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ORIGINAL ARTICLE

Blockade of integrin β3 signals to reverse the stem-like phenotype and drug resistance in melanoma

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Abstract

Purpose Cancer cells with stem-like phenotype are frequently proliferative and show high resistance to chemotherapeutic agents. Specific cell markers to identify the cancer stem cells and reverse the drugs resistance are urgent needs in clinic cancer treatment.

Methods To identify the potential role of integrin β3 in melanoma stem cells. Flow cytometry and immunofluorescence were performed to detect the expression levels of integrin β3 and integrin β3 related signal molecules. qRT-PCR and western blotting were used to detect the signaling pathways induced by integrin β3. Colony formation analysis and melanoma-bearing mice treatment by chemotherapeutic agents and integrin β3 inhibitors were used to detect the curative effects.

Results We proved that integrin β3 could serve as a marker of stem-like cancer cells in melanoma, along with the acquired chemotherapeutic drugs resistance. Furthermore, we observed that the membrane–proximal complex of integrin β3 with KRAS and Galectin-3 on the surface of melanoma cancer cells could recruit the RalB, resulting in the activation of TBK1. The phosphorylated TBK1 facilitates the activation of NF-κB signaling pathway, leading to the stem-like phenotype and drug resistance development in melanoma. Herein, the combination of cilengitide, an integrin β3 inhibitor, and chemotherapeutic agents were capable of suppressing the tumor growth and reversing the drug resistance induced by integrin β3.

Conclusion These findings identified integrin β3 as a driver of melanoma stem-like cells with drug resistance and revealed an innovative strategy in clinic melanoma treatment.

Keywords Integrin β3 · Stem-like phenotype · Drug resistance · Melanoma

Introduction

Melanoma is one of the most common malignancies with high metastasis worldwide [1]. Due to the clinical advances of early diagnoses and combination of surgical resection and adjuvant chemotherapy, many melanoma patients show positive responses [2]. Unfortunately, high percentage of the treated melanoma patients recur with increased drug resistance [3, 4]. Traditional treatment methods cannot eliminate cancer cells completely, especially those stem-like cancer cells with high drug resistance, which eventually cause tumor recurrence and progression [5, 6]. Therefore, innovative therapeutic methods to enhance melanoma treatment effects are highly desirable.

Integrin families, formed by 18 α-subunits and 8 β-subunits, are heterodimeric cell surface receptors, which could mediate the adhesion to the extracellular matrix and signal transduction [7]. Accumulating evidences indicate that the expression of integrins is associated with the initiation, progression and metastasis of various solid tumors [8]. The foundational functions of integrins are known as coordination of cell–matrix communication or regulating intracellular signaling transduction [9, 10]. Recent studies reveal that αvβ3 participates in the tumor stemness maintain in breast cancer [11]. Moreover, it has been demonstrated that the expression of αvβ1 in tumor cells is capable

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of triggering cell migration and cancer metastasis. Furthermore, Seguin et al. found that integrin β3 could regulate the tumor stemness through the KRAS and Galectin-3 complex to regulate the downstream signals in lung cancer [12, 13]. However, the specific mechanisms of integrin-associated tumor progressions in melanoma still remain unclear and efficient inhibitors to target integrins to influence tumor cell survival could be a crucial determinant in clinical melanoma cancer therapy.

In our study, we reported that the subpopulation of integrin β3-positive melanoma cells showed enhanced stem-like phenotype and high resistance to chemotherapeutic agents compared with the integrin β3-negative melanoma cells. Furthermore, our results revealed that the membrane–proximal complex of integrin β3 with KRAS and Galectin-3 on the surface of cancer cells could activate the RalB/TBK1/NF-κB signaling pathway, leading to the enhanced stem-like phenotype and drug resistance in melanoma. Concomitantly, the application of integrin β3 inhibitor could efficiently suppress the tumor growth or drug resistance development, and combination of chemotherapeutic agents and integrin β3 inhibitor might be a potential strategy in clinical melanoma treatment.

Materials and methods

Cell lines and reagents

Murine melanoma cancer cell line B16 and human melanoma cancer cell line were purchased from ATCC (USA). The D119A mutant A375 cells were purchased from KAIGEN (Wuhan, China). All cells were maintained in 1640 complete culture medium containing 10% fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The MTT assay kit was purchased from Solarbio (China). Doxorubicin (DOX) and methotrexate (MTX) were purchased from Sigma (USA). The integrin β3 inhibitor cilengitide was purchased from Absin (China). The AKT inhibitor MK-2206 and EPR inhibitor SCH772984 were purchased from Selleck (USA).

Cell viability analysis and colony formation

The cell viability was determined by MTT assay. Briefly, 5000 B16 or A375 cells were seeded into 96-well culture plates. After 12 h, cells were treated with DOX, MTX and cilengitide. After 24 h, cell growth was measured after addition of 10 μL 0.5 mg/mL MTT solution. After 4 h incubation at 37 °C, the medium was replaced with 100 μL dimethylsulfoxide and vortexed for 10 min. Absorbance (A) was measured at 570 nm by a microplate reader (Bio-Rad, USA). Each experiment was performed for at least three times. The colony formation analysis was performed as described. Briefly, 1000 B16 or A375 cells were sorted and seeded into 6-well plates. Cells were cultured with 1640 complete medium without fetal calf serum at 37 °C. After 10 days, cells were stained with crystal violet (Solarbio, Beijing, China).

RNA interference

For KRAS, NRAS Galectin-3 knockdown in tumor cells, 2×10^5 A375 cells were seeded in a six-well plate and starved in an antibiotic-free growth medium for 24 h before transfection. KRAS, NRAS or Galectin-3 siRNA (4 mL; 0.5 mg) or a mock siRNA solution was performed for 48 h according to the manufacturer's protocol. The transfection efficacy was validated by western blot. The primers were designed and constructed by Gene Pharma Company. Then the cells were sorted with flow cytometry for integrin β positive and negative cells. SiRNA-KRAS sense: 5′-GUGCAA UGAAGGGACCAGUA-3′ and 5′-GUCUCUUGGAUA UUCUCGA-3′. SiRNA-Galectin-3 sense: 5′-GAAGAAAGA CAGTCGGTTT-3′ and 5′-GCAATACAAAGCTGGATA A-3′. SiRNA-NRAS sense: 5′-CGAGAAGAGUACAGU GCCAUG-3′ and 5′-CAAGAAGAGUACAGUGCCAUG-3′.

Flow cytometry

To isolate the integrin β3-positive and negative populations in cancer cells, anti-CD61 (integrin β3) human antibody (eBioscience, USA) was added to the cell suspension. After incubating for 30 min at room temperature, samples were sorted by DAKO cytomation (USA). Isotype was stained as negative control.

Real-time PCR

2 μg cDNA were used as the template to do the quantitative real-time PCR for the detection of the target genes (SYBR™ Green Real time PCR master mixes, ThermoFisher Scientific, USA). The GAPDH was used as the internal control and three independent experiments in each sample were performed. The relative expression was quantified by normalizing the target gene level to the GAPDH by the ΔΔCt method. The primer pairs used are listed as followed: human GAPDH forward primer 5′-GGAGCG AGATCCCTCCAAAAT-3′, reverse primer 5′-GGCTGT TGTCATACTTCTCATGG-3′; human HRAS forward primer 5′-ATGACGGAATATAAGCTGGTGGT-3′ and reverse primer 5′-GGCACGTCTCCCCATCAATG-3′; human KRAS forward primer 5′-ACAGAGAGTGGAGGA TGCTTT-3′ and reverse primer 5′-TTTCACACAGCC AGGAGTCTT-3′; human NRAS forward primer 5′-ATG ACTGAGTACAAACTGGTGGT-3′ and reverse primer

5′-CATGTATTGGTCTCTCATGGCAC-3′; human RRAS forward primer 5′-GACCCCACTATTGAGGACTCC-3′ and reverse primer 5′-CGGTCGTTAATGGCGAACAC-3′.

ELISA analysis

The level of activated GTP-KRAS was detected by ELISA. The sorted cells were washed twice with PBS and incubated with the lysate. Then the supernatants were collected and stored at − 80 °C for further use. Human GET-KRAS ELISA kit (Lanji, Guangzhou, China) was used to detect the level of activated GTP-KRAS in A375 cells, according to the manufacturer's recommended protocol. Each experiment was performed in triplicate.

Western blotting

Samples were solubilized with an equal volume of loading buffer (125 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol) and were boiled for 10 min, then samples were separated by SDS-PAGE, followed by transferring to PVDF membranes and detecting by immunoblotting with primary antibodies against integrin β3 (1:500, abcam, USA), KRAS (1:500, abcam, USA), Galectin-3 (1:400, abcam, USA), pTBK1 (1:300, abcam, USA), total TBK1 (1:500, abcam, USA), RalB (1:500, abcam, USA) and NF-κB (1:500, abcam, USA), respectively, at 4 °C overnight. Then HRP-conjugated secondary antibody (1:1000, abcam, USA) was incubated for 1 h at room temperature, and visualized by ECL detection kit (CST, USA). β-actin (1:1000, abcam, USA) was used as an internal control.

Immunofluorescence

To examine the expression of NF-κB and pTBK1, integrin β3, KRAS and Galectin-3 in integrin β3-positive and negative populations, A375 cells were fixed and permeabilized. Then the cells were labeled with anti-NF-κB (1:200, abcam, USA), pTBK1 (1:200, abcam, USA), integrin β3 (1:100, abcam, USA), KRAS (1:200, abcam, USA) and Galectin-3 (1:500, abcam, USA) followed by Alexa 488 goat anti-FITC antibodies (1:800, abcam, USA) or Alexa 594 goat anti-FITC antibodies (1:800, abcam, USA). Nuclei were labeled with DAPI. To ensure the specificity of IF staining, primary antibodies were substituted with isotype-matched non-specific IgGs in the experiment. All immunofluorescent images were captured from FV1000 (Leica, Germany) laser scanning confocal microscope.

Cellular reactive oxygen species (ROS) level detection

The cellular ROS level was detected by CellROX™ Deep Red Reagent (Thermo, USA). Briefly, the sorted integrin β3 A375 cells treated with cilengitide (10 nM, 12 h) or not was collected and resuspended with PBS. Then 1 nM CellROX™ Deep Red Reagent was added into the cells suspension. After 30-min culture in incubator, the cells were washed with PBS and detected by a flow cytometry (BD, USA). Each experiment was performed in triplicate.

Macropinosome analysis

The sorted A375 cells were pre-treated with cilengitide (10 nM, 12 h) or not. Then the macropinosomes were marked by a DQ-Green BSA (Thermo, USA) at a concentration of 1 mg/mL at 37 °C after 60 min, cells were washed with cold PBS and fixed in 4% formaldehyde. Cells images were captured using an Olympus confocal microscope (Tokyo, Japan) and analyzed by ImageJ 6.0. Particle. Each experiment was performed in triplicate.

Animal protocols

Female C57 mice (6–8 weeks) and female nude mice (6–8 weeks) were purchased from Huafukang (China). To evaluate the anticancer effects of cilengitide combined with chemotherapeutic agents. 2×10^6 A375 cells or sorted β3+ A375 cells were subcutaneously injected into the nude mice (ten mice each group). When the tumor volume reached 5 mm × 5 mm, PBS, DOX (2 mg/kg), cilengitide (5 mg/kg) and cilengitide (5 mg/kg) combined with DOX (2 mg/kg) were used to treat the mice by tail intravenous injection every 3 days. Mice were treated for 2 weeks. Tumor growth was recorded with the length (L) and width (W) of tumors by vernier calipers, and the tumor volume (V) was calculated by the formula $V = (L \times W2)/2$. The data were monitored every 2 days and the survival of tumor-bearing mice was observed every day from the day 10.

Statistical analysis

All data were presented as mean ± SEM. Graph Pad Prism 6.0 was performed to analyze ($P < 0.05$). Student's t test was used to analyze the difference between two means and ANOVA studies were performed for multiple groups. Survival analysis was performed by Kaplan–Meier method and evaluated using the log-rank test. $P < 0.05$ was considered significant.

Results

Integrin β3 drives a stem-like cell phenotype and drugs resistance in melanoma

It has been reported that the integrin expression in tumor cells is linked to various tumor progression, including increased metastasis, sustained tumor growth and so on [14, 15]. To assess a potential role of αvβ3 in melanoma growth, we sorted the integrin β3-positive and negative population in B16 and A375 melanoma cells. We found that enhanced colony formation capability in integrin β3-positive population compared with the integrin β3-negative population in B16 (Fig. 1a) and A375 (Fig. 1b). We next assessed whether the integrin β3 expression could impact the ability of tumorigenicity in melanoma. In line with our colony formation result, the integrin β3-positive population of melanoma showed dramatic increase of tumorigenicity in B16 (Fig. 1c) and A375 (Fig. 1d). Furthermore, the integrin β3-positive tumor cells revealed enhanced tumor growth ability and reduced

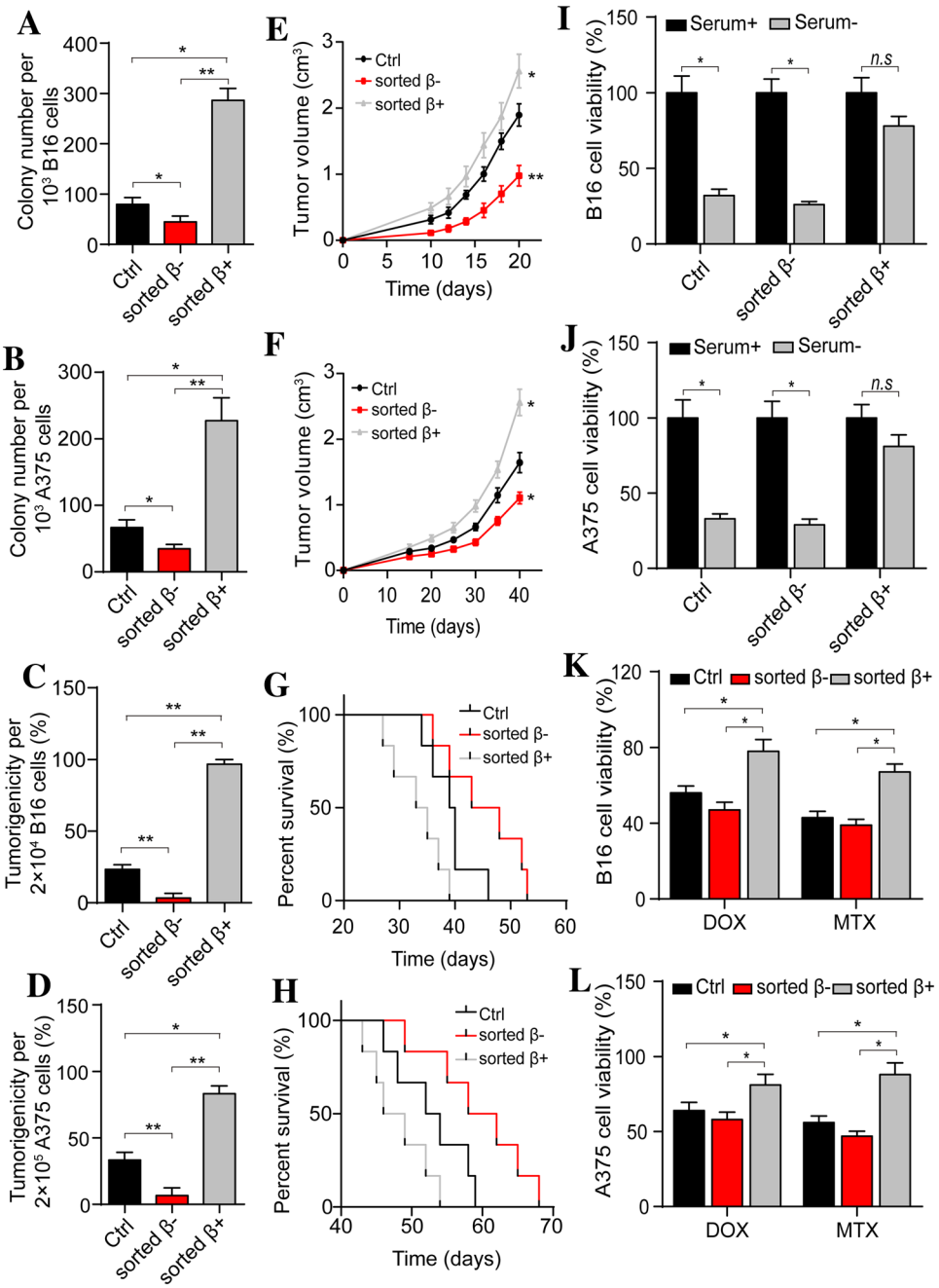

Fig. 1 Integrin β3 expression in melanoma drives a stem-like cell phenotype and drug resistance. **a** Quantification of colonies formed by the control, the β3− and the β3+ populations from B16 cells. **b** Quantification of colonies formed by the control, the β3− and the β3+ populations from A375 cells. **c** Tumorigenicity of the control, the β3− and the β3+ populations from B16 cells. **d** Tumorigenicity of the control, the β3− and the β3+ populations from A375 cells. **e** Tumor volume of mice subcutaneously injected with 2×105 control, sorted β3−, sorted β3+ B16 cells. **f** Tumor volume of mice subcutaneously injected with 2×106 control, sorted β3−, sorted β3+ A375 cells. **g** Survival rate of mice subcutaneously injected with 2×105 control, sorted β3−, sorted β3+ B16 cells. **h** Survival rate of mice subcutaneously injected with 2×106 control, sorted β3−, sorted β3+ A375 cells. **i, j** Effect of serum deprivation on sorted β3− and β3+ B16 and A375 cells. Cells were grown in 96-well plates in media containing 10% serum or 0% serum. **k, l** Cell viability of sorted β3− and β3+ B16 and A375 cells treated with DOX (1 μg/mL) or MTX (1 μg/mL) for 24 h. The data were presented as the means ± SEM from three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$; *ns* not statistically significant

survival time was observed in those mice compared with integrin β3-negative group (Fig. 1e–h). Those results indicate that the expression of integrin β3 is sufficient to induce the stem-like properties in melanoma cells.

It has been reported that stem-like cancer cells are resistant to cells stresses, including multi-drug resistance [16, 17] and nutrient deprivation [18]. Indeed, the integrin β3-positive tumor cells showed enhanced survival advantages compared to the integrin β3-negative or control cells (Fig. 1i, j). Accordingly, we observed that the integrin β3-positive cancer cells show enhanced resistance to the clinical chemotherapeutic agents such as MTX and DOX (Fig. 1k, l). Together, these results indicate that the integrinβ3 expression in melanoma cells drive a stem-like phenotype and facilitate the resistance to chemotherapeutic agents.

Integrin β3 induces the stem-like phenotype and drug resistance by the activation of KRAS and Galectin-3

The RAS family is known to be necessary for the signal transmission of integrins [19, 20]. Herein, we investigated the expression of four RAS family members in mRNA level. The significantly increased expression of KRAS was observed in integrin β3-positive A375 cells compared to the integrin β3-negative group while the HRAS, NRAS and RRAS show no differences in these cells (Fig. 2a). Notably, the ability of colony formation and tumorigenicity was abolished when silence KRAS by siRNA (Fig. 2b, c). Accordingly, silencing KRAS also reversed the drug resistance in integrin melanoma β3-positive cells (Fig. 2d, e), indicating that integrin β3 and KRAS might cooperate to induce the stem-like phenotype and drug resistance in melanoma cells. Previous evidence indicates that NRAS could activate the KRAS and facilitate the KRAS expression in several tumors. To further investigate the role of NRAS in KRAS up-regulation in melanoma, we used siRNA to silence the NRAS in A375 and then sorted the integrin β3-positive cells. However, silence of NRAS did not induce the alteration of KRAS expression, indicating that the KRAS expression in integrin β3-positive melanoma cells was NRAS independent (Fig. S1A). Additionally, we also analyze the activated KRAS level of the integrin β3-positive A375 cells. We found that the increased level of GTP binding KRAS in integrin β3-positive A375 cells compared to the integrin β3-negative A375 cells (Fig. S1B), indicating that the integrin β3-positive melanoma cells also reveal enhanced activated KRAS level.

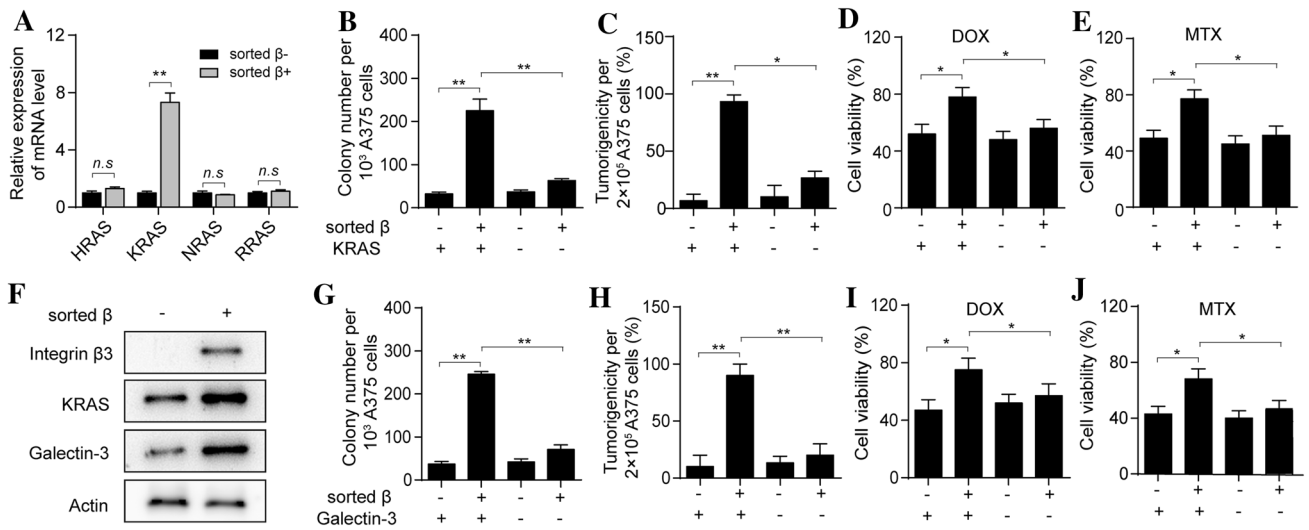

Fig. 2 Activation of integrin β3/Galectin-3/KRAS results in the stem-like phenotype and drug resistance. **a** The mRNA expression of HRAS, KRAS, NRAS and RRAS in the β3− and the β3+ populations from A375 cells. **b** Quantification of colonies formed by the β+/KRSA+, β+/KRAS−, β−/KRAS+ and β−/KRAS− populations from A375. **c** Tumorigenicity of the β+/KRSA+, β+/KRAS−, β−/KRAS+ and β−/KRAS− populations from A375 (2×105). **d** Cell viability of the β+/KRSA+, β+/KRAS−, β−/KRAS+ and β−/KRAS− populations from A375 treated with DOX (1 μg/mL) for 24 h. **e** Cell viability of the β+/KRSA+, β+/KRAS−, β−/KRAS+ and β−/KRAS− populations from A375 treated with MTX (1 μg/mL) for 24 h. **f** Western blotting of integrin β3, KRAS, Galectin-3 and actin of sorted β3− and the β3+ populations from A375 cells. **g** Quantification of colonies formed by the β+/Galectin-3+, β+/Galectin-3−, β−/Galectin-3+ and β−/Galectin-3− populations from A375. **h** Tumorigenicity of the β+/Galectin-3+, β+/Galectin-3−, β−/Galectin-3+ and β−/Galectin-3− populations from A375 (2×105). **i** Cell viability of the β+/Galectin-3+, β+/Galectin-3−, β−/Galectin-3+ and β−/Galectin-3− populations from A375 treated with DOX (1 μg/mL) for 24 h. **j** Cell viability of the β+/Galectin-3+, β+/Galectin-3−, β−/Galectin-3+ and β−/Galectin-3− populations from A375 treated with MTX (1 μg/mL) for 24 h. The data were presented as the means ± SEM from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *ns* not statistically significant

Galectin-3, one of the β-galactoside lectin proteins family members, has been reported to interact with integrin and RAS family [21]. Herein, we supposed that the Galectin-3 might facilitate the interaction between integrin β3 and KRAS, resulting in the stem-like phenotype and drug resistance. In our study, we found the up-regulation of KRAS and Galectin-3 in integrin β3-positive cells compared to the integrin β3-negative group (Fig. 2f). Furthermore, we also observed the co-location of integrin β3 with KRAS and Galectin-3 (Fig S1C and D), indicating that integrin β3, KRAS and Galectin-3 form complex to regulate the biological activities in integrin β3-positive melanoma cells. Moreover, knockdown of Galectin-3 prevented the colony formation of integrin β3-positive A375 cells and reduced the tumorigenicity in nude mice (Fig. 2g, h). Accordingly, the knockdown of Galectin-3 reversed the resistance to chemotherapeutic agents in integrin β3-positive A375 cells (Fig. 2i, j). To further investigate whether the integrin β3/KRAS/Galectin-3 complex formation is independent of integrin β3 RGD binding domain or not, we used integrin D119A mutant A375 cells to detect the colony formation ability and drug resistance. In our studies, we also observed the enhanced colony formation ability and drug resistance in integrin β3-positive D119A mutant A375 cells. Furthermore, the addition of integrin β3 inhibitor cilengitide could efficiently reverse the phenotype, indicating that integrin β3/KRAS/Galectin-3 complex formation is independent of the RGD binding domain. Together, those results reminded that integrin β3/KRAS/Galectin-3 complex could induce the stemness and drug resistance in melanoma.

Integrin β3 activates the RalB/TBK1/NF-κB signaling pathway to induce stem-like phenotype and drug resistance

Seguin et al. has demonstrated that integrin β3, KRAS and Galectin-3 complex could induced tumor cells ROS inhibition and micropinocytosis promotion to facilitate tumor growth [12]. Here, we also found that the integrin β3-positive cells revealed enhanced micropinocytosis (Fig. S3A) and reduced ROS level (Fig. S3B), demonstrated that the integrin β3 expression enables tumor cells enhanced capability of nutrition intake and self-regulation. Next, we wondered to figure out the downstream of integrin β3 to induce the stemness and drugs resistance. Downstream effectors, such as JAK/STAT3 and AKT/PIK3, are necessary for the cellular functional changes induced by the membrane receptors [22, 23]. AKT or ERK, serving as the downstream of KRAS, has been reported to participate in various tumor progressions. Thereby, we used AKT and ERK inhibitors, MK-2206 and SCH772984, to treat the integrin β3-positive A375 cells. however, blockade of AKT or ERP could inhibit the colony formation of those melanoma cells (Fig. S3C), but failed to reverse the drug resistance induced by the integrin β3 (Fig. S3D), reminding us that AKT and ERK might participate in the melanoma stemness regulation through other signaling pathways and other signals are involved in the integrin β3 pathway. NF-κB, a transcription factor protein, which is reported be associated with cell stemness maintain and drug resistance development in cancer cells [24, 25]. In our study, we found the up-regulation of NF-κB (c-rel) in integrin β3-positive A375 cells (Fig. 3a), indicating the activation of NF-κB signaling pathway. Furthermore, the application of SN50, a NF-κB inhibitor [26], efficiently suppressed the colony formation and the tumorigenicity of integrin β3-positive A375 cells (Fig. 3b, c). Besides, the drug resistance of the integrin β3-positive A375 cells was reversed in presence of the NF-κB inhibitor (Fig. 3d, e), suggesting that the integrin β3 drives the stem-like phenotype and drug resistance via the activation of NF-κB signaling pathway.

It has been reported that NF-κB (c-rel) activation is induced by the RalB/TBK1 signaling pathway [13, 27]. And the enhanced expression of phosphorylated TBK1 was observed in integrin β3-positive A375 cells compared with the integrin β3-negative group (Fig. 3f). In addition, the up-regulation of integrin β3/RalB/pTBK1/NF-κB was found in β3-positive A375 cells by western blotting (Fig. 3g). Together with these results, we concluded that the integrin β3 activates the RalB/TBK1/NF-κB signaling to regulate the melanoma cells stem-like phenotype and drug resistance.

Blockade of integrin β3 reverses the stem-like phenotype and drug resistance to enhance anticancer effects

Considering the drug resistance induced by the integrin β3 in melanoma, we postulated the combination of integrin β3 inhibitor and chemotherapeutic agents might serve as potential strategy in clinical melanoma therapy. Here, we used cilengitide, an integrin β3 and β5 inhibitor [28], to treat the integrin β3-positive A375 cells and found that cilengitide could efficiently inhibit the ability of colony formation (Fig. 4a) and tumorigenicity (Fig. 4b) of integrin β3-positive cells. Moreover, addition of cilengitide facilitates the cytotoxicity of DOX and MTX to integrin β3-positive A375 cells (Fig. 4c). Those results indicate that blockade of integrin β3 could effectively reverse the stem-like phenotype and drug resistance in melanoma.

To further evaluate the integrin β3 inducing drug resistance in vivo, we generated a xenograft mouse model using A375 cells injected subcutaneously and treated with PBS, cilengitide, DOX and cilengitide combined with DOX. Tumor volume data showed single DOX or cilengitide treatment slightly inhibited tumor growth, while the combination significantly reduced the tumor volume compared with the

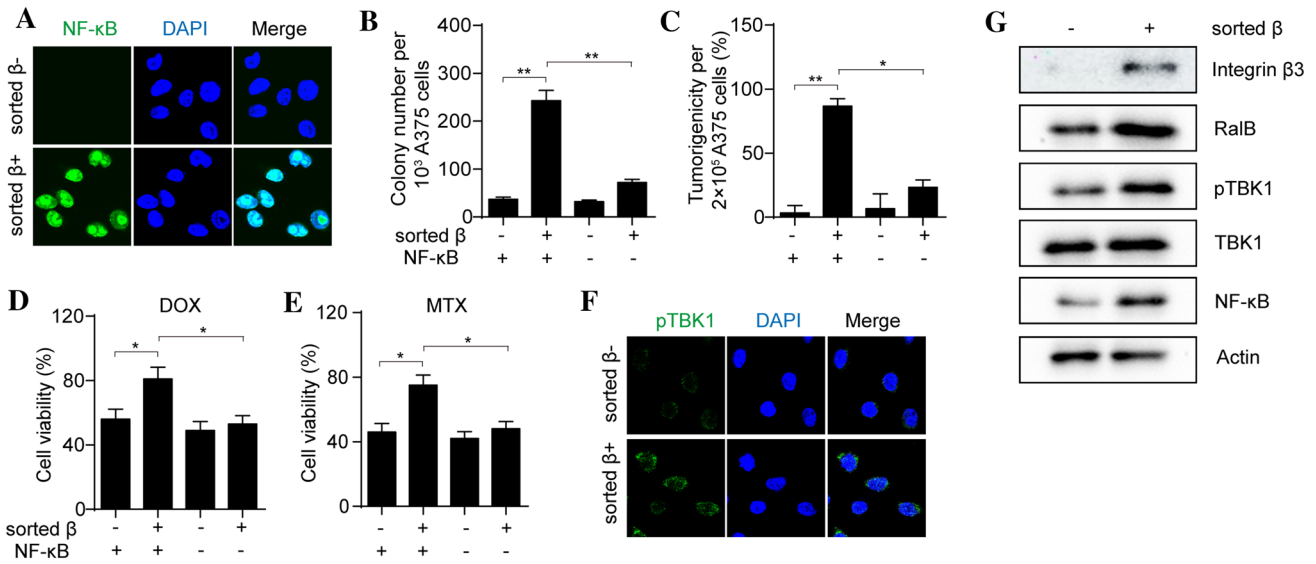

Fig. 3 Integrin β3 induces stem-like cell phenotype and drug resistance through the RabB/TBK1/NF-κB signaling pathway activation. **a** Immunofluorescence of NF-κB in sorted β3− and the β3+ populations from A375 cells. **b** Quantification of colonies formed by the β+/NF-κB+, β+/NF-κB-, β−/NF-κB+ and β−/NF-κB− populations from A375. **c** Tumorigenicity of the β+/NF-κB+, β+/NF-κB-, β−/NF-κB+ and β−/NF-κB− populations from A375. **d** Cell viability of the β+/NF-κB+, β+/NF-κB-, β−/NF-κB+ and β−/NF-κB− populations from A375 treated with DOX (1 μg/mL) for 24 h. **e** Cell viability of the β+/NF-κB+, β+/NF-κB-, β−/NF-κB+ and β−/NF-κB− populations from A375 treated with MTX (1 μg/mL) for 24 h. **f** Immunofluorescence of pTBK1 in sorted β3− and the β3+ populations from A375 cells. **g** Western blotting of integrin β3, RalB, pTBK1, TBK1, NF-κB and actin in sorted β3− and the β3+ populations from A375 cells. The data was presented as the means ± SEM from three independent experiments. $*p<0.05$; $**p<0.01$; $***p<0.001$; *ns* not statistically significant

PBS treatment (Fig. 4d). In line with the data, combining treatment significantly extended the mice life compared with the single DOX or cilengitide (Fig. 4e). We also generated a integrin β3 inducing drug resistance xenograft mouse model using integrin β3-positive A375 injected subcutaneously and treated with PBS, cilengitide, DOX and cilengitide combined with DOX. Importantly, the single DOX treatment could not inhibit the tumor growth or prolong the survival time of integrin β3-positive A375 tumor-bearing mice, while cilengitde or cliengitide combined with DOX groups show enhanced anticancer effects (Fig. 4f, g). Overall, these results suggest the therapeutic potency of chemotherapy combined with integrin β3 inhibitor in clinical melanoma treatment.

Discussion

Cancer stem-like cells are associated with various tumor progressions, including tumorigenicity, metastasis and drugs resistance development [16, 29]. It has been demonstrated that several cell surface markers, such as CD133, CD24 and CD44, are determined as the cancer stem cells markers in particular cancers [30, 31]. However, the expression of those cell surface markers does not necessarily or sufficiently correspond to the stem-like phenotypes [32]. Moreover, the underlying mechanisms of the stem-like phenotypes induced by the cell surface markers still remain unclear [33]. Herein, we identified integrin β3 as the membrane surface receptor to facilitate stem-like phenotype and drug resistance development in melanoma cells. Notably, the combination of integrin β3 inhibitor and chemotherapeutic agents reveals potential application in melanoma therapy.

Recent reports have indicated that integrin family serve as the cancer stem cells markers and facilitate the tumor progressions and drug resistance development in a wide range of tumors [34, 35]. Our study further demonstrated the stem-like phenotype and drug resistance in melanoma were resulted by the integrin β3, which discloses a more fundamental role of integrin in tumor development and cancer cell remodeling. We reported that the β3/Galectin-3/KRAS complex might result in a stem-like phenotype and chemotherapeutic drugs resistance by recruitment and activation of RalB, which is capable of driving the TBK1 to activate the NF-κB signaling pathway. Those results are in line with the previous reports of Seguin et al. in lung cancer cells [13]. However, we further expounded that the expression upregulation of KRAS in integrin β3-positive melanoma cells and described the signaling pathway in melanoma. Those results imply the role of RAS family and indicate that TKB1 are crucial in the pro-survival signaling pathway activation in melanoma, moreover, highlight the potency of blocking

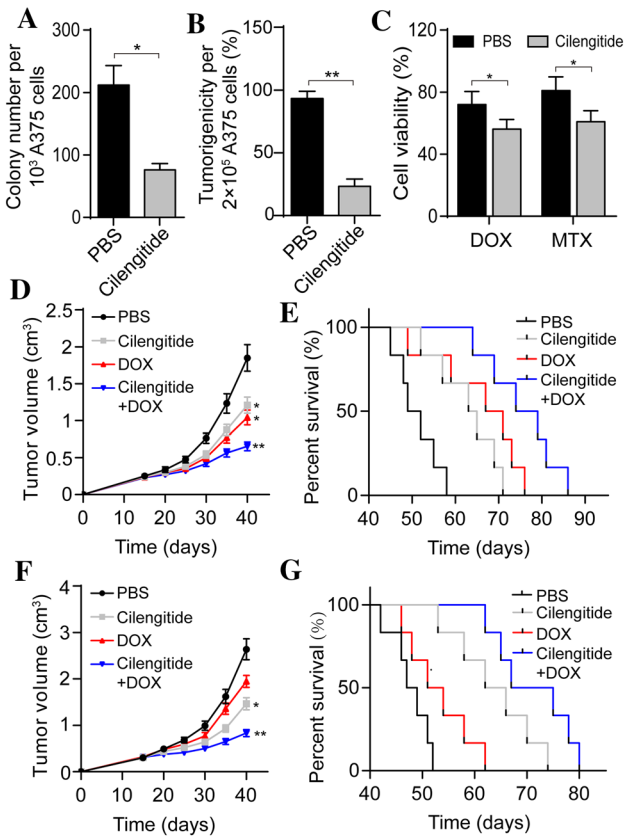

Fig. 4 Blockade of integrin β3 suppresses the tumor growth and relives the drug resistance in melanoma. **a** Quantification of colonies formed by the β3+ population from A375 cells treated with PBS and cilengitide (10 nM). **b** Tumorigenicity of the β3+ population from A375 cells pre-treated with PBS and cilengitide (10 nM) for 24 h. **c** Cell viability of sorted β3+ A375 cells treated with DOX (1 μg/mL), DOX (1 μg/mL) combined with cilengitide (10 nM), MTX (0.5 μg/mL) and MTX (0.5 μg/mL) combined with cilengitide (10 nM) for 24 h. **d** Tumor volume of mice subcutaneously injected with 2×106 A375 cells treated with PBS, cilengitide, DOX and DOX combined with cilengitide. **e** Survival rate of mice subcutaneously injected with 2×106 A375 cells treated with PBS, cilengitide, DOX and DOX combined with cilengitide. **f** Tumor volume of mice subcutaneously injected with 2×106 sorted β3+ A375 cells treated with PBS, cilengitide, DOX and DOX combined with cilengitide. **g** Survival rate of mice subcutaneously injected with 2×106 sorted β3+ A375 cells treated with PBS, cilengitide, DOX and DOX combined with cilengitide. The data was presented as the means ± SEM from three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$; *ns* not statistically significant

RAS or TBK1 could disrupt the pro-survival pathways in melanoma.

In previous Seguin et al. studies [12, 13], the NF-kB inhibitor was applied for the lung cancer treatment. However, the NF-kB signal plays crucial roles in biological development and functional stem cells. Blockade of NF-kB signal is prone to influence the normal cells functions and cause potential systemic toxicity. In our study, according to the crucial role of integrin β3 in stem-like phenotype and drug resistance development, targeting integrin β3 to selectively kill β3-positive tumor cells might efficiently suppress the tumor growth and reverse the drug resistance in melanoma [36]. However, the underlying mechanisms that integrins induced tumor progressions still remain unclear [11]. Previous reports have demonstrated that the integrin β3 expression in tumor cells is associated with various factors, such as extracellular matrix or hypoxia in microenvironment [37, 38], which might serve as the potential targets in tumor treatments. Also, it should be valid to target the upstream transcription factors of integrin β3, such as FOSL149, to prevent the stem-like phenotype and reverse the drug resistance. Here, we reported the chemotherapeutic agents combined with the integrin β3 inhibitor cilengitide to suppress the melanoma, which eradicated the stem-like phenotype and reversed the drug resistance in melanoma cells. However, A randomized phase II study of cilengitide to metastatic melanoma patients revealed that cilengitide failed to provide benefit in those naive or chemo-resistant melanoma patients and the mechanism of the cilengitide for melanoma is still unclear [39]. Our study indicated that integrin β3 could induce stem-like phenotype and drug resistance in melanoma cells. However, blockade of integrin β3 by cilengitide could reverse the drug resistance and stem-like phenotype instead of killing tumor cells directly. Moreover, our animal experiments also indicated that single cilengitide administration could not efficiently suppress the tumor growth compared to other groups (Fig. 4d, f). Those results might explain the failure of cilengitide in the phase II clinical trial and reminded us that the combination of cilengitide and chemotherapeutic agents is necessary to improve the curative effects in melanoma therapy.

In conclusion, we define the integrin β3 as the driver of cancer stem-like phenotype and drug resistance in melanoma. The integrin β3 could contract to Galectin-3 and KRAS to form the integrin β3/Galectin-3/KRAS complex to recruit RalB. Then the RalB result in the activation of pro-survival NF-κB signaling pathways via the induction of TBK1. The combination of integrin β3 and chemotherapeutic agents reveals a promising and feasible strategy in melanoma therapy.

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Compliance with ethical standards

Conflict of interest Author Yi Tang has received research grants from Health and Family Planning Commission of Zhejiang Province in China. Author Xiaoxia Zhu declares that he has no conflict of interest. Author Xiaohua Tao declares that he has no conflict of interest. Author

Wei Lu declares that she has no conflict of interest. Author Yang Ding declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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