the suppression of tumor progression in Huh7- and Hep3B-derived LCSCs. To determine whether this enhanced effect was synergistic or merely additive, we performed combination index (CI) analysis using the Chou–Talalay method. The CI values were consistently less than 1 across a range of concentrations, confirming a synergistic interaction between myricetin and chloroquine (Supplementary Fig. 1). This cotreatment promoted apoptosis and effectively downregulated stemness regulators, primarily through the inhibition of STAT3 activation (Fig. 10). These results suggest that myricetin is a promising therapeutic agent for impeding the growth and progression of LCSCs.

The regulation of cell death pathways involves intricate interactions, and the simultaneous induction of multiple pathways can enhance the efficiency of cancer cell death by utilizing diverse mechanisms (Ohno *et al*., 2008). In our recent study, low doses of the ethyl acetate extract of *Hovenia dulcis* (EAHDT) induced RIPK3/MLKL-mediated necroptosis, while high doses triggered caspase-dependent apoptosis in Huh7-derived LCSCs, thereby inhibiting LCSC proliferation (Kwon and Jung, 2023). Caspases, key enzymes in cellular apoptosis, also modulate autophagy. Early activation of caspase-3 during apoptosis can help cells regulate the autophagy-related protein Beclin-1 as a protective mechanism (Wu *et al*., 2014). The anti-apoptotic protein Bcl-2 also interacts with Beclin-1 to inhibit autophagy, suggesting that disrupting the Bcl-2/Beclin-1 complex can activate autophagy (Marquez and Xu, 2012). Based on our findings, myricetin may induce both apoptosis and autophagy by interfering with the Bcl-2/Beclin-1 complex in Huh7- and Hep3B-derived LCSCs.

Additionally, the upstream PI3K/AKT/mTOR signaling pathway enhances the survival and resistance of CSCs by regulating both apoptosis and autophagy (Zhu *et al*., 2020; Han *et al*., 2022). A prior study reported that rottlerin, a plant-derived chemotherapeutic agent, induced apoptosis and autophagy in prostate CSCs by inhibiting the PI3K/AKT/mTOR pathway (Kumar *et al*., 2014). Similarly, in this study, myricetin activated extrinsic and intrinsic apoptotic pathways and stimulated autophagy by inactivating the PI3K/AKT/mTOR signaling pathway in Huh7- and Hep3B-derived LCSCs.

Autophagy plays a dual role in cancer, as it can either promote cell death or protect tumor cells depending on the context. In some cases, autophagy stimulates caspase activation and apoptosis, while in others, it provides a cytoprotective function, enabling cancer cells to survive chemotherapy and sustain tumor progression (Shin, 2020; Wang *et al*., 2022). Inhibiting autophagy has shown potential to enhance chemotherapy responsiveness and suppress tumor growth. Chloroquine, a late-stage autophagy inhibitor that impairs the fusion of autophagosomes with lysosomes, has been widely used in malaria treatment (Lee *et al*., 2023). Increasing evidence indicates that chloroquine also exhibits significant anticancer

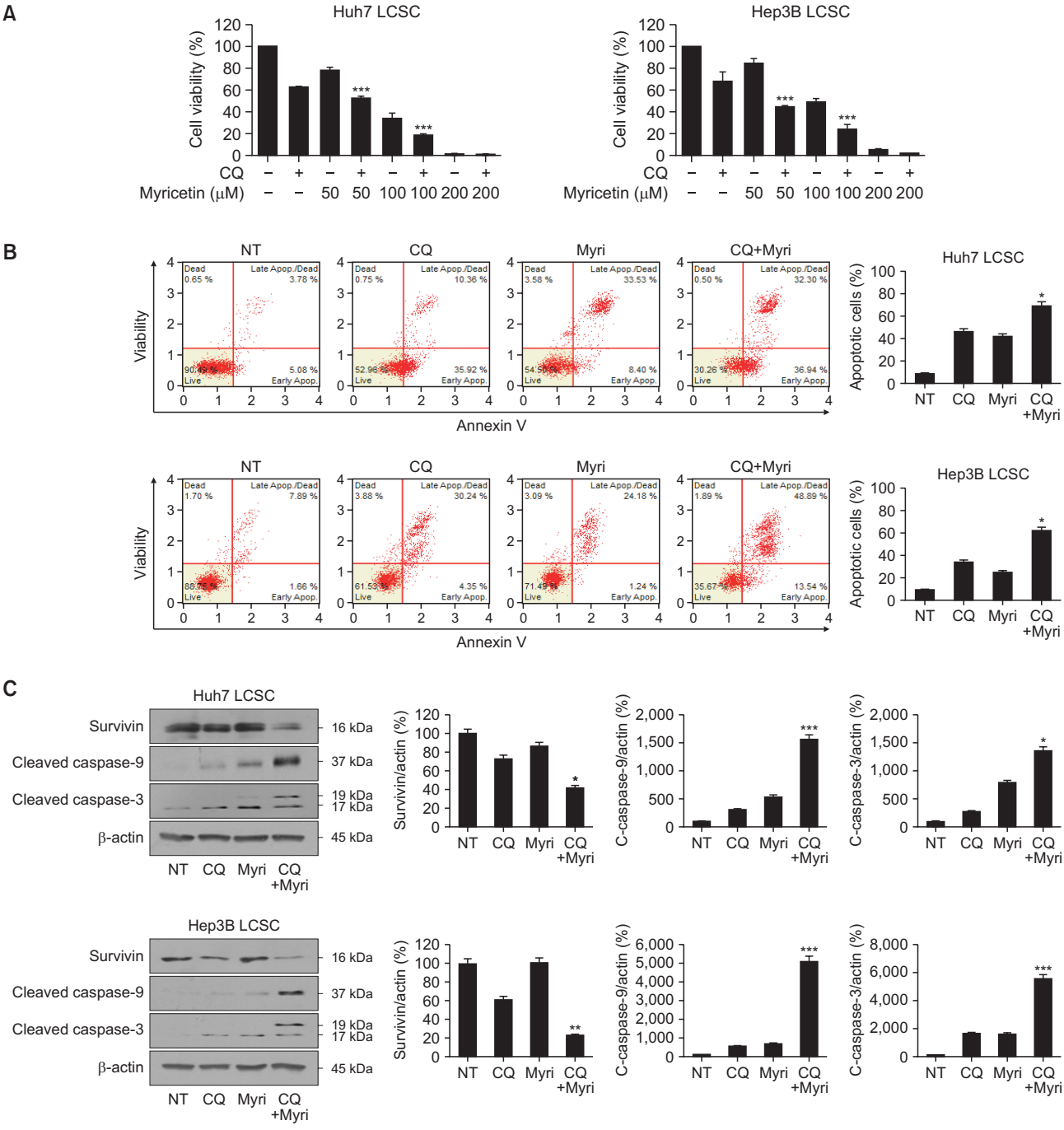

Fig. 8. Chloroquine enhances the apoptotic effects of myricetin in Huh7- and Hep3B-derived LCSCs. (A-C) Huh7- and Hep3B-derived LCSCs were pretreated with chloroquine for 1 h, followed by myricetin treatment for 24 h. (A) Cell viability was assessed using a luciferase-based ATP luminescence assay after co-treatment with myricetin (50, 100, 200 μM) and chloroquine (50 μM). (B) Apoptotic cell death was analyzed by flow cytometry following treatment with myricetin (200 μM) and chloroquine (200 μM). (C) Expression of apoptosis markers was evaluated by western blotting after co-treatment with myricetin (100 μM) and chloroquine (200 μM). Data are presented as mean ± SD (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to the myricetin-treated group. LCSC, liver cancer stem cell; NT, non-treated; CQ, chloroquine; Myri, myricetin.

potential, either alone or in combination with chemotherapeutic agents. Chloroquine has been shown to downregulate DNA methyltransferase 1 and other proteins involved in activating the JAK2/STAT3 pathway, thereby disrupting key survival and proliferation signals in breast CSCs (Choi *et al*., 2014). It also enhanced the susceptibility of breast CSCs to paclitaxel by inhibiting autophagy. Additionally, chloroquine mitigated chemoresistance to cisplatin in ovarian cancer cells by upregu-

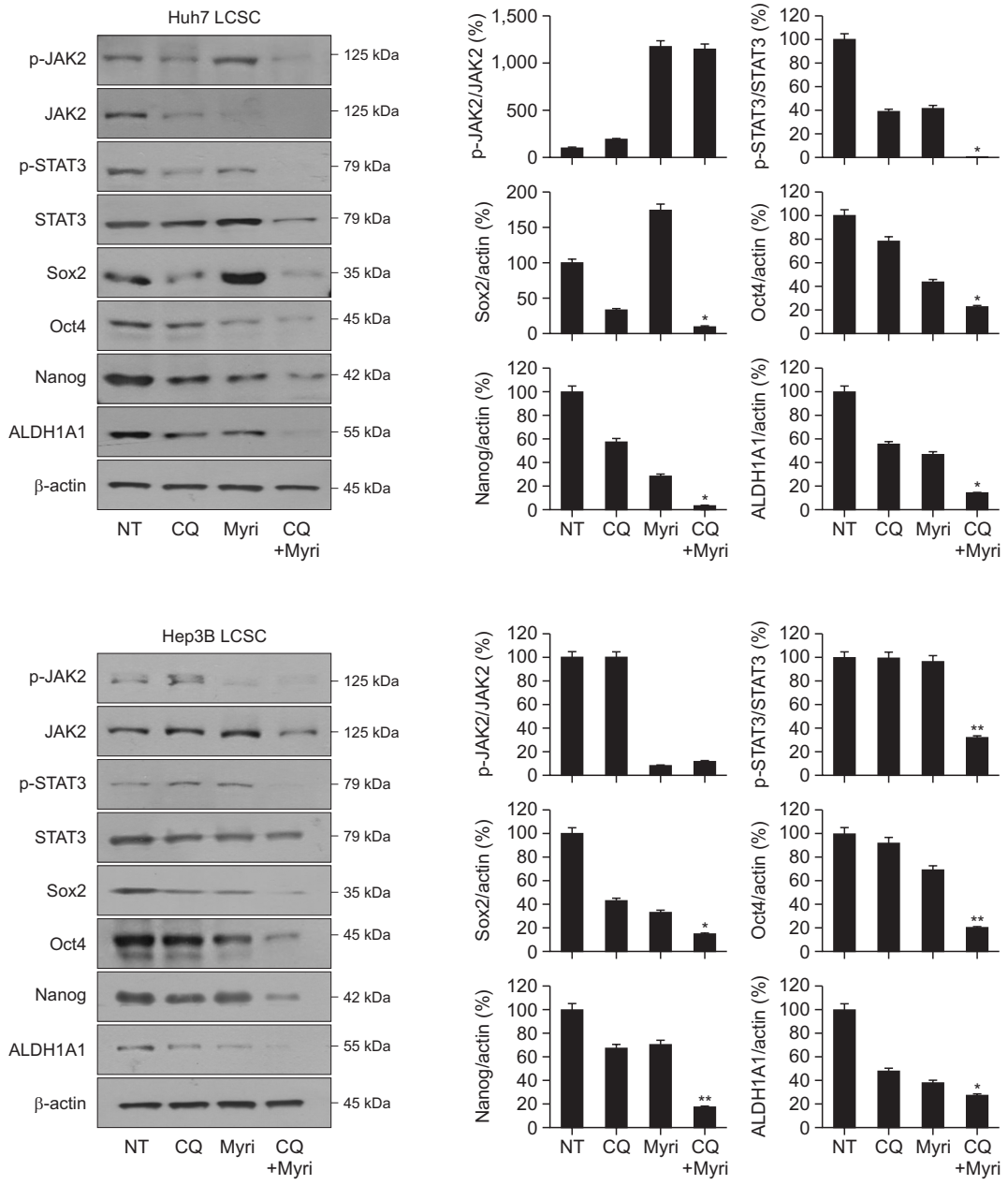

Fig. 9. Effects of co-treatment with chloroquine and myricetin on the JAK2/STAT3 pathway and stemness markers in Huh7- and Hep3B-derived LCSCs. Huh7- and Hep3B-derived LCSCs were pretreated with chloroquine (200 μM) for 1 h, followed by myricetin (100 μM) treatment for 24 h. Protein expression levels were determined by western blotting and are presented as the normalized ratio of each target protein (or phosphorylated protein) to β-actin or the corresponding total protein. Data are presented as mean ± SD (n=3). **p*<0.05, ***p*<0.01 compared to the myricetin-treated group. LCSC, liver cancer stem cell; NT, non-treated; CQ, chloroquine; Myri, myricetin; p-, phosphorylated; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3; Sox2, SRY-box transcription factor 2; Oct4, octamer-binding transcription factor 4; Nanog, Nanog homeobox; ALDH1A1, aldehyde dehydrogenase 1 family member A1..

lating p21WAF1/CIP1 expression and suppressing autophagy (Hwang *et al*., 2020). In triple-negative breast cancer cells, chloroquine prevented resistance to PI3K/AKT inhibitors (ipatasertib and taselisib) and enhanced their antitumor effects when combined with paclitaxel (Cocco *et al*., 2022). Furthermore, the combination of chloroquine with salidroside, a plant-derived bioactive compound, increased apoptosis in liver cancer cells by downregulating salidroside-induced autophagy (Jiang *et al*., 2023). In this study, myricetin induces cytoprotective autophagy in LCSCs, indicating that autophagy inhibition could enhance its anticancer efficacy against LCSCs. Our study demonstrated that the combined treatment of myricetin and chloroquine promoted apoptosis in LCSCs by mitigating the autophagy-inducing effects of myricetin. This co-treatment significantly reduced STAT3 phosphorylation without markedly affecting JAK2 phosphorylation, resulting in a further de-

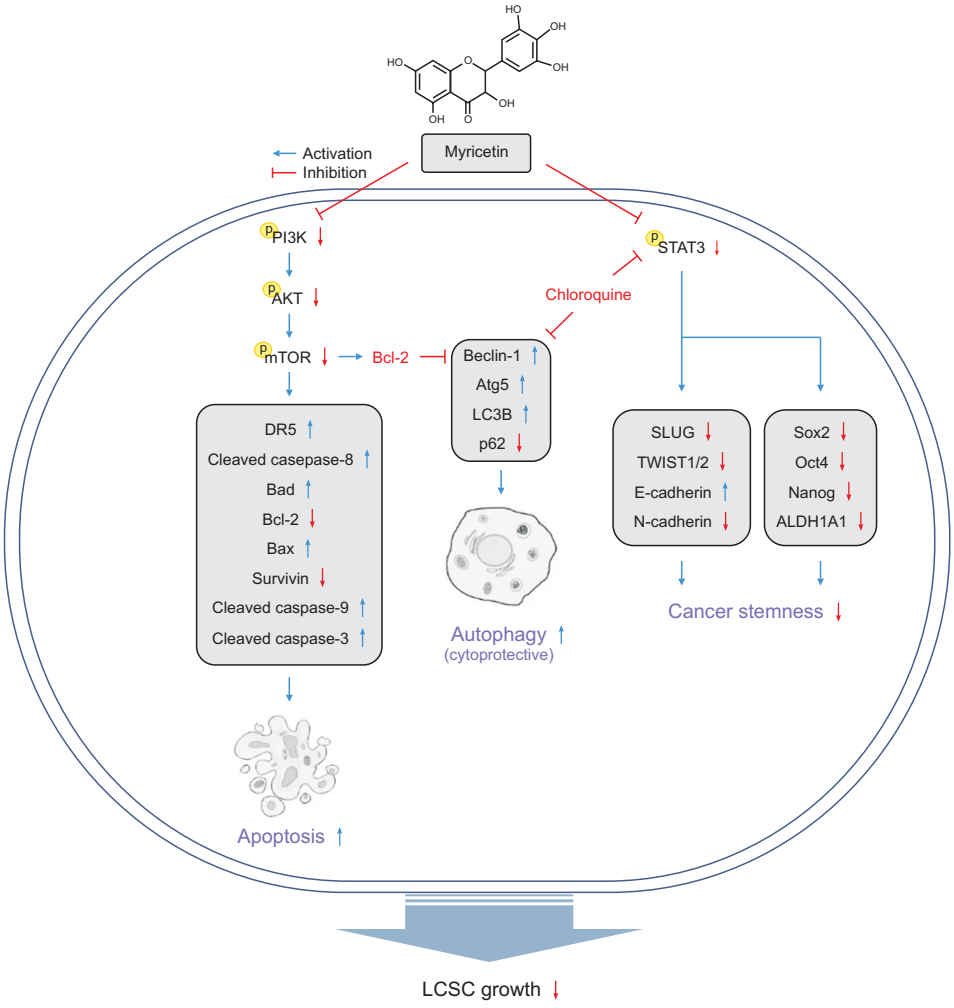

Fig. 10. Proposed molecular mechanism of myricetin's growth-inhibitory effects on Huh7- and Hep3B-derived LCSCs. Myricetin suppresses the growth of LCSCs through the regulation of apoptosis, autophagy, and stemness. p-, phosphorylated; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; DR5, death receptor 5; Bad, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Atg5, autophagy-related gene 5; LC3B, microtubule-associated protein 1 light chain 3 beta; p62, sequestosome 1; STAT3, signal transducer and activator of transcription 3; SLUG, Zinc finger protein SNAI2; TWIST1/2, twist-related protein 1/2; E-Cadherin, epithelial cadherin; N-cadherin, neural cadherin; Sox2, SRY-box transcription factor 2; Oct4, octamer-binding transcription factor 4; Nanog, Nanog homeobox; ALDH1A1, aldehyde dehydrogenase 1 family member A1.

crease in the expression of stemness-related markers such as Sox2, Oct4, Nanog, and ALDH1A1 in LCSCs. These findings suggest that the combination of chloroquine and myricetin enhances myricetin's inhibitory effects on the stem-like properties of LCSCs primarily through STAT3 inactivation, rather than through broad suppression of the JAK2/STAT3 signaling axis. Therefore, this combination therapy, targeting both apoptosis induction and autophagy inhibition, strengthens the anti-CSC effects of myricetin, making it a promising strategy for overcoming chemotherapy resistance and improving the efficacy of HCC treatment.

Nevertheless, the effective dose range of myricetin for LCSCs was relatively high in this study. Previous reports have shown that myricetin exerts anticancer effects across a broad concentration range (50-250 μM), depending on the cancer cell type and experimental conditions (Kumar *et al.*, 2023), underscoring the variability in its effective dosage. The use of a 3D tumorsphere culture model for LCSCs may have further contributed to the higher dose requirement compared to conventional 2D monolayer systems.

It is true that the IC_{50} values of myricetin in CSCs are relatively high compared to those of conventional chemotherapeutic agents. However, myricetin is a natural flavonoid with low systemic toxicity and has been reported to modulate multiple oncogenic pathways with minimal adverse effects. Importantly, its combination with chloroquine, an autophagy inhibitor, significantly enhanced its anti-CSC efficacy in this study, suggesting the potential for dose reduction and a more favorable therapeutic index. These findings highlight the therapeutic advantage of utilizing natural compounds like myricetin in combination regimens to overcome limitations related to high single-agent dosage.

Although Huh7 and Hep3B LCSCs exhibited broadly similar responses to myricetin, chloroquine, and their combination, it

is important to note that intrinsic genetic differences may have influenced subtle variations in drug sensitivity. Specifically, Hep3B cells are p53-null and harbor integrated HBV DNA, whereas Huh7 cells contain a mutant form of p53 and lack HBV integration. These distinctions may affect cellular stress responses, autophagic capacity, or apoptotic susceptibility, potentially modulating the magnitude of treatment effects. Future studies employing isogenic cell lines or p53 reconstitution approaches will be necessary to further elucidate the contribution of these genetic contexts to therapeutic outcomes.

Moreover, myricetin exhibited consistent dose- and time-dependent inhibitory effects across all *in vitro* assays, suggesting that its suppressive activity against LCSCs is not merely a consequence of general cytotoxicity. Although the *in vivo* efficacy of myricetin was preliminarily assessed using a CAM assay, additional validation using xenograft mouse models will be essential to confirm its therapeutic potential and safety in targeting LCSC-driven tumor growth.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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