Original Research

YAP activation in melanoma contributes to anoikis resistance and metastasis

Bei Zhao[1], Jun Xie[1], Xiyuan Zhou[1], Lixia Zhang[1], Xiankui Cheng[2] and Chenglin Liang[1]

[1]Institute of Dermatology and Venereology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu 610072, China; [2]Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, China
Corresponding author: Chenglin Liang. Email: liangchenglin@med.uestc.edu.cn

Impact statement

This study reveals a novel role of aberrantly activated YAP in anoikis resistance and metastasis of melanoma cells.

Abstract

Melanoma is inherently heterogeneous, providing resistance to apoptosis. Anoikis resistance is a hallmark feature of metastatic melanoma to escape apoptosis when cells lose contact with adjacent cells or extracellular matrix. The yes-associated protein transcription co-activator is the effector of Hippo pathway. Herein, we investigated the function of yes-associated protein in anoikis resistance of melanoma cells. When melanoma cells were grown under anchorage-independent condition, anoikis-resistant cells displayed higher levels of yes-associated protein activation than the cells that were attached to the basement membrane, as evidenced by downregulated phosphorylated yes-associated protein at Ser127 and higher expression of downstream genes *BCL2* and *MCL-1*. Yes-associated protein overexpression directly enhanced the anoikis resistance and metastatic potential of melanoma cells. Conversely, yes-associated protein inhibitor CA3 exhibited Dose-dependent induction of anoikis in resistant melanoma cells and exerted great inhibition on cell migration. Knockdown of yes-associated protein expression by shRNA also rendered melanoma cells susceptible to anoikis and interrupted cell invasiveness. Yes-associated protein inhibition in anoikis-resistant cells also reduced the number of metastatic nodules in the lung sections of SCID mice. Clinically, higher yes-associated protein level in the lung metastasis tissues correlated with higher BCL2 and MCL1 expressions compared with the non-metastasis tissues. Overall, our finding suggests that the aberrant activation of yes-associated protein exerts important role on anoikis resistance and metastatic capability of melanoma cells.

Keywords: Melanoma, anoikis resistance, metastasis, hippo signaling, yes-associated protein

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Introduction

Melanoma is the most aggressive form of malignant skin cancer with high metastatic propensity. It typically migrates through lymph nodes to distant sites of the body, especially to lungs, liver, and brain at late stage of melanoma.[1] Metastasis may be present at the initial diagnosis and such cases are resistant to radiotherapy, chemotherapy, and targeted therapy.[2] Metastatic melanoma accounts for approximately 80% of skin cancer-related deaths. The median overall survival time of patients with metastatic melanoma is less than one year, and only approximately 10% of these patients survive more than five years after diagnosis.[3] Systemic chemotherapy remains the mainstay of melanoma treatment in clinic. As melanoma is highly heterogeneous, conventional therapies often fail to inhibit melanoma invasion and metastasis. Up to date, the management of melanoma is evolving rapidly owing to the profound understanding of the molecular heterogeneity of this disease.

Increasing evidences show that anoikis resistance is a crucial step for tumor progression and metastatic colonization.[4] Anoikis is an anchorage-independent cell death.[5] Epithelial cells highly rely upon appropriate cell-matrix and cell–cell interaction for cell anchorage and survival.[6] As an obstacle to metastases, cells usually undergo classical apoptosis upon losing contact with surrounding matrix or neighboring cells.[7] Thus, anoikis acts as a vital mechanism in impeding adherent-independent cell growth and attachment to an inappropriate matrix, thus avoiding cell dissemination to distant organs. However, malignant tumor cells can develop different mechanism to counteract anoikis and thereby maintain survival after detaching from primary sites and while migrating through the circulatory and lymphatic systems.[8] Tumor cells overexpressing anti-apoptotic

proteins such as Bcl2 and FLIP, become resistant to anoikis.[5,9] Thus, anoikis resistance presents as a critical characteristic of metastatic tumors, whereas the precise mechanisms of anoikis resistance are mainly unclear.

Melanoma cells resistant to anoikis are apt to metastasize. Previous studies showed that B-RAF and PI-3 kinase signaling can render melanoma cells resistant to anoikis.[10] RhoA inhibition directly enhanced anoikis of aggregated B16F10 melanoma cells.[11] Depletion of Mcl-1 using RNA interference, conferred mutant B-RAF melanoma cells sensitive to anoikis.[12] Moreover, it has been found that anoikis-resistant melanoma cells display significantly higher expression and phosphorylation of STAT3 at Y705 than those cells attached to the basement membrane. STAT3 silence can decrease anoikis resistance of melanoma cells and reduce their metastatic potential.[13] Timp1 assembling with CD63 and β1-integrin is another important molecule, which confers anoikis resistance to melanoma cells by activating PI3K signaling during tumorigenic progression.[14]

The yes-associated protein (YAP) transcription coactivator is an effector of the Hippo signaling pathway, and the Hippo tumor suppressor pathway phosphorylates and inhibits YAP.[15] One influential function of YAP is to mediate the transduction of cell structural features, including polarity, shape, and cytoskeletal organization. YAP as a mechanosensitive factor, is important in control of cell function, such as growth, apoptosis, migration, and communication.[16] In the current study, we have revealed a crucial role of YAP activation in anoikis resistance of melanoma *in vitro* and *in vivo*. Targeting YAP by small molecular inhibitor or genetic manipulation could decrease the malignant potential of melanoma cells to resist anoikis and to invade. Thus, our finding provides a novel insight into the therapy of melanoma via blocking YAP-induced anoikis resistance.

Materials and methods

Reagents

Poly(2-hydroxyethyl) methacrylate (poly-HEMA), G418, and puromycin were obtained from Sigma-Aldrich (St Louis, MO, USA). CA3 were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against YAP, LAST1, cleaved PARP, and phosphorylated YAP (Ser127), phosphorylated MST1/2 (Thr180) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for PARP-1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The YAP-expressing plasmid and vector control, the shRNA targeting YAP, and the non-target control were synthesized by GeneChem Biochemistry (Shanghai, China).

Cell culture

The human melanoma cell lines A375 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mM L-glutamine under a humidified atmosphere containing 5% CO_2 maintained at 37°C.

Anoikis assay

Adherent culture was maintained on Petri dishes (Corning). In order to induce anoikis under anchorage-independent condition, culture dishes were coated with poly-HEMA. Anoikis assay was performed as described previously.[13] Approximately 1×10^6 cells were plated in poly-HEMA-coated Petri dishes. After the desired treatment time was achieved, suspended melanoma cells were centrifuged and uniformly divided in dishes or wells for adherent culture. These viable cells which survived in three-round anoikis induction, were stored and termed as anoikis-resistant cells.

Flow cytometry analysis

For cell apoptosis assay, apoptotic cells were measured by an Annexin-V apoptosis detection kit (MultiSciences Biotech, China) according to the manufacturer's protocol. Cells incubated under anchorage-dependent or -independent conditions were collected, washed once with PBS, resuspended in 200 μL binding buffer at the concentration of 1×10^6 cells/mL, stained with 10 μL Annexin V-FITC stock solution and 5 μL PI working solution, and incubated at 4°C in the dark condition for 30 min. Then, cells were washed by PBS and were analyzed by flow cytometry (BD-FACS Canto) to determine the viability (Annexin V and PI negative), early apoptosis (Annexin V positive and PI negative), or late apoptosis (Annexin V and PI positive). A minimum of 1×10^4 cells were collected and analyzed.

Wound healing assay

Wound healing assays were performed on anoikis-resistant cells or control cells always cultured under adherent condition. Cells were seeded in 6-well plates. When monolayer cells grew to about 80% confluence, a wound was made across it by scratching with 1 mL sterile pipette tip. Cells were washed to remove displaced and floating cells. The migration of the cells at the edge of the scratch was analyzed at 12 h. Images were captured under a microscope (Olympus Corporation, Japan).

Transwell assay

For transwell migration assay, Boyden's chamber (BD Biosciences, Bedford, MA) were pre-coated with matrigel; 1×10^5 cells (in DMEM medium with 1% FBS) were applied into the upper compartment of the chamber. The bottom wells contained 600 μL of culture medium with 10% FBS, and were left un-agitated in the cell incubator until observation. After incubation for 24 h, cells in the bottom compartment were fixed with methanol, and stained with 0.1% crystal violet. Images were captured and the number of migrated cells in five randomly selected counted under microscopy.

Transfection

8×GTIIC-luc and pcDNA4/HisMaxB-YAP1 plasmids were obtained from Addgene. shRNA-YAP and control plasmids were purchased from Genechem (GENECHEM, Shanghai,

China). shRNA-BCL2 and control plasmids were purchased from Genechem (GENECHEM, Shanghai, China). shRNA-MCL-1 and control plasmids were purchased from Genechem (GENECHEM, Shanghai, China). Transient transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instruction. The transfected cells were selected and enriched under growth medium containing 500 μg/mL G418 (for YAP overexpression) or 5 μg/mL puromycin (for shRNA-transfected cells).

Luciferase assay

For the luciferase reporter assay, melanoma cells were seeded in 12-well plates. The 8×GTIIC reporter and renilla-Luc plasmids were co-transfected into melanoma cells with Lipofectamine 2000 (Life Technologies); 12 h after transfection, cells were digested and cultured under detached or adherent conditions condition. Then, cells were lysed and measured using the Dual-Glo luciferase assay system (Promega) under a Perkin Elmer EnVision plate reader. All firefly luciferase activities were normalized to renilla luciferase activities.

Immunoblotting

Cells (1×10^6) were lysed on ice in 100 μL modified RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 2 mM EDTA). The protein content was quantitated using BCA Protein Assay Kit (Beyotime Institute of Biotechnology), and approximately 30 μg of total protein was separated by SDS-PAGE electrophoresis, and transferred to PVDF membranes (Millipore Corporation, USA). Proteins were detected using the indicated primary antibody and the signal was detected using peroxidase-conjugated secondary antibody followed by ECL chemiluminescence detection (Beyotime Institute of Biotechnology).

RNA isolation and real-time PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). cDNA was synthesized by reverse transcription using random hexamers and subjected to real-time PCR with gene-specific primers in the presence of SYBR Green (Applied Biosystems). The level of expression of each target gene was calculated using $2^{-\Delta\Delta Ct}$ method. The relative amount of each mRNA has normalized to *GAPDH*. Each sample has been examined in triplicate. RT-PCR analysis was performed with the following primers: *BCL2* 5′-TCGCCCTGTGGATGACTGA-3′ (forward), 5′-CAGAGACAGCCAGGAGAAATCA-3′ (reverse); *MCL-1* 5′-CCAAGGCATGCTTCGGAAA-3′ (forward), 5′-TCACAATCCTGCCCCAGTTT-3′ (reverse); *GAPDH* 5′-CTCACCGGATGCACCAATGTT-3′ (forward), 5′-CGCGT TGCTCACAATGTTCAT-3′ (reverse).

Experimental tumor metastasis in SCID mice

NOD/SCID mice were randomly allocated into two groups; six mice each. Melanoma A375 cells stably expressing non-target control (NC Ctrl) or shRNA targeting YAP (shRNA #3) in logarithmic growth phase (4×10^5/200 μL PBS) were slowly injected into the tail vein. Mice were humanely sacrificed by cervical dislocation under anesthesia 35 days later, lungs were rapidly excised and dissected, photographed, and stored in liquid nitrogen for further analysis. For metastasis quantification, tumor nodules were counted in whole lungs. For real-time PCR, tissues from three mice were homogenated and lysed with Trizol, and total RNA was applied for cDNA synthesis and qPCR detection. Each experiment was triplicated.

Colony formation assay

For colony formation assay, A375 cells were trypsinized to single cell suspensions and seeded into fresh 6-well plates at 1000 cells/well. Colonies were fixed with absolute methanol for 15 min and then stained with 0.1% crystal violet for 20 min. After washing with PBS three times, the colonies with a diameter over 2 mm were visualized by a scanner.

Immunohistochemistry

Detection of YAP, MCL-1, and BCL-2 was achieved by using the DAKO EnVision$^+$ System (Angilent, Carpinteria, CA, USA). Briefly, tissues were fixed with 4% formaldehyde and permeabilized with Triton X-100. After blocked with BSA, cells were incubated with anti-YAP or anti-MUC-1 or anti-BCL-2 antibody at 4°C for overnight, followed by incubation with HRP secondary antibodies for 30 min at room temperature and nucleus counterstaining with hematoxylin. Imaging was performed using a microscope (model IX71; Olympus, Tokyo, Japan).

Statistical analysis

All data in this study were displayed as means ± SD. Comparisons were analyzed by Student's *t*-test for comparison between two groups or one-way ANOVA among three groups and above. The significance was analyzed with SPSS10.0 software and a *P*-value <0.05 was considered to be statistically significant.

Results

Melanoma cells with anoikis resistance display higher YAP activity

We initially evaluated human cutaneous melanoma A375 cells for susceptibility to anoikis. A375 cells were cultured under detached (anchorage-free, suspension) or adherent conditions for 48 h. Then the detached cells were cultivated on an adherent plate where only the anoikis-resistant cells attached and survived, after which cell apoptosis was assessed by flow cytometry. We found that A375 cells underwent prominent anoikis when cultured under anchorage-free conditions compared with the adherent conditions (Figure 1(a)). Importantly, about 63% of cells

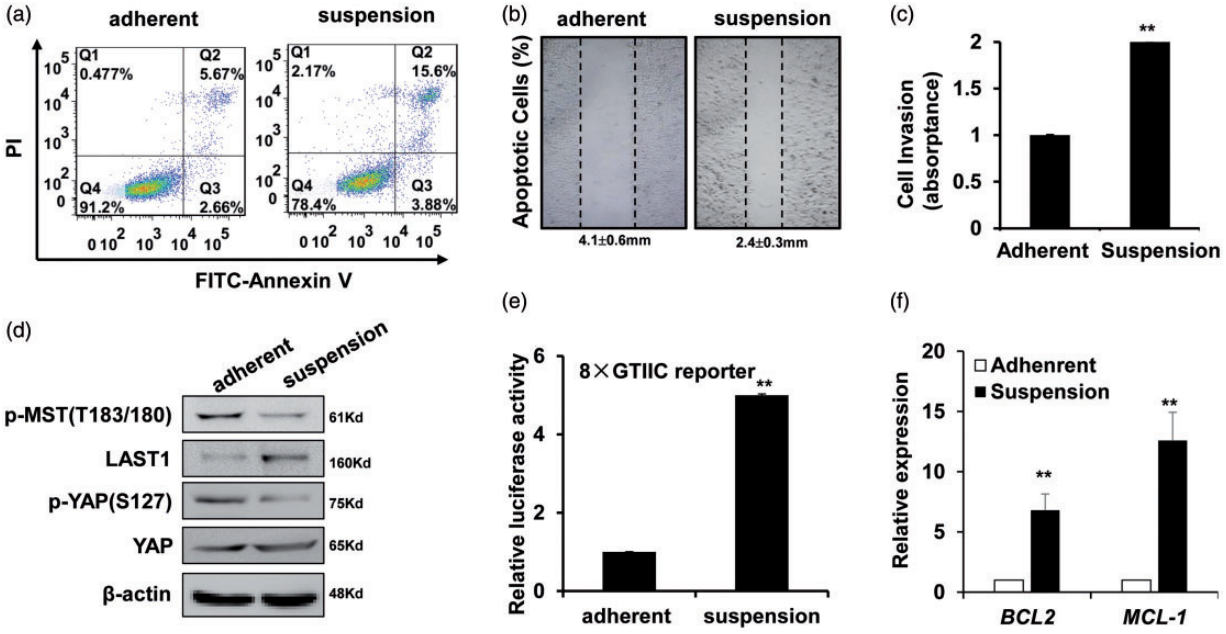

Figure 1. Upregulated YAP activity in anoikis-resistant melanoma cells. (a) A375 cells were cultivated under detached condition in the plates coated with poly-HEMA for 48 h and then allowed to attach for further culture. After suspension culture for 48 h, the cell apoptosis was evaluated using flow cytometry detection. The cell apoptosis was compared with the cells cultured under adherent conditions for same time period. (b) Wound healing assays were performed on anoikis-resistant cells or control cells always cultured under adherent condition. Confluent monolayers were scratched with 1 mL pipette tip. Wounds were allowed to heal for 12 h and imaged by microscope. Numbers under each photo represent the mean±SD (mm) for three independent experiments with each one triplicated. (c) Cell invasion was analyzed by Transwell assay. Anoikis-resistant or control A375 cells were allowed to invade through matrigel for 24 h. Results were shown as mean number of invaded cells ± SD ($n = 3$). $^{**}P < 0.01$, vs. the control. (d) Immunoblotting assay was used to detect the MST1/2 phosphorylation at Thr180/183, LAST1, YAP phosphorylation at Ser127, and total YAP in anoikis-resistant cells or control cells. (e) A375 cells were transfected with 8×GTIIC reporter and renilla-luc plasmids and were cultured under detached or adherent conditions for 48 h. Then the relative firefly/renilla luciferase activity was determined. Values are plotted as mean ± S.D. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with adherent group. (f) Expressions of Hippo signaling pathway target genes, BCL2 and MCL-1 in A375 cells under adherent or suspension conditions were detected by real-time PCR. Data represent mean ± SD from three independent experiments. Each experiment was repeated at least three times with similar results. $^{**}P < 0.01$, adherent vs. suspension. (A color version of this figure is available in the online journal.)

resisted anoikis and survived under anchorage independent conditions for 48 h. These viable cells which survived in three-round anoikis induction were stored and termed as anoikis-resistant cells. Subsequently, we performed wound healing assay to evaluate the migration ability of anoikis-resistant cells. As shown in Figure 1(b), anoikis-resistant cells healed the wound at much higher rate than control cells (Figure 1(b)). Anoikis-resistant cells were more invasive as compared to their respective adherent controls, as demonstrated by transwell assay (Figure 1(c)). Hence, these results indicate that anoikis-resistant A375 cells were highly migratory and invasive.

Convincing evidences have established a solid link of YAP oncoprotein with tumor growth and metastasis.[17] In the current study, we investigated the role of YAP as well as the up- and down-stream mediators in anoikis resistance of melanoma cells. As compared to adherent cells, the phosphorylation level of YAP at Ser127 but not the upstream mediator MST1/2 at Thr183/180 and was significantly decreased, and the total YAP was increased in anoikis-resistant melanoma cells, indicating the abnormal activation of YAP transcription cofactor (Figure 1(d)). After transfection with a YAP responsive luciferase (8×GTIIC-luc) reporter, A375 cells were then cultured under suspension or adherent conditions for 48 h. Anoikis-resistant A375 cells surviving under suspension conditions exhibited increased transcriptional activity of YAP (Figure 1(e)), concomitant with the mRNA level of the downstream target genes of YAP/TAZ, BCL2, and MCL-1 were significantly augmented (Figure 1(f)) Taken together, our results implicate that the abnormal activation of YAP is involved in the acquisition of anoikis resistance.

YAP overexpression enhances anoikis resistance

To delineate the association of YAP activity and anoikis resistance, we established A375 cells stably overexpressing YAP gene (Figure 2(a)). After incubation under detached condition for 48 h, A375 cells were stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. As shown in Figure 2(b), YAP-overexpressing A375 cells generated stronger resistance to anoikis and the number of viable cells was upregulated after suspension culture (Figure 2(b)). Decreased cleavage of PARP also indicated that YAP overexpression reduced the induction of anoikis by detachment culture (Figure 2(c)). As previous reports showed that YAP regulates the expression of anti-apoptotic *BCL2* and *MCL-1* genes,[18] we employed real-time PCR and demonstrated that YAP overexpression augmented the expression of *BCL2* and *MCL-1* genes (Figure 2(d)). To further confirm YAP induces anoikis resistance through BCL2 and MCL1, we silenced *BCL2 and MCL-1* expression in YAP-overexpressing melanoma cells using shRNA (Figure 2(e)). After incubation under detached condition for 48 h, solo silencing of BCL2 or MCL-1 led to much

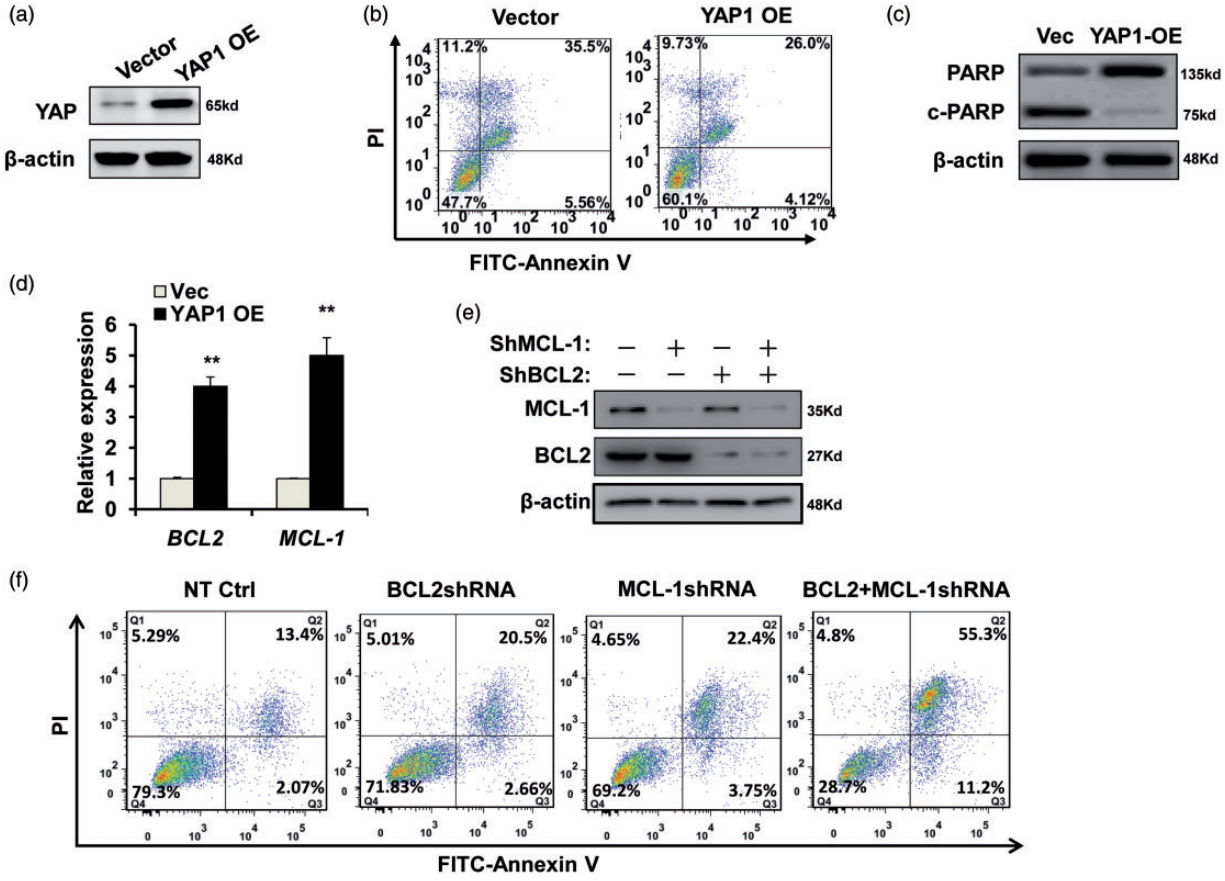

Figure 2. YAP overexpression renders anoikis resistance. (a) A375 cells transfected with YAP (YAP OE) or empty plasmids (vector) were subjected for Western blot to detect the level of YAP. (b) A375 cells stably expressing YAP were cultured under detached condition for 48 h. Flow cytometry analysis of apoptosis was determined using Annexin V-FITC/PI staining. Data are typical of three independent experiments. (c) Immunoblotting assay was used to detect the cleavage of PARP as indicative of cell apoptosis. (d) The expression of YAP target genes *Bcl-2* and *Mcl-1* was determined by real-time PCR. Data represent mean ± SD from three independent experiments (**$P < 0.01$, YAP OE vs. vector control). (e) A375 cells transfected with BCL2 shRNA, MCL-1 shRNA, or empty plasmids (vector) were subjected for Western blot to detect the level of BCL2, MCL-1. (f) A375 cells stably expressing BCL2 and MCL-1 were cultured under detached condition for 48 h. Flow cytometry analysis of apoptosis was determined using Annexin V-FITC/PI staining. Data are typical of three independent experiments. (A color version of this figure is available in the online journal.)

higher apoptosis rate, and double knockdown of both genes drove synergetic pro-apoptotic effect on YAP-overexpressing A549 cells (Figure 2(f)). Collectively, these data suggested YAP-mediated acquisition of anoikis resistance may attribute to upregulated expression of survival genes such as *BCL2* and *MCL-1*.

YAP inhibitor reduces anoikis resistance

CA3 is a small molecule that has potent inhibitory activity on YAP/TEAD transcriptional activity.[19] Next, we estimated the effects of YAP inhibitor CA3 on anoikis resistance of melanoma cells. As shown in Figure 3(a), anoikis-resistant A375 cells were treated with increasing concentrations CA3 (0, 1, 5, 10 μM) under detached condition for 48 h. We found that CA3 significantly induced apoptosis in a concentration-dependent manner, as analyzed by flow cytometry (Figure 3(a)). Western blot also confirmed enhanced amount of cleaved PARP along with the attenuated YAP protein level after CA3 treatment in anoikis-resistant A375 cells (Figure 3(b)), and the downregulation of YAP target genes *BCL2* and *MCL-1* (Figure 3(c)). Furthermore, we observed that 10 μM CA3 was sufficient to induce anoikis in YAP-overexpressing A375 cells (Figure 3(d). Overall, these results strongly suggest that YAP inhibitor CA3 significantly reduces anoikis resistance in melanoma cells via blocking YAP activity.

YAP knockdown reverses anoikis resistance

Further, we silenced *YAP* expression in anoikis-resistant melanoma cells using shRNA. We firstly screened three different shRNAs targeting different regions of YAP mRNA, and found all of them could suppress the expression of YAP but the shRNA3 had the best efficacy with approximately 70% silencing of YAP was achieved (Figure 4(a)). Consistently, the expression of *BCL2* and *MCL-1* genes was also decreased after YAP knockdown (Figure 4(b)). Subsequently, anoikis-resistant A375 cells stably transfected with *YAP* shRNA or non-target shRNA as controls were subjected for anoikis induction by suspension culture. As shown in Figure 4(c), *YAP* knockdown partially reversed anoikis resistance and induced more apoptotic cells (Figure 4(c)). On the other hand, *YAP* knockdown cells also displayed great reduction of cell migration ability in the wound-healing assay (Figure 4(d)).

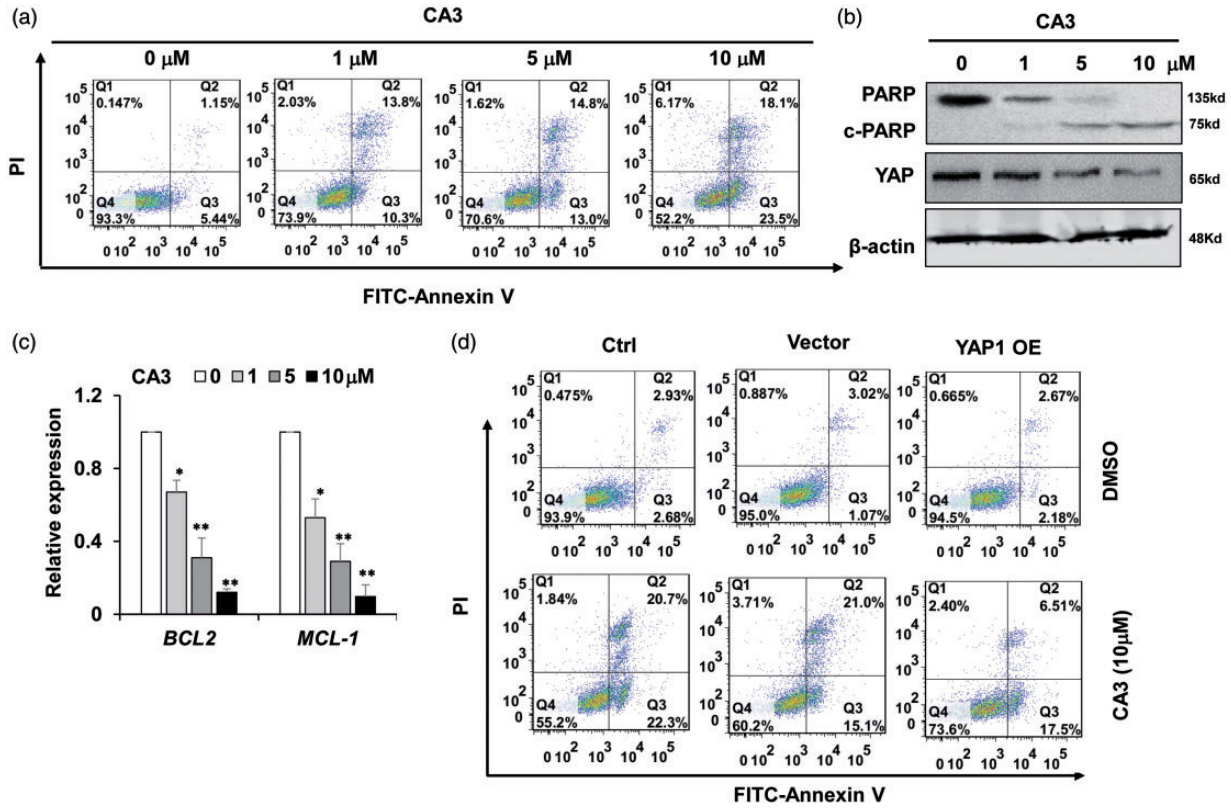

Figure 3. YAP inhibitor enhances anoikis. (a) Anoikis-resistant A375 cells were cultured in plates coated with poly-HEMA as suspension culture and treated with DMSO or various concentrations of CA3. After 48 h, cell apoptosis was analyzed by flow cytometry. (b) Immunoblotting assay was used to detect the expression of YAP, and the cleavage of PARP in CA3-treated cells. (c) qPCR to detect the YAP downstream target genes *BCL2* and *MCL-1*. GAPDH was used as the endogenous control, and data represent mean ± SD from three independent experiments, $^{**}P < 0.01$, $^{*}P < 0.05$, vs. the control. (d) YAP-overexpressing A375 cells were treated with CA3 or DMSO control under suspension condition for 48 h and then cell apoptosis was determined by flow cytometry. Representative graphs for three independent experiments show the percentage of anoikis in different treatment conditions. (A color version of this figure is available in the online journal.)

In addition, the colony formation ability of YAP1-overexpressed A375 cells was significantly stronger than the vector control one (Figure 4(e) and (f)). The results indicated that anoikis-resistant melanoma cells are prone to metastasis. Taken together, these results indicate that *YAP* knockdown not only reverses anoikis resistance but also attenuates the migratory potential in anoikis-resistant melanoma cells.

YAP knockdown inhibits tumor metastasis in vivo

To validate the modulatory role of YAP in anoikis resistance in the *in vivo* model, we performed tumor xenograft experiments and compared lung metastases of melanoma cells stably expressing non-target control (NC Ctrl) or YAP shRNA (YAP RNAi) following tail-vein injection in SCID mice. As shown in Figure 5(a), macroscopic examination of lungs showed massive metastatic nodules in mice from the anoikis-resistant cells transfected with scrambled shRNA (Figure 5(a)). *YAP* knockdown in anoikis-resistant A375 cells significantly decreased the number and size of lung metastases. Quantification analysis also confirmed a drastic reduction in total metastasis area per lung in *YAP* knockdown group compared with the control group (Figure 5(b)). In addition, the real-time PCR results also indicated that the downstream target of YAP, *BCL2*, and *MCL-1* gene expressions were all significantly impaired in the YAP interfered A375 cells-derived metastatic tissues (Figure 5(c)), also evidenced by the suppressed protein levels of BCL2 and MCL-1 (Figure 5(d)). To further confirm the correlation of YAP levels and the target gene expressions, we detected levels of these molecules in the metastatic tissues in lung and the non-metastasis tissues, and found that metastatic tissues expressed obvious higher level of YAP, BCL-2, and MCL-1than the non-metastatic ones, and higher YAP correlated with higher levels of BCL2 and MCL-1 (Figure 5(e)). Therefore, *YAP* has close correlation with the incidence and the growth of lung metastases by intravenously inoculated anoikis-resistant melanoma cells.

Discussion

There have been studies revealing the important role of YAP in multiple cancer metastasis, and only a few reports concerning the relationship between YAP and anoikis, but so far no published study is reported to reveal the relationship between YAP and anoikis in melanoma. In this study, we demonstrated the association of YAP with anoikis resistance and metastasis in melanoma. Abnormal activation of YAP contributed to cell survival under anchorage-independent conditions, and increased cell migration capability. YAP blockade promoted anoikis of melanoma cells and repressed tumor growth *in vitro* and *in vivo*.

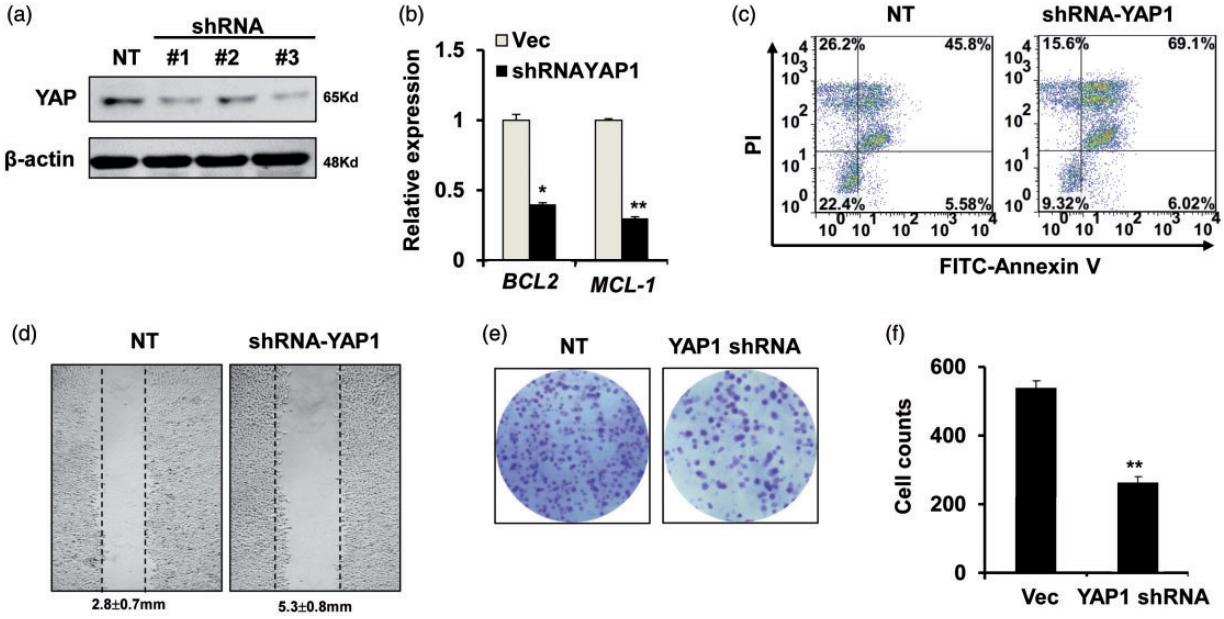

Figure 4. YAP knockdown promotes anoikis. (a) Immunoblotting assay showed the knockdown efficiency of three shRNAs targeting different regions of *YAP* mRNA. (b) The expression of YAP target genes *BCL2* and *MCL-1* in *YAP* knockdown (shRNA #3) and control groups was determined by real-time PCR. * $P < 0.05$,** $P < 0.01$, vs. the control. (c) A375 cells silencing YAP gene were subjected for suspension culture to induce anoikis. After 48 h, cell apoptosis was analyzed by flow cytometry. (d) Wound healing assays were performed on anoikis-resistant cells with *YAP* knockdown. Representative images were taken after treatment for 12 h. (e) Representative images of colony-forming assay of A375 cells forcedly expressed with YAP1 knockdown (YAP1 shRNA) or non-target shRNA (NT), and (f) is the quantification of colony formation of A375 cells with YAP1 knockdown (YAP1 shRNA) or non-target shRNA (NT). Numbers under each photo represent the mean±SD (mm) for three independent experiments with each one triplicated. (A color version of this figure is available in the online journal.)

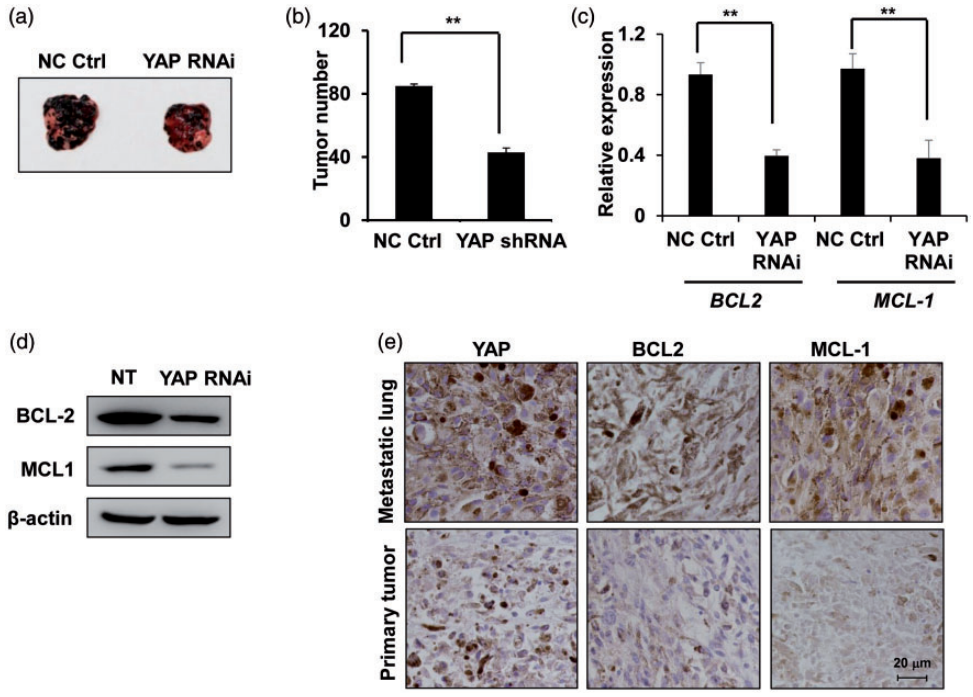

Figure 5. YAP knockdown inhibits lung metastasis of anoikis-resistant A375 cells following tail-vein injection in SCID mice. (a) Representative macroscopic images of lungs from SCID mice, 35 days after tail-vein injection of 4×10^5 A375 cell stably expressed NC Ctrl or YAP shRNA #3. The numbers of metastases were counted in whole lungs. (b) Quantification analysis of the metastasis numbers in lung tissues. (c) qPCR to detect the YAP downstream target genes *BCL2* and *MCL-1* in metastatic tissues from lung derived from A375 cell stably expressing NC Ctrl or YAP shRNA. *GAPDH* was used as the endogenous control, and data represent mean ± SD from three independent experiments, ** $P < 0.01$, * $P < 0.05$, vs. the control. ** $P < 0.01$, vs. the NC control. (d) Immunoblotting assay was used to detect BCL-2, MCL-1 in the metastatic tissues generated from A375 cells stably expressing NC Ctrl or YAP shRNA. (e) Immunohistochemistry staining showed the level of YAP, BCL-2, and MCL-1 in metastatic melanoma tissues in lung and non-metastatic tissues from patients.

Thus, our results defined YAP as a positive modulator for melanoma survival and metastasis.

YAP as well as transcription activator with PDZ binding motif (TAZ) are important effectors of Hippo signaling.[17] The Hippo pathway is composed of versatile serine/threonine kinases including LATS1/2 and MST1/2. LATS1/2 and MST1/2 kinases modulate YAP activity via phosphorylating YAP at Ser127. When the Hippo pathway is active, stimulated MST1/2 kinases phosphorylate and activate downstream kinases LATS1/2. Subsequently, LATS1/2 kinases phosphorylate and inhibit YAP. In addition, YAP is also an autophagy substrate, and autophagy inhibition retains YAP stabilization.[20] When the Hippo signaling is blocked, YAP protein becomes dephosphorylated and is delivered into the nucleus. Active YAP combines with TEA-domain (TEAD) transcription factors to constitute functional transcription complexes, thus stimulating the expression of target genes. In this way, YAP fine-tunes diverse cell events, such as cell growth, apoptosis, migration, cell–cell junction, communication, and inflammation.[21,22] The Hippo/YAP pathway possesses the unique capacity to give rise to tumorigenesis. YAP expression and nuclear localization were found to be elevated in various tumors, including prostate tumor, hepatocellular carcinoma, ovary tumor, breast tumor, colon tumor, and glioblastoma.[15] Increasing evidences have validated the oncogenic action of YAP during three main stages of tumor initiation, promotion, and progression.[23] YAP located in the nucleus as a transcriptional co-activator transactivates the expression of multiple cell proliferative genes such as *CTGF*, *FGF1*, and *BDNF*. YAP can also promote malignant proliferation and invasion of human glioma cells through classic Wnt signaling.[24] Moreover, YAP is highly abundant in hematopoietic stem cells, and participates in the expression of stemness genes such as *OCT4, NANOG*, *SOX2*, and *KLF4*.[25] YAP is also involved in the modulation of epithelial-to-mesenchymal transition (EMT), a fundamental process for tumor metastasis. YAP can also affect the expression of *E-Cadherin, N-Cadherin*, and *Vimentin* genes by itself or through the cooperation with SNAIL and TWIST and ZEB1 transcription factors, thus modifying the EMT process.[26] These compelling evidences pronounce that the Hippo/YAP pathway plays an important role in tumor growth and metastasis. In the current study, we revealed a previously not disclosed function of YAP in anoikis resistance of melanoma cells. When melanoma cells were grown under anchorage-independent condition, anoikis could be detected. However, anoikis-resistant cells also originated due to the high heterogeneity of melanoma, and we found that anoikis-resistant melanoma cells displayed higher levels of YAP activation than the cells that were attached to the basement membrane. Mechanistically, our results indicated that anoikis downregulated phosphorylated YAP at Ser127 to activate the transcriptional activity of YAP drive expressions of downstream genes *BCL2* and *MCL-1*, consequentially enhancing tumor metastasis. However, the current work did not provide direct evidence for relationship between YAP-mediated anoikis resistance and YAP-mediated metastasis. Despite of the known downstream genes promoting tumor progression like BCL2 and MCL-1, further studies are necessary to investigate the relationship.

Indeed, previous studies have indicated that YAP in melanoma can drive tumor growth and metastasis. The metastatic potential of melanoma cells is strongly correlated with the transcriptional activity of YAP-TEAD complex.[27] YAP overexpression directly enhances the invasive and metastatic capacity of melanoma cells.[28] Non-receptor tyrosine kinase (SRC) enhances YAP/TAZ activity and the expression of YAP downstream genes in human breast cancer and melanoma cells.[29] Verteporfin, an agent which disrupts YAP-TEAD interactions, can overcome BRAF inhibitor resistance and reduce tumor growth in melanoma cancer stem cells.[30] Therefore, the Hippo/YAP pathway may represent a worthy target for therapeutic intervention of melanoma. In our study, we found that YAP could promote metastasis. Our findings argues that YAP promotes melanoma metastasis may anoikis interdependent, neither by receptor-ligand interactions nor chemokine receptors, but through intrinsic gene expression. However, more work is needed to do about how YAP doing this, either by interact other signaling or direct promote anoikis resistance gene expression. Since A375 is a BRAF mutant cell line, it is necessary to expand the study to other cell lines with or without BRAF/NRAS mutant and evaluate the potential cross-talk between MAPK and Hippo signaling that could help us to better understand the real mechanism of melanoma development and progression.

Inactivation of YAP oncoprotein by the Hippo signaling can restore cell contact inhibition and inhibit tumor growth.[31] Platelets reduce anoikis in tumor cells via RhoA-MYPT1-PP1-mediated YAP dephosphorylation and its nuclear translocation.[32] Thus, we hypothesize that YAP activity is closely linked with anoikis, detachment induced cell apoptosis. Herein, according to findings in our study, we provided evidence that melanoma cells resistant to anoikis usually harbor higher YAP activity and downstream gene expression. Melanoma cells transfected with YAP plasmids exhibit high potential of anoikis resistance and migration to facilitate their seeding and invading to distant organs. Our results demonstrated increased expression of anti-apoptotic *Bcl-2* and *Mcl-1* in YAP-overexpressing melanoma cells. Thus, our results implicate that abnormal activation of YAP is an important mechanism to evade anoikis in melanoma and promote metastasis. However, the exact molecular mechanism how suspension cells acquire YAP activation is not known, and whether YAP drives migration or invasion directly or by promoting anoikis need further clarification and investigation. Deciphering the regulatory mechanisms of YAP activity under cell detachment is important for potential interception of tumor.

Furthermore, our results showed that YAP inhibitor CA3 is potent to induce anoikis in resistant melanoma cells and impair cell migration. The silence of YAP gene by shRNA also rendered melanoma cells susceptible to anoikis and interrupted cell invasiveness. Stable YAP knockdown in anoikis-resistant melanoma cells dramatically reduced lung metastases in mice following tail-vein injection. Therefore, YAP inhibition can be used as a rational

therapeutic strategy to prevent the growth and metastasis of melanoma.

AUTHORS' CONTRIBUTIONS

BZ and XC performed the research and analyzed the data; XC and XZ designed the research study; and BZ and XZ prepared the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Chenglin Liang https://orcid.org/0000-0002-8375-307X

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