Apigenin regulates the migration, invasion, and autophagy of hepatocellular carcinoma cells by downregulating YAP

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Apigenin is an edible flavonoid with anticancer properties; however, the underlying mechanisms in hepatocellular carcinoma (HCC) remain to be clarified. In the present study, we demonstrated that apigenin decreased the viability of both SMMC-7721 and SK-Hep1 cells in a dose-dependent manner, and inhibited the migration and invasion of HCC cells with different metastatic potential by regulating actin cytoskeletal rearrangements. Moreover, we showed that apigenin decreased the expression of YAP, and subsequently reduced migration and invasion by modulating the expression of the epithelial-mesenchymal transition (EMT) markers, and promoted the autophagy of HCC cells by regulating the expression of autophagy-related genes. Collectively, the present findings might provide a novel mechanism for the therapeutic application of apigenin in HCC.

Key words: hepatocellular carcinoma, apigenin, Hippo signaling pathway, migration, invasion, autophagy

Hepatocellular carcinoma (HCC) is the second leading cause of tumor-related mortality worldwide, representing the third most common malignancy and a major cause of cancer-related mortality in China, and its incidence and mortality rates are getting increase [1, 2]. The high rate of recurrence and metastasis leads to the poor prognosis of HCC patients, resulting in over 500,000 deaths annually [3]. Despite the recent advances in treatments for HCC, the outcomes of HCC are still undesirable [4]. Thus, it is crucial to develop effective therapeutic agents for human HCC treatment.

Abundant evidence has demonstrated that loss of the Hippo signaling or aberrant activation of its core downstream effector, yes-associated protein (YAP), has been implicated in cancer pathogenesis across multiple human cancers including HCC [5, 6]. Therefore, the Hippo/YAP signaling pathway has emerged as an attractive target for cancer therapeutics. Apigenin is an edible flavonoid enriched in fruits, vegetables, and drinks [7]. Accumulating evidence has demonstrated that apigenin has low cytotoxicity on normal cells, and plays an essential role in antioxidant and anti-inflammatory activities [8, 9]. Notably, apigenin exhibits cell growth arrest, apoptosis, and inhibition of cell migration and invasion in different types of tumors through regulating several signaling pathways [10], suggesting that it has the potential to be used in cancer prevention and therapy. However, the function of apigenin in HCC remains largely elusive.

Thus, in the present study, we examined whether apigenin mediates its anti-tumor effect by targeting the Hippo/YAP signaling in HCC cells. Herein, we demonstrated that apigenin decreased cell viability, migration, and invasion, and induced autophagy via downregulating YAP in HCC cells.

Materials and methods

Cell lines and culture conditions. Human HCC cell lines SMMC-7721 and SK-HEP1, purchased from the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China), were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone),

supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin, and incubated in a humid incubator at 37 °C with 5% CO_2. To silence the expression of YAP, lentiviruses expressing shYAP or shNC were constructed in our lab [11] and used to transduce into SMMC-7721 cells. After selection with 0.5 μg/ml puromycin for 14 days, stable YAP knockdown SMMC-7721 cells were generated and verified by western blotting. Adenoviruses encoding YAP and control were obtained from Vigene Biosciences (Vigene, China).

Cell viability assay. Cell viability assay was performed using a cell counting kit-8 (CCK-8) (Vazyme, Nanjing, China) according to the manufacturer's instructions. In brief, 100 μl of cell suspension of SMMC-7721 or SK-HEP1 cells were seeded in a 96-well plate with six duplications at a density of 1×10^5 cells/well. After 48 h incubation with indicated concentrations of apigenin, which was obtained from Selleck Chemicals (S2262, Houston, TX), 10 μl of CCK-8 solution was added into each well of the plate, followed by incubation for 45 min at 37 °C in the incubator. The absorbance at 450 nm was detected by using a microplate reader (Multiskan Spectrum, Thermo Scientific, USA).

Wound-healing assay. SMMC-7721 or SK-HEP1 cells were grown in 6-well cell plates and starved in serum-free DMEM overnight. The confluent monolayer was scratched with a sterile 200 μl pipette tip and washed with PBS. Cells were incubated in a serum-free medium in the presence or absence of apigenin at 37 °C for the indicated time. At 24 h and 48 h, images were obtained under an inverted light microscope (Nikon, Japan).

Transwell cell migration and invasion assays. A 24-well Transwell chamber (diameter 6.5 mm; pore size 8.0 μm; Corning Inc., Corning, NY, USA) was used to assess cell migration and invasion as described previously [11]. In brief, cells were serum-starved overnight, and then 4×10^4 SMMC-7721 cells or 2×10^4 SK-HEP1 cells in a 200 μl serum-free DMEM were seeded into the uncoated or Matrigel (100 μg/ml, BD Biosciences)-coated upper chamber for migration and invasion assays, respectively, which were then placed into the lower chambers containing 600 μl of fresh DMEM supplemented with 10% FBS. After incubation at 37 °C with 5% CO_2 for 24 h, the migrated or invaded cells on the lower surface of the chambers were fixed in methanol at room temperature for 15 min and stained with 0.1% crystal violet for 20 min, and counted from five randomly selected microscopic fields at 200× magnification per chamber. All experiments were performed in triplicate from three independent experiments.

RNA-sequencing (RNA-seq). Total RNA was extracted with TRIzol reagent (Invitrogen, USA), and the library construction and RNA-seq analysis were performed by Wuhan igenebook Co., Ltd. (Wuhan, China). The criteria for differential genes were set up with a fold change greater than 2-fold and an FDR <0.05. Differentially expressed genes were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cell samples by using the TRIzol reagent (Invitrogen, USA), followed by cDNA synthesis using the PrimeScriptTM RT Master Mix (TaKaRa, Dalian, China). Real-time PCR was performed using the SYBR Green qPCR system (TaKaRa, Dalian, China) on a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). The qPCR thermocycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 34 s. Primers used in the present study were synthesized by Tsingke Biological Technology (Nanjing, China) and listed as follows: YAP forward, 5'-CTCGAACCCCAGATGACTTC-3' and reverse, 5'-CCAGGAATGGCTTCAAGGTA-3', transcriptional coactivator with PDZ-binding motif (TAZ) forward, 5'-CTTGGATGTAGCCATGACCTT-3' and reverse, 5'-TCAATCAAAACCAGGCAATG-3'; Connective tissue growth factor (CTGF) forward, 5'-AGGAGT-GGGTGTGTGACGA-3' and reverse, 5'-CCAGGCAGTT-GGCTCTAATC-3'; Cysteine-rich angiogenic inducer 61 (CYR61) forward, 5'-AGCCTCGCATCCTATACAACC-3' and reverse, 5'-TTCTTTCACAAGGCGGCACTC-3'; N-cadherin forward, 5'-CAGTGTACAGAATCAGTG-3' and reverse, 5'-CAACAGTAAGGACAAACA-3'; E-cadherin forward, 5'-CAAGTGACCACCTTAGAG-3' and reverse, 5'-GAATTTGCAATCCTGCTT-3'; β-catenin forward, 5'-ACCTATACTTACGAAAAACTAC-3' and reverse, 5'-CCACCAGCTTCTACAATA-3'; Vimentin forward, 5'-GACGCCATCAACACCGAGTT-3' and reverse, 5'-CTTTGTCGTTGGTTAGCTGGT-3'; Microtubule-associated protein 1 light chain 3 Beta (LC3B) forward, 5'-AGCAGCATCCAACCAAAATC-3' and reverse, 5'-TGTGTCCGTTCACCAACAG-3'; SQSTM1/p62 forward, 5'-ATCGGAGGATCCGAGTGT-3' and reverse, 5'-TGGCTGTGAGCTGCTCTT-3'; Unc-51 like autophagy activating kinase 1 (ULK1) forward, 5'-AAGATCGCTGA-CTTCGGCTT-3' and reverse, 5'-TTCTCGTAGAACAGGC GCAG-3'; and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. The relative mRNAs amount normalized against GAPDH was calculated by the $2^{-\Delta\Delta Ct}$ method. The experiment was independently repeated four times.

Western blotting. Western blotting was performed following standard methods. In brief, cells were washed twice with cold PBS and lysed in RIPA lysis buffer containing protease inhibitors mix and phosphatase inhibitors (Roche Diagnostics, Switzerland). After quantification of protein with a BCA kit (Thermo Scientific, USA), samples were boiled and resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, USA). Membranes were immunoblotted with corresponding primary antibodies followed by incubation with the horseradish peroxidase (HRP)-conjugated secondary

antibodies. The immunoblots were visualized with the Pierce ECL Plus Western blotting substrate (Thermo Fisher Scientific, Inc., USA), and GAPDH was used as the loading control. The following antibodies were used in the present study: rabbit anti-YAP (#14074), rabbit anti-TAZ (#4883), rabbit anti-LC3B (#3868), rabbit anti-ULK1 (#8054), rabbit anti-β-catenin (#8480), rabbit anti-Vimentin (#5741), rabbit anti-SQSTM1/p62 (#39749), and HRP-linked anti-rabbit IgG (#7074) and anti-mouse IgG (#7076) purchased from Cell Signaling Technology (Danvers, MA, USA); and mouse anti-GAPDH purchased from Kang Chen Bio-tech (Shanghai, China).

Immunofluorescence analysis. Immunofluorescence analysis was performed as described previously [11]. In brief, cells grown on sterile coverslips in 24-well plates were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.5% TritonX-100 for 15 min, blocked with Image-iT FX Signal Enhancer (Invitrogen), and then incubated with rhodamine-conjugated phalloidin (Sigma-Aldrich, USA) for 2 h in the dark at room temperature. After washing with PBST, the nuclei were counterstained with 0.5 μg/ml DAPI (Sigma-Aldrich, USA). Images were captured using a fluorescence microscope.

Autophagic flux assay. SMMC-7721 or SK-HEP1 cells retrovirally transduced with GFP-LC3-RFPLC3ΔG (Addgene Inc.) were grown on sterile coverslips in 24-well plates. After incubation with apigenin for 48 hours, images were captured under a fluorescence microscope.

Statistical analysis. Data were presented as means ± standard deviation (SD). Statistical significance was determined by the Student's t-test, and p-values less than 0.05 were considered statistically significant.

Results

Apigenin decreased cell viability, migration, and invasion of HCC cells. Apigenin has been shown to exhibit cell growth arrest, apoptosis, and anti-metastasis in HCC [12–14], however, its underlying mechanisms remain largely unknown. In the present study, we first performed the CCK-8 assay to investigate the effect of apigenin on the cell viability of HCC cell lines SMMC-7721 and SK-HEP1. As expected, an incubation with different concentrations of apigenin for 48 h decreased the cell viability of both SMMC-7721 and SK-HEP1 cells in a dose-dependent manner (Figures 1A, 1B). Then, we performed a wound-healing assay and Transwell assay to assess the effect of apigenin on the migration and invasion of HCC cells. In comparison with the controls (0 μM), treatment with indicated concentrations of apigenin in SMMC-7721 cells with low metastatic potential, and in SK-HEP1 cells with high metastatic potential, decreased wound healing, migratory and invasive ability in a dose-dependent manner (Figures 1C–1H). Furthermore, HCC cells were exposed to a fixed concentration of apigenin (30 μM for SMMC-7721 cells and 20 μM for SK-HEP1 cells)

for 24 h, 48 h, or 72 h, and Transwell assays demonstrated that apigenin decreased cell migration and invasion in a time-dependent manner (Figures 1I, 1J). Together, these results indicate that apigenin reduced the motility of both SMMC-7721 and SK-HEP1 cells, which was consistent with other studies [15–18]. Considering that the assembly of the actin cytoskeleton serves an important role in cell migration and invasion [19], we performed rhodamine-labeled phalloidin to stain the actin cytoskeleton in SMMC-7721 and SK-HEP1 cells in the presence or absence of apigenin. The results showed that incubation with 30 μM of apigenin for SMMC-7721 cells and 20 μM of apigenin for SK-HEP1 cells resulted in an increased number of stress fibers and decreased the formation of lamellipodia and filopodia at the cell edge in both SMMC-7721 and SK-HEP1 cells (Figure 2A), suggesting that apigenin-induced reduction of the migration and invasion of HCC cells may be mediated by the rearrangement of the actin cytoskeleton.

Apigenin downregulated YAP and its targets in HCC cells. To gain insights into the regulatory mechanism by which apigenin exerted anti-tumor activity in terms of comprehensive gene expression, RNA-seq was employed to compare the transcriptional expression profiles in SMMC-7721 cells in the presence or absence of apigenin. A total of 713 upregulated genes and 956 downregulated genes were differentially expressed at least 2-fold in apigenin-treated cells (Figures 2B, 2C). Notably, the KEGG analysis showed the Hippo signaling pathways highly altered in the apigenin-treated cells (Figure 2D), suggesting that the Hippo signaling might be implicated in apigenin-induced reduction of the motility of HCC cells. Among the Hippo signaling-related genes, CTGF, CYR61, and YAP, but not TAZ, exhibited remarkable reduction. Given that CTGF and CYR61 are two well-characterized downstream targets of YAP/TAZ, we then performed RT-qPCR to examine the mRNA levels of CTGF, CYR61, YAP, and TAZ in SMMC-7721 and SK-HEP1 cells in the presence or absence of apigenin. In agreement with the RNA-seq findings, the mRNA levels of CTGF, CYR61, and YAP showed a significant reduction in apigenin-treated cells (Figures 2E, 2F). The western blotting analysis also demonstrated the downregulation of YAP (but not TAZ) in both apigenin-treated SMMC-7721 and SK-HEP1 cells (Figure 2G). These findings demonstrated that apigenin might regulate the YAP expression and its transcriptional activity, suggesting that apigenin might exhibit its anti-tumor activity through downregulation of the YAP.

Apigenin regulated the migration, invasion, and autophagy of HCC cells through downregulating YAP. In order to narrow down the scope of downstream genes, RNA-seq analysis was also employed to analyze the transcriptional alterations in stable YAP knockdown SMMC-7721 cells (Figures 3A, 3B). We integrated both data from RNA-seq analysis for apigenin treatment and YAP knockdown to establish the gene set overlaps. A total of 785 genes were demonstrated by the Venn diagram, including YAP, CTGF, CYR61,
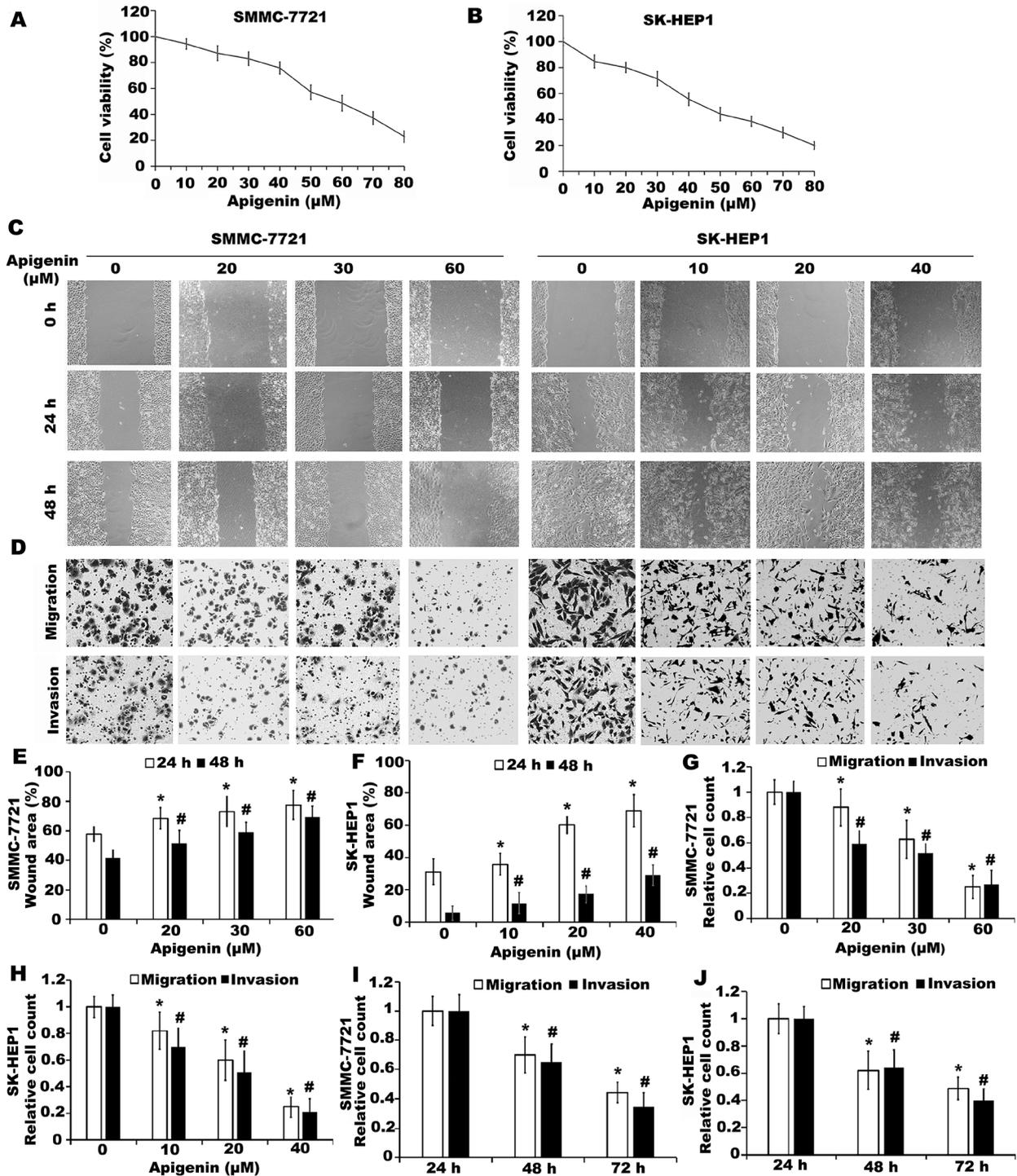
Figure 1. Effects of apigenin on cell viability, migration, and invasion of SMMC7721 and SK-HEP1 cells. A, B) Cell viability of SMMC7721 (A) and SK-HEP1 cells (B) after incubation with different concentrations of apigenin for 48 h was assessed by CCK-8 assay; n=3. C) Representative the wound-healing assay to assess the migration of HCC cells after incubation with the indicated dose of apigenin for SMMC-7721 cells and SK-HEP1 cells was shown (magnification, ×100); n=3. D) Representative Transwell cell migration and invasion assays in SMMC-7721 and SK-HEP1 cells after incubation with apigenin were shown (magnification, ×200); n=3. E, F) Quantification of relative wound healing for SMMC-7721 (E) and SK-HEP1 cells (F) was shown. Data presented as means ± SD; n=3, *p<0.05 and #p<0.05 vs. the indicated control (0 μM). G, H) Quantification of relative numbers of migrated and invaded cells for SMMC-7721 (G) and SK-HEP1 cells (H) was shown. Data presented as means ± SD; n=3, *p<0.05 and #p<0.05 vs. the indicated control (0 μM). I, J) Quantification of relative numbers of migrated and invaded cells for SMMC-7721 cells after incubated with 30 μM of apigenin (I) and SK-HEP1 cells after incubated with 20 μM of apigenin (J) at indicated time point was shown. Data presented as means ± SD; n=3, *p<0.05 and #p<0.05 vs. the indicated cells at 24 h.
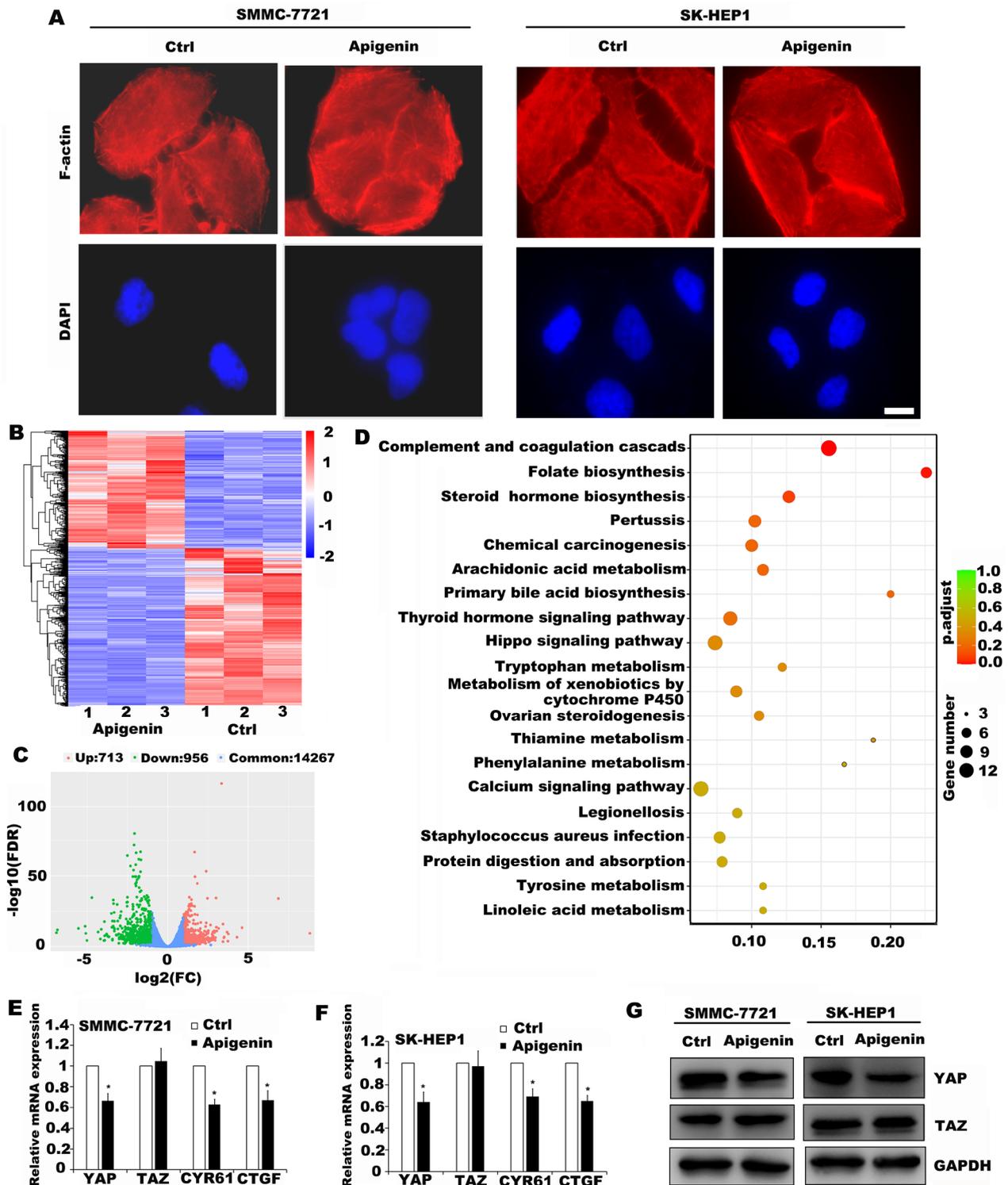
Figure 2. Apigenin-modulated gene expression and signaling pathways in HCC cells. A) Representative images of the actin cytoskeleton and DAPI staining. Scale bar; 20 μm. B) Clustered heatmap of the differentially expressed genes in SMMC-7721 cells in the presence or absence of apigenin by RNA-seq analysis was shown. Each sample contained a mixture of three repeats. C) Volcano plot of the differentially expressed genes identified in control and apigenin-treated SMMC7721 cells. D) Pathway enrichment analysis of the differentially expressed genes regulated by apigenin reveals 20 significant enriched. E, F) Quantitative analysis of the mRNA levels of YAP, TAZ, CYR61, and CTGF in SMMC7721 (D) and SK-HEP1 cells (E) after incubation with apigenin for 48 h by RT-qPCR, and normalized against GAPDH. Data presented as means ± SD; n=4, *p<0.05 vs. the indicated control (Ctrl). G) Protein levels of YAP and TAZ were determined by western blotting from SMMC7721 and SK-HEP1 cells after incubation with apigenin for 48 h, and GAPDH was used as a loading control. Representative blots are shown, n=3.

some EMT markers, and autophagy-related genes (Figure 3C). Mounting evidence reveals that YAP is involved in the EMT, which contributes to cancer cell migration, invasion, and cancer stemness [20]. Thus, we conducted RT-qPCR and western blotting to validate the impact of apigenin on some EMT markers in both SMMC-7721 and SK-HEP1 cells. The results revealed consistent significant upregulation of E-cadherin (E-CAD) and β-catenin (β-CAT), and downreg-

ulation of N-cadherin (N-CAD) and Vimentin (VIM) in the apigenin-treated HCC cells compared to the control cells (Figures 3D–3F), indicating that apigenin reduced the motility of HCC cells by inhibiting the EMT process.

In recent years, the autophagy involved in the tumorigenesis and metastatic progression of HCC has drawn research attention. Therefore, we subsequently performed RT-qPCR and western blotting to validate the impact of apigenin on
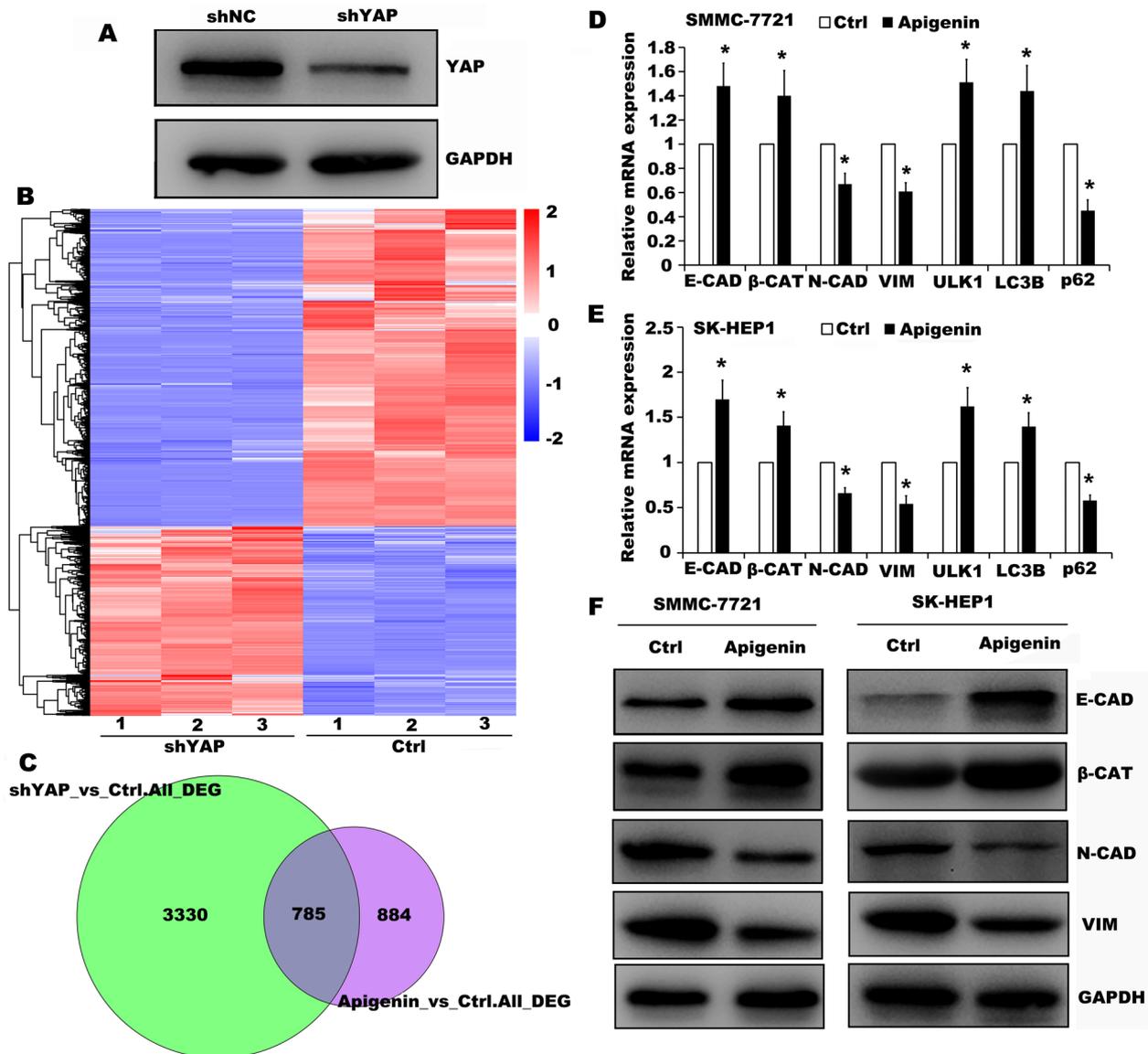
Figure 3. Apigenin regulated the expression of EMT markers and autophagy-related genes in HCC cells. A) Stable YAP knockdown (shYAP) and control (shNC) in SMMC-7721 cells were verified by western blotting. GAPDH served as the internal control, n=3. B) Clustered heatmap of the differentially expressed genes in SMMC-7721 cells with stable YAP knockdown or control by RNA-seq analysis was shown. Each sample contained a mixture of three repeats. C) A total of 785 genes were identified through the intersection of data from RNA-seq analysis for apigenin treatment and YAP knockdown in SMMC7721 cells. D, E) The expression of E-cadherin (E-CAD), β-catenin (β-CAT), N-cadherin (N-CAD), Vimentin (VIM), LC3B, ULK1, and SQSTM1/p62 (p62) was detected by RT-qPCR in SMMC7721 (D) and SK-HEP1 cells (E) after incubation with apigenin for 48 h. GAPDH served as the internal control, n=3, *p<0.05 vs. the indicated control (Ctrl). F) The expression of E-cadherin (E-CAD), β-catenin (β-CAT), N-cadherin (N-CAD), and Vimentin (VIM) was detected by western blotting in SMMC7721 and SK-HEP1 cells after incubation with apigenin for 48 h. GAPDH served as the loading control, n=3.

the expression of some autophagy-related genes in both SMMC-7721 and SK-HEP1 cells. Data from RT-qPCR confirmed the significant upregulation of LC3B and ULK1, two well-established markers of autophagy induction, and downregulation of SQSTM1/p62, an indicator of autophagic degradation, in both apigenin-treated SMMC-7721 and SK-HEP1 cells compared to the control cells (Figure 3D, 3E). Meanwhile, the western blotting analysis also showed that treatment with apigenin increased the amount of LC3B-II and ULK1, and decreased the accumulation of SQSTM1/p62 in both SMMC-7721 and SK-HEP1 cells (Figure 4A). To further validate the impact of apigenin on autophagy, the autophagic flux was evaluated by using a GFP-LC3-RFP-LC3ΔG reporter construct to detect GFP-LC3-RFP-LC3ΔG fluorescence and co-localization as described previously [21]. As shown in Figure 4B, treatment with apigenin displayed fewer green signals in both SMMC-7721 and SK-HEP1 cells than those in indicated control cells, suggesting the increased autophagic flux. Collectively, these results clearly suggested that apigenin induced autophagy in HCC cells.

Considering that apigenin led to the downregulation of YAP and the reduction of its transcriptional activity as mentioned above, we postulated that apigenin-induced reduction of the migration and invasion of HCC cells probably accounted for the regulating YAP expression. To verify this postulate, we generated YAP overexpressing SMMC-7721 and SK-HEP1 cells by infection with adeno-virus encoding YAP (AdYAP) or control (Adc) (Figure 5A). Then, Transwell assays showed that, compared with the controls (Adc), YAP overexpression significantly increased the migration and invasion of both SMMC-7721 and SK-HEP1 cells, whereas treatment with apigenin partially abrogated the migratory and invasive capacity of both SMMC-7721 and SK-HEP1 cells induced by YAP overexpression (Figures 5B, 5C). Moreover, RT-qPCR and western blotting analysis indicated that YAP overexpression downregulated E-cadherin, β-catenin, LC3B, and ULK1, and upregulated N-cadherin, Vimentin, and SQSTM1/p62 in SMMC-7721 cells compared with those in controls (Adc), whereas treatment with apigenin partially exhibited
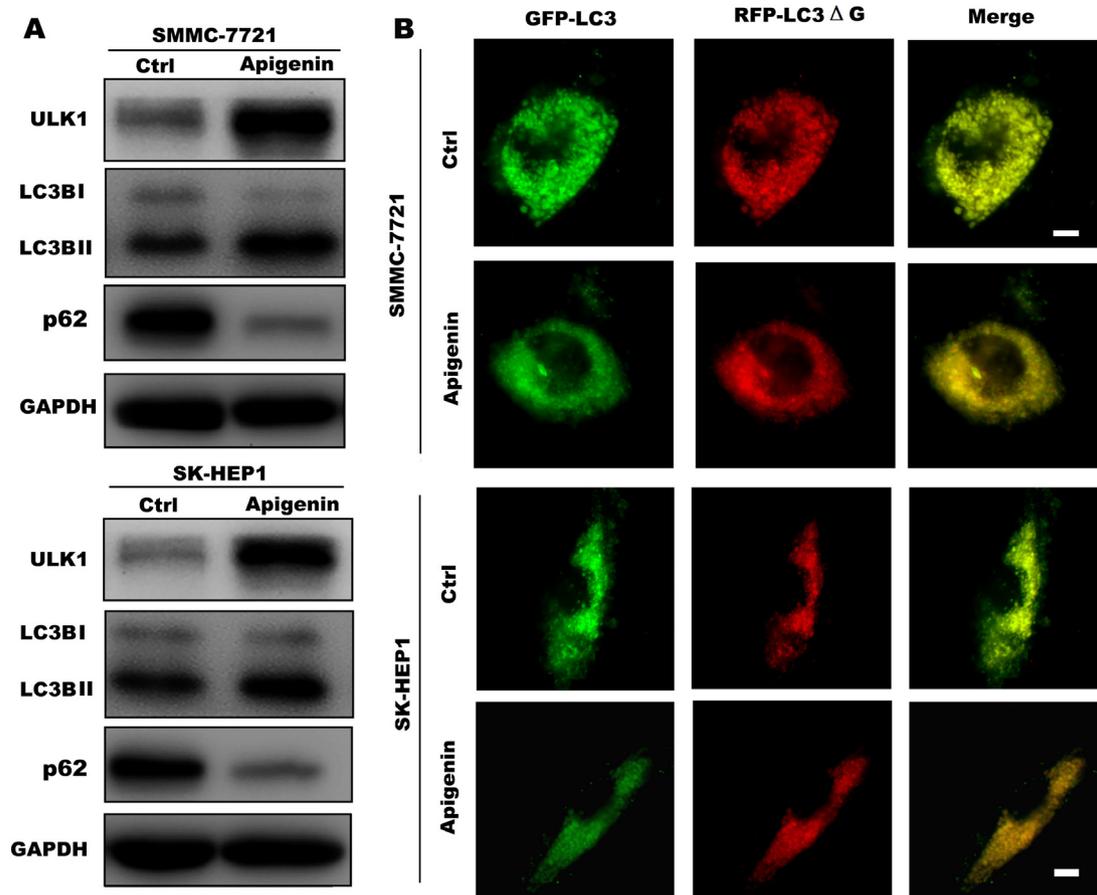
Figure 4. Apigenin induced autophagy in SMMC-7721 and SK-HEP1 cells. A) The level of LC3B, ULK1, and SQSTM1/p62 (p62) was detected by western blotting in SMMC7721 and SK-HEP1 cells after incubation with apigenin for 48 h. GAPDH served as the loading control, n=3. **B)** The autophagic flux was observed in apigenin-treated SMMC-7721 and SK-HEP1 cells after transduction with GFP-LC3-RFP-LC3ΔG construct, and representative images are shown, n=3. Scale bar 10 μm.
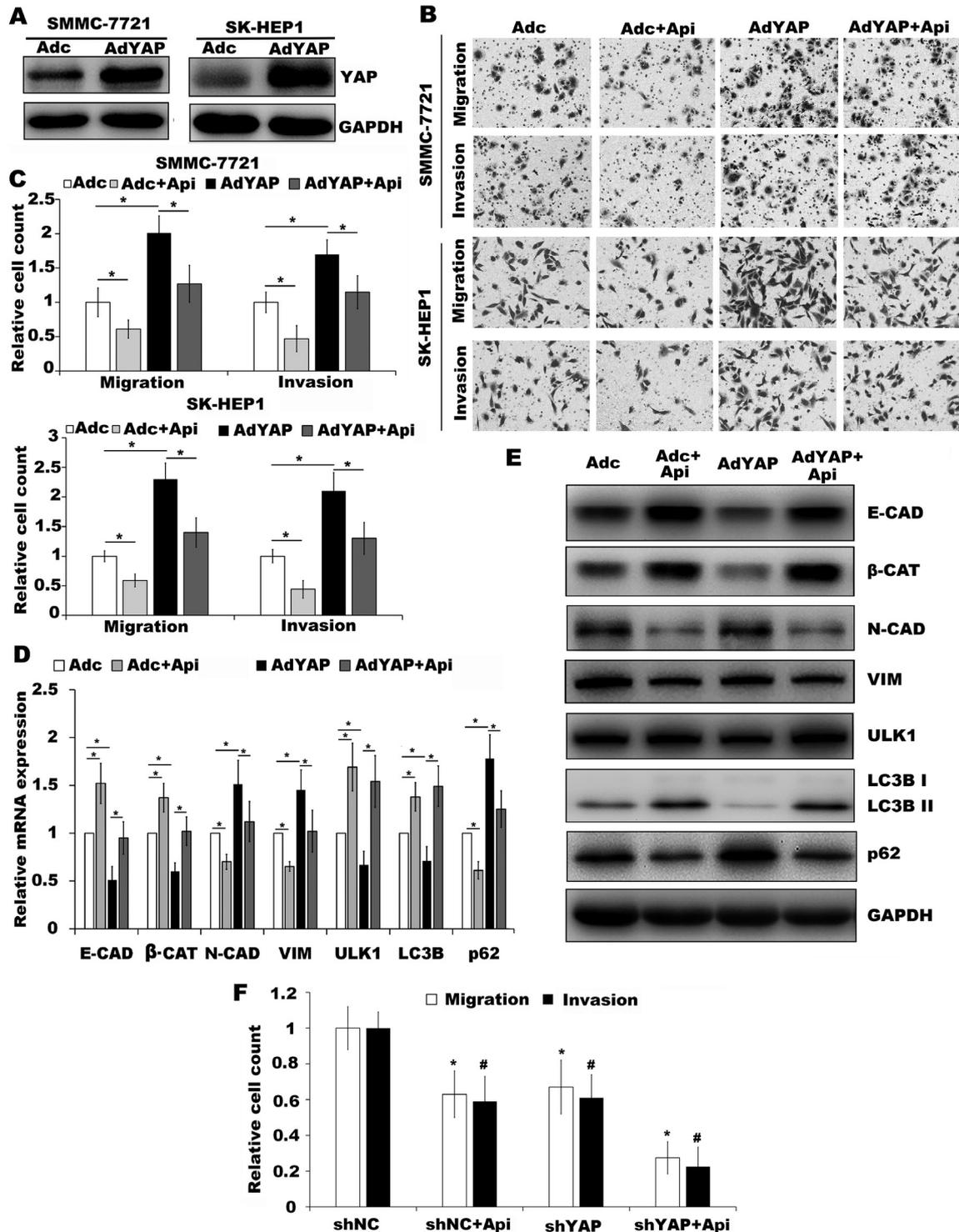
Figure 5. YAP reversed the inhibitory effect of apigenin on SMMC7721 and SK-HEP1 cells. A) Overexpression of YAP in SMMC7721 and SK-HEP1 cells after infection with adenovirus encoding YAP (AdYAP) or control (AdC) was verified by western blotting. GAPDH served as the loading control, n=3. B, C) Representative Transwell cell migration and invasion assays in SMMC-7721 and SK-HEP1 cells (B, magnification, ×200), and quantification of relative numbers of migrated and invaded cells (C) was shown. Data presented as means ± SD, n=3, *p<0.05. D, E) The expression of E-cadherin (E-CAD), β-catenin (β-CAT), N-cadherin (N-CAD), Vimentin (VIM), LC3B, ULK1, and SQSTM1/p62 (p62) was detected by RT-qPCR (D) and western blotting (E) in SMMC7721 cells. GAPDH served as the internal control, n=3, *p<0.05. F) Quantification of relative numbers of migrated and invaded cells for stable YAP knockdown SMMC-7721 cells (shYAP) and control (shNC) after incubation with or without apigenin was shown. Data presented as means ± SD, n=3, *p<0.05 and #p<0.05 vs. the indicated control (shNC).

the reverse effects in YAP overexpressing SMMC-7721 cells (Figure 5D, 5E). In addition, data from Transwell assays further demonstrated that treatment with apigenin in stable YAP knockdown SMMC-7721 cells induced synergistic effects for inhibition of the cell migratory and invasive ability (Figure 5F). Taken together, these results indicate that apigenin might promote autophagy and inhibit cell migration and invasion by regulating the expression of YAP.

Discussion

Apigenin is a common plant-derived flavonoid with anti-cancer properties and has been reported as an anti-cancer agent in various types of cancers, including breast, lung, skin, blood, colon, prostate, pancreatic, cervical, oral, and gastric as well as liver cancer [14]. This natural compound exhibits its anti-cancer properties by inducing apoptosis and inhibiting cell proliferation, migration, invasion, or angiogenesis via altering several signaling pathways [8, 14]. Apigenin induced cell cycle arrest and inhibition of proliferation of HCC cells by regulating the expression of CDK4 and Cyclin D1 [12]. Notably, apigenin exerts its anti-metastatic activity by downregulation of matrix metallopeptidases-2 (MMP-2), MMP-9, Snail, and Slug, and upregulation of E-cadherin in lung cancer cells, ovarian cancer cells, or HCC cells [22–25]. In the current study, we demonstrated that apigenin decreased the expression of YAP, and subsequently reduced migration and invasion, and induced autophagy of HCC cells. Our findings identified a novel molecular event in HCC cells after treatment with apigenin.

The biology and regulation of YAP and TAZ, two closely related transcriptional regulators, are receiving increasing attention owing to their fundamental roles in organ growth, tissue repair, and tumorigenesis [5]. In particular, the widespread activation and the crucial role of YAP in cancers are demonstrating YAP as a novel promising therapeutic target for designing anti-cancer drugs [26]. Although a few YAP inhibitors have been verified in some cancers [27, 28], inhibitors specifically targeting YAP for anti-cancer therapeutic reagents remain very limited. In the present study, we selected low metastatic potential SMMC-7721 and high metastatic potential SK-HEP1 cell line to evaluate the effect of apigenin on the cell viability, migration, and invasion by CCK-8 assay and Transwell assays. As expected, and in agreement with previous publications that apigenin decreased the viability, migration, and invasion of both SMMC-7721 and SK-HEP1 cells. Moreover, the data further indicated that apigenin could inhibit the migration and invasion of HCC cells by regulating actin cytoskeletal rearrangements. These rearrangements induced by apigenin include decreased the formation of lamellipodia and filopodia at the leading edge in both SMMC-7721 and SK-HEP1 cells. This result is consistent with the results of apigenin inhibiting the proliferation, migration, and invasion of HCC cells reported in other studies [15, 18].

To better understand the role of apigenin-induced inhibition of the viability, migration, and invasion of HCC cells, RNA-seq was employed to ascertain the altered expression of mRNA transcripts induced by treatment with apigenin. Hierarchical clustering showed a total of 1,669 differentially expressed genes in apigenin-treated cells, and the KEGG pathway analysis revealed that the differentially expressed genes were enriched in various pathways, including the Hippo signaling pathway. Among the Hippo signaling-related genes, CTGF and CYR61, two well-characterized downstream targets of YAP/TAZ, exhibited remarkable reduction. Then, we performed RT-qPCR to examine the mRNA levels of CTGF, CYR61, YAP, and TAZ in both SMMC-7721 and SK-HEP1 cells in the presence or absence of apigenin. The mRNA levels of CTGF, CYR61, and YAP showed a significant reduction in apigenin-treated cells. The western blotting analysis further demonstrated the downregulation of YAP (but not TAZ) in apigenin-treated cells, suggesting that apigenin induced anti-tumor activity probably by downregulation of the YAP.

We combined the data from RNA-seq between apigenin-treated cells and stable YAP knockdown cells to reveal that there is a total of 785 differentially expressed genes in the gene set overlaps, including YAP, CTGF, CYR61, some EMT markers, and some autophagy-related genes. Therefore, we next validated the effect of apigenin on these EMT markers by RT-qPCR and western blotting. We demonstrated that apigenin significantly upregulated E-cadherin and β-catenin, and downregulated N-cadherin and Vimentin in both SMMC-7721 and SK-HEP1 cells, indicating that apigenin reduced cell motility of HCC cells probably by inhibiting the EMT process. Given that autophagy has been proposed to regulate the motility of cancer cells [29] and apigenin altered the expression of some autophagy-related genes from RNA-seq data, we subsequently evaluate the effect of apigenin on autophagy. Results from RT-qPCR and western blotting analysis showed that apigenin significantly upregulated ULK1 and LC3BII, and downregulated SQSTM1/p62 in both SMMC-7721 and SK-HEP1 cells. Furthermore, data from autophagic flux assay, which was based on the equimolar release of GFP-LC3 and RFP-LC3ΔG upon cleavage by endogenous ATG4 in the cells transduced with GFP-LC3-RFP-LC3ΔG construct, and GFP-LC3 was degraded by autophagy, whereas RFP-LC3ΔG remained in the cytosol, serving as an internal control [21], demonstrated that treatment with apigenin obviously induced autophagic flux in both SMMC-7721 and SK-HEP1 cells, indicating that apigenin promoted the autophagy of HCC cells by modulating the expression of autophagy-related genes.

Next, we speculated that apigenin-induced inhibition of the migration and invasion, and promotion of autophagy might be mainly caused by downregulating YAP, thereby affecting the expression of some EMT markers and autophagy-related genes in HCC cells. To test this hypothesis, SMMC7721 and SK-HEP1 cells were infected with adeno-

virus encoding YAP or control. Results from Transwell cell migration and invasion assays showed that apigenin could partially reverse the promoting effects of YAP overexpression on cell migration and invasion. Meanwhile, RT-qPCR and western blotting results also showed that apigenin could partially reverse the effects of YAP overexpression on the expression of the EMT markers and autophagy-related genes. In addition, we also observed that treatment with apigenin in YAP knockdown SMMC-7721 cells displayed synergistic effects for inhibition of the cell migration and invasion. Taken together, these results provided evidence that treatment with apigenin induced autophagy, and inhibited cell migration and invasion at least in part by downregulating the expression of YAP in HCC cells.

In summary, our study demonstrated that the inhibition of cell viability, migration, and invasion, and induction of autophagy in HCC cells by apigenin might be, at least, partly through the downregulation of YAP, which provides a novel mechanism for the therapeutic application of apigenin in HCC patients.

References

[1] FORNER A, REIG M, BRUIX J. Hepatocellular carcinoma. Lancet 2018; 391: 1301–1314. https://doi.org/10.1016/S0140-6736(18)30010-2

[2] MCGLYNN KA, PETRICK JL, LONDON WT. Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. Clin Liver Dis 2015; 19: 223–238. https://doi.org/10.1016/j.cld.2015.01.001

[3] TORRE LA, BRAY F, SIEGEL RL, FERLAY J, LORTET-TIEULENT J et al. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87–108. https://doi.org/10.3322/caac.21262

[4] YONG KJ, GAO C, LIM JS, YAN B, YANG H et al. Oncofetal gene SALL4 in aggressive hepatocellular carcinoma. N Engl J Med 2013; 368: 2266–2276. https://doi.org/10.1056/NEJMoa1300297

[5] GUO L, TENG L. YAP/TAZ for cancer therapy: opportunities and challenges (review). Int J Oncol 2015; 46: 1444–1452. https://doi.org/10.3892/ijo.2015.2877

[6] MOROISHI T, HANSEN CG, GUAN KL. The emerging roles of YAP and TAZ in cancer. Nat Rev Cancer 2015; 15: 73–79. https://doi.org/10.1038/nrc3876

[7] YAN X, QI M, LI P, ZHAN Y, SHAO H. Apigenin in cancer therapy: anti-cancer effects and mechanisms of action. Cell Biosci 2017; 7: 50. https://doi.org/10.1186/s13578-017-0179-x

[8] ŞIRIN N, ELMAS L, SEÇME M, DODURGA Y. Investigation of possible effects of apigenin, sorafenib and combined applications on apoptosis and cell cycle in hepatocellular cancer cells. Gene 2020; 737: 144428. https://doi.org/10.1016/j.gene.2020.144428

[9] OZÇELIK B, KARTAL M, ORHAN I. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. Pharm Biol 2011; 49: 396–402. https://doi.org/10.3109/13880209.2010.519390

[10] SHARMA A, GHANI A, SAK K, TULI HS, SHARMA AK et al. Probing into Therapeutic Anti-cancer Potential of Apigenin: Recent Trends and Future Directions. Recent Pat Inflamm Allergy Drug Discov 2019; 13: 124–133. https://doi.org/10.2174/1872213X13666190816160240

[11] ZHOU W, SHEN Q, WANG H, YANG J, ZHANG C et al. Knockdown of YAP/TAZ Inhibits the Migration and Invasion of Fibroblast Synovial Cells in Rheumatoid Arthritis by Regulating Autophagy. J Immunol Res 2020; 2020: 9510594. https://doi.org/10.1155/2020/9510594

[12] LI Y, CHENG X, CHEN C, WU H, ZHAO H et al. Apigenin, a flavonoid constituent derived from P. villosa, inhibits hepatocellular carcinoma cell growth by CyclinD1/CDK4 regulation via p38 MAPK-p21 signaling. Pathol Res Pract 2020; 216: 152701. https://doi.org/10.1016/j.prp.2019.152701

[13] QIN Y, ZHAO D, ZHOU HG, WANG XH, ZHONG WL et al. Apigenin inhibits NF-κB and snail signaling, EMT and metastasis in human hepatocellular carcinoma. Oncotarget 2016; 7: 41421–41431. https://doi.org/10.18632/oncotarget.9404

[14] IMRAN M, ASLAM GONDAL T, ATIF M, SHAHBAZ M, BATOOL QAISARANI T et al. Apigenin as an anticancer agent. Phytother Res 2020; 34: 1812–1828. https://doi.org/10.1002/ptr.6647

[15] TONG J, SHEN Y, ZHANG Z, HU Y, ZHANG X, et al. Apigenin inhibits epithelial-mesenchymal transition of human colon cancer cells through NF-κB/Snail signaling pathway. Biosci Rep 2019; 39: BSR20190452. https://doi.org/10.1042/BSR20190452

[16] ZHANG E, ZHANG Y, FAN Z, CHENG L, HAN S et al. Apigenin Inhibits Histamine-Induced Cervical Cancer Tumor Growth by Regulating Estrogen Receptor Expression. Molecules 2020; 25: 1960.

[17] CHEN X, XU H, YU X, WANG X, ZHU X et al. Apigenin inhibits in vitro and in vivo tumorigenesis in cisplatin-resistant colon cancer cells by inducing autophagy, programmed cell death and targeting m-TOR/PI3K/Akt signalling pathway. J BUON 2019; 24: 488–493.

[18] CARDENAS H, ARANGO D, NICHOLAS C, DUARTE S, NUOVO GJ, et al. Dietary Apigenin Exerts Immune-Regulatory Activity in Vivo by Reducing NF-κB Activity, Halting Leukocyte Infiltration and Restoring Normal Metabolic Function. Int J Mol Sci 2016; 17: 323. https://doi.org/10.3390/ijms17030323

[19] ZHAO H, JIAO Y, ZHANG Z. Deguelin inhibits the migration and invasion of lung cancer A549 and H460 cells via regulating actin cytoskeleton rearrangement. Int J Clin Exp Pathol 2015; 8: 15582–15590.

[20] NOGUCHI S, SAITO A, NAGASE T. YAP/TAZ Signaling as a Molecular Link between Fibrosis and Cancer. Int J Mol Sci 2018; 19: 3674. https://doi.org/10.3390/ijms19113674

[21] KAIZUKA T, MORISHITA H, HAMA Y, TSUKAMOTO S, MATSUI T et al. An Autophagic Flux Probe that Releases an Internal Control. Mol Cell 2016; 64: 835–849. https://doi.org/10.1016/j.molcel.2016.09.037

[22] CHANG JH, CHENG CW, YANG YC, CHEN WS, HUNG WY et al. Downregulating CD26/DPPIV by apigenin modulates the interplay between Akt and Snail/Slug signaling to restrain metastasis of lung cancer with multiple EGFR statuses. J Exp Clin Cancer Res 2018; 37: 199. https://doi.org/10.1186/s13046-018-0869-1

[23] HE J, XU Q, WANG M, LI C, QIAN X et al. Oral administration of apigenin inhibits metastasis through AKT/P70S6K1/MMP-9 pathway in orthotopic ovarian tumor model. Int J Mol Sci 2012; 13: 7271–7282. https://doi.org/10.3390/ijms13067271

[24] LIN CM, CHEN HH, LIN CA, WU HC, SHEU JJ et al. Apigenin-induced lysosomal degradation of β-catenin in Wnt/β-catenin signaling. Sci Rep 2017; 7: 372. https://doi.org/10.1038/s41598-017-00409-z

[25] PAN FF, ZHENG YB, SHI CJ, ZHANG FW, ZHANG JF et al. H19-Wnt/β-catenin regulatory axis mediates the suppressive effects of apigenin on tumor growth in hepatocellular carcinoma. Eur J Pharmacol. 2021; 893: 173810. https://doi.org/10.1016/j.ejphar.2020.173810

[26] ZANCONATO F, BATTILANA G, CORDENONSI M, PICCOLO S. YAP/TAZ as therapeutic targets in cancer. Curr Opin Pharmacol 2016; 29: 26–33. https://doi.org/10.1016/j.coph.2016.05.002

[27] LI W, CAO Y, XU J, WANG Y, LI W et al. YAP transcriptionally regulates COX-2 expression and GCCSysm-4 (G-4), a dual YAP/COX-2 inhibitor, overcomes drug resistance in colorectal cancer. J Exp Clin Cancer Res 2017; 36: 144. https://doi.org/10.1186/s13046-017-0612-3

[28] FISHER ML, CIAVATTONE N, GRUN D, ADHIKARY G, ECKERT RL. Sulforaphane reduces YAP/ΔNp63α signaling to reduce cancer stem cell survival and tumor formation. Oncotarget 2017; 8: 73407–73418. https://doi.org/10.18632/oncotarget.20562

[29] CHOI KS. Autophagy and cancer. Exp Mol Med 2012; 44: 109–120. https://doi.org/10.3858/emm.2012.44.2.033