Received: 3 May 2018 | Revised: 24 August 2018 | Accepted: 26 August 2018

DOI: 10.1111/cas.13786

ORIGINAL ARTICLE

WILEY Cancer Science

ROCK inhibitor enhances the growth and migration of BRAF-mutant skin melanoma cells

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81572681

Rho-associated protein kinase (ROCK) plays crucial roles in the proliferation and migration of different types of cells. ROCK inhibitor Y-27632 was previously reported to inhibit melanoma cell growth, and ROCK signaling was suggested to be a therapeutic target for treating melanoma. However, the negative effect of Y-27632 on melanoma cells was mainly seen in studies on murine B16 melanoma cells. Here, we reported that ROCK inhibitor actually promoted human melanoma cell growth and migration in vitro. Y-27632 increased the growth and migration of BRAF-mutated melanoma cells but had a negative effect on wild-type melanoma cells or primary melanocytes. We discovered that Y-27632 enhanced the growth of BRAF-mutated melanoma cells through increased ATK and ERK activity. The in vivo study further confirmed the in vitro finding. These data suggested that the effect of ROCK inhibitor on melanoma cells is cell-context dependent, and the application of ROCK inhibitor in the treatment of melanoma requires further study.

KEYWORDS
AKT, BRAF, ERK, melanoma, ROCK inhibitor

1 | INTRODUCTION

Rho-associated protein kinase (ROCK), a serine-threonine kinase belonging to the AGC (PKA/PKG/PKC) family, regulates cell shape and migration by acting on the cytoskeleton; it also plays an important role in modulating cell survival, proliferation and apoptosis through multiple downstream targets.[1,2] The 2 known mammalian ROCK isoforms, ROCK1 and ROCK2, are 62% homologous overall and 92% homologous in their kinase domain.[1,2] Because ROCK affects multiple biological processes, ROCK inhibitors have been tested for clinical effects, including on cancer.[3] The ROCK inhibitor Fasudil was approved for clinical treatment of cerebral vasospasm in Japan and China.[1] Y-27632, another well-established ROCK inhibitor, has been widely used to study the biological and pharmacological functions of ROCK in vitro and in vivo.[1-3] A majority of studies have shown that ROCK functions as a positive regulator in tumor cell growth, migration, metastasis and survival.[1] For instance, Y-27632 decreased breast cancer cell invasion/migration in vitro and metastasis in vivo[4]; Y-27632 was reported to reduce invasive potential of ovarian cancer[5,6] and colon cancer cells[7]; Y-27632-was shown to suppress progression of hepatocellular carcinoma through negative regulation of its proliferation and migration.[8] In contrast to these beneficial findings, other studies have shown that ROCK inhibitors can stimulate tumor functions. Y-27632 was reported to increase migration and invasion of MCF-7 breast cancer cells, to enhance the migration of SW480 colon cancer cells and to promote the proliferation of both colon and pancreatic cancer cells.[9-12] Taken together, the biological consequence of ROCK inhibition has been shown to

depend on cell type, cell-context and the involved microenvironments.

Melanoma is the most dangerous and aggressive skin cancer.[13] Finding a cure for metastatic melanoma remains a challenge, although recent immunotherapeutic applications appear promising.[13-15] The mutation of BRAF gene ($BRAF^{V600E}$), a major growth driver, occurs in 65% of aggressive melanomas.[16] The effect of ROCK inhibitors on melanoma formation has not been studied in detail. Whatever we know about the ROCK inhibitor effect on melanoma was learned from studies of mouse B16 melanoma cells; these studies suggest that the inhibition of ROCK by Y-27632 could block melanoma cell proliferation and migration in vitro and in vivo[17-20]; however, the underlying molecular mechanism remains unclarified. Therefore, we investigated the effect of Y-27632 on human melanoma cells. Interestingly, we found that Y-27632 actually enhances both human melanoma cell growth and migration in vitro and in vivo, rather than blocking it, as previously reported. We found that the enhancement of Y-27632 depends on the mutation status of the BRAF gene of melanoma through increased AKT activity.

2 | MATERIALS AND METHODS

2.1 | Melanoma cell culture and drug treatments

All melanoma cells and primary melanocytes (HEM) were cultured in DMEM supplemented with 10% FBS and .1% penicillin/streptomycin. Y-27632 (Sigma Chem Co., St. Louis, MO, USA) was dissolved in the distilled H_2O to make 10 mmol/L of stock solution; cells were treated with 10 μmol/L Y-27632.

2.2 | Cell proliferation assay

Melanoma cells were seeded in 96-well plates at a density of 7.5×10^4 cells/mL treated with or without Y-27632 and collected at the indicated time. The proliferation was determined using a cell counting kit-8 (CCK8) as previously described.[21]

2.3 | Wound healing and migration assay

In vitro wound healing was studied as previously described.[22] In brief, melanoma cells were seeded onto 6-well plates, grown to 100% confluence, wounded with a sterile pipette tip to remove cells by linear scratches, and treated with PBS, Y-27632 or Fasudil. Wound closure was digitally photographed immediately following the scratch and 24 hours later. The percentage of wound healing was reported as the healed area width divided by the total wounded area width.

For the effect of Y-27632 on melanoma cell migration, 1×10^5 cells in 200-μL DMEM containing .1% FCS were cultured in the upper chamber. The lower wells were supplemented in 500-μL DMEM containing 10% FBS. The chambers were incubated for 24 hours at 37°C. After the removal of non-migrated cells on top of the filter, cells that had migrated through the membrane were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), washed, and then stained with .1% crystal violet (Sigma-Aldrich). The number of cells that had migrated into the lower chamber was counted in 6 randomly selected high-power microscopic fields.

2.4 | ROCK activity assay

The activity of ROCK was measured by using an ELISA kit (Cell Biolabs, STA-416, San Diego, CA, USA) and followed the manufacturer's instructions. Briefly, melanoma cells with different conditions as described in the figure legends were lysed and the cell lysates were placed in MYPT-1-precoated wells and incubated for 1 hour at 30°C and anti-Phospho-MYPT-1 (Thr696) antibody was added for another 1 hour. Then, the HRP-conjugated secondary antibody was added into the solution. After another 1-hour incubation, the plates were washed, and the substrate solution was added for incubation of 10 minutes. Absorbance at 450 nm reflected the relative amount of ROCK activity in the sample, which was calculated relative to the total protein content of each sample.

2.5 | Western blot analysis

The Western blots were carried out according to the standard protocol. The following primary antibodies were used: phospho-Akt (Thr308) rabbit mAb (13038), phospho-Akt rabbit mAb (Ser 473) (4060), anti-Akt (pan) rabbit mAb (4691), phospho-P70s6k rabbit mAb (9205), P70s6k rabbit mAb (2708), phospho-4ebp1 (#13396, monoclonal), anti-4ebp1 (#9644), anti-phospho-B-Raf (#2696, polyclonal), anti-B-Raf (#9433, monoclonal), anti-phospho-Erk (#4370, monoclonal), anti-Erk (#4695, monoclonal) (all from Cell Signaling Technology, Beverly, MA, USA). The band density was quantified using ImageJ software (NIH, Rockville, MD, USA).

2.6 | siRNA transfection

Melanoma cells were seeded onto 6-well plates, grown to 50% confluence, transfected with siRNA ROCK1 or siRNA ROCK2 or double siRNA ROCK1/ROCK2 using lipofectamine 3000 (Life Technology, Carlsbad, CA, USA), and the control cells were transfected with scrambled siRNA. At 72 hours, the cells were collected for RT-PCR analysis. The oligo sequences of siRNA are as follows:

ROCK1-3354: Sense 5′-GCAGAUGAAACAGGAAAUATT-3′, Antisense UAUUUCCUGUUUCAUCUGCTT,
ROCK1-2751: Sense CCAGCUGCAAGCUAUAUUATT, Antisense UAAUAUAGCUUGCAGCUGGTT
ROCK1-3843: Sense GGCAGAGGAAGAAUAUAAATT, Antisense UUUAUAUUCUUCCUCUGCCTT
ROCK2-2226: Sense GCAGCUGGAAUCUAACAAUTT, Antisense AUUGUUAGAUUCCAGCUGCTT
ROCK2-1669: Sense CUGCCUUUCAUCGGAUUUATT, Antisense UAAAUCCGAUGAAAGGCAGTT

ROCK2-2583: Sense GGCACGACUAGCAGAUAAATT, Antisense UUUAUCUGCUAGUCGUGCCTT

2.7 | Real time RT-PCR analysis

Total RNA was extracted from melanoma cells using TRIzol (Ambion: Austen, TX, USA, Life Technologies). The PCR were performed with Takara SYBRR Premix Ex TaqTM II (Takara Bio, Kusatsu, Shiga, Japan) with LightCycler 480 II (Roche Diagnostics Mannheim, Germany). The sequences of the primers were as follows: ROCK1: forward, 5′-TGCCTTCCTTACTGACAGGG-3′, reverse, 5′-CCAAGCC CACTGGTCATTT-3′; ROCK2: forward, 5′-GCACAGTTTGAGAAG-CAGCT-3′, reverse, 5′-TACCACGCTTGACAGGTTCT-3′; 36B4: forward, 5′-GTGTAGGGGTCAAAGCACGA-3′, reverse, 5′-GCAATGTTG CCAGTGTCTGT-3′.

2.8 | In vivo tumor formation

Eight-week-old nude/nude mice (Charles River Laboratories, Wilmington, MA, USA) were randomly assigned to the control group (PBS only; N = 3 mice) or drug treatment groups (Y-27632 in PBS; N = 3 mice). One million UACC257 cells were injected subcutaneously into the backside skin of mice as described previously[23], and each mouse received 6 injections. Then, Y-27632 (10 mg/kg) was intraperitoneally injected, and administrated 3 times a week for 2 weeks. At 3 weeks after grafting, mice were killed, and the resultant tumors were collected and weighed.

2.9 | Ethics statement

The animal study was approved by the ethics committee of Stomatological Hospital Shandong University, (Protocol No. 2015120402, Date: 12-05-2015). The animal procedures were performed in this study in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3 | RESULTS

3.1 | ROCK inhibitor promoted melanoma cell growth and migration

To test the effect of Y-27632 on melanoma cell growth, we treated melanoma cell line UACC257 with 10 μmol/L Y-27632. In contrast to the previous report, we found that Y-27632 did not inhibit UACC257 growth but enhanced it, as measured by the CCK8 proliferation assay (Figure 1A). Because this result was inconsistent with the previous reports, we tested another human melanoma cell line, UACC62, in the same way; once again, we found the same result (Figure 1B). Next, in an in vitro wound healing assay, we found that Y-27632-treated cells, both UACC257 and UACC62, closed the wounds significantly faster than the control cells (Figure 1C-E). This result was further confirmed by transwell migration assay, which showed that Y-27632 could clearly promote melanoma cell migration (Figure 1F). Taken together, these data suggest that Y-27632 enhances human UACC257 and U62 melanoma cell growth and migration.

3.2 | Knockdown of ROCK1/2 promoted melanoma cell growth and migration

Y-27632 inhibits ROCK activity by targeting the ATP-dependent kinase domain of both isoforms ROCK1 and ROCK2, and we confirmed that Y-27632 could significantly reduce ROCK activity in UACC257 cells by ELISA (Figure 2A). To further investigate whether the above effects on melanoma cells by Y-27632 were through the inhibition of ROCK, we blocked ROCK1 and ROCK2 expression by siRNA of ROCK1/ROCK2. Figure 2B shows the efficient knockdown of ROCK1/ROCK2 expression by siRNA, and the decreased ROCK activity in the cells with knockdown of ROCK is shown in Figure 2C. The proliferation assay ROCK showed that the downregulation of either ROCK1 or ROCK2 or both ROCK1/ROCK2 promotes melanoma cell growth (Figure 2D). Moreover, in vitro scratch assay showed that reducing ROCK expression, especially double knockdown of ROCK1 and ROCK2, significantly enhanced melanoma cell wound healing (Figure 2E). These data suggested that the knockdown of ROCK recapitulates the effect induced by Y-27632 on melanoma cells, indicating that Y-27632 promotes melanoma cell growth and migration by blocking the ROCK pathway. To establish that the effect was not unique to Y-27632, we treated UACC257 cells with another ROCK inhibitor, Fasudil, and we found that Fasudil also enhances melanoma cell growth and migration, as for Y-27632 (Figure 2F-G). This result further confirmed that ROCK inhibitor could enhance both UACC257 and UACC62 cell growth and migration.

3.3 | ROCK inhibitor promotes BRAF-mutant melanoma cell growth and migration

The effect of Y-27632 on melanoma cell growth and migration was inconsistent with a previous report which showed that Y-27632 inhibited the proliferation and migration of B16F1 mouse cells, a widely used mouse melanoma cell line.[18] Therefore, we checked whether Y-27632 produced the same effect on B16F1 cells in our culture system. We treated B16F1 with 10 μmol/L of Y-27632 and found that our result agreed with the previous finding: The treatment of Y-27632, indeed, blocked B16F1 cell growth (Figure 3A) and migration (Figure 3B). This result suggested that the effect of ROCK inhibitor is likely cell-specific. Recognizing the importance of BRAF mutation to melanoma formation, we noticed that both UACC257 and UACC62 cells carry BRAF mutations ($BRAF^{V600E}$), while B16F1 cells do not. Therefore, we hypothesized that the effect of Y-27632 on melanoma cells could depend on the mutation status of the BRAF gene. To test this hypothesis, first, we tested a human melanoma cell line, MeWo, which does not have a BRAF mutation, and found that Y-27632 inhibited MeWo cell migration (Figure 3C). Second, we found that Y-27632 inhibited the growth of normal

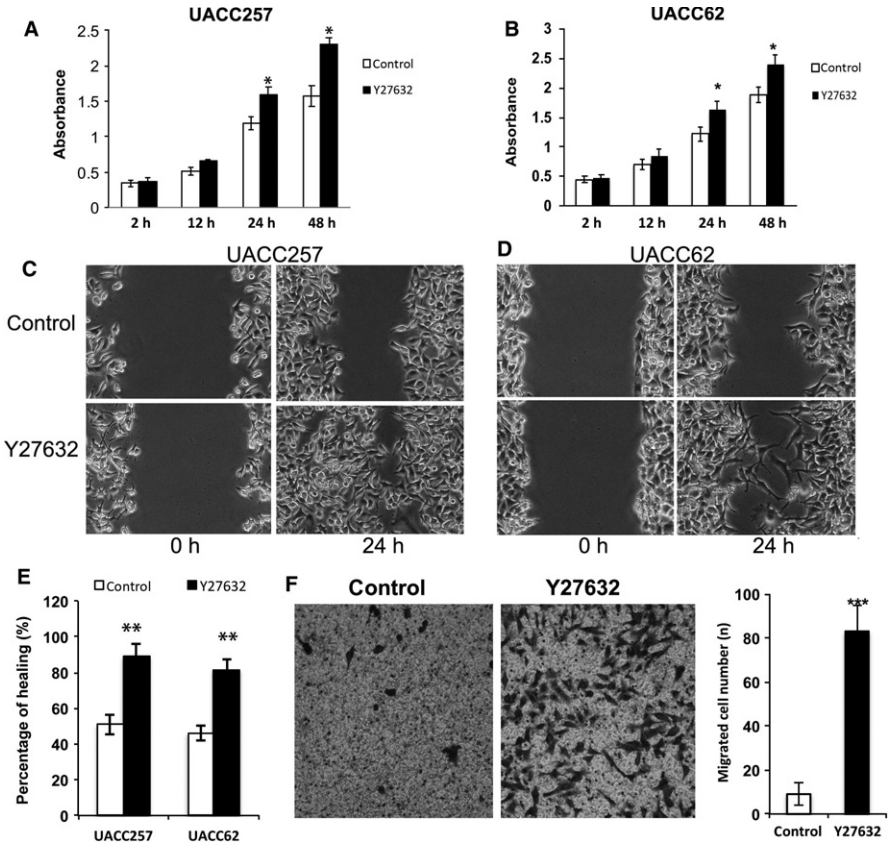


FIGURE 1 Y-27632 positively regulates the proliferation and migration of human melanoma cells. A-B, Either UACC257 (A) or UACC62 (B) melanoma cells were treated with 10 μmol/L Y-27632, with the untreated cells as control. At different time points as indicated in the figure, cells were analyzed for the proliferation using a cell counting kit (CCK 8). *Indicates $P < .05$ when comparing Y-27632-treated cells with the control group; C-D, Both confluent UACC257 (C) and UACC62 (D) cells with or without treatment of Y-27632 were scratched using plastic tips for in vitro wound healing assay; the representative images of cells right after scratching (0 h) and at 24 h after scratching. E, The quantification data of healing percentage (%) of (C-D). **Indicated $P < .01$ when comparing Y-27632-treated cells with the control cells. F, The representative images of migrated cells at 24 h of the migration assay with or without Y-27632, and the quantification of migrated cell number is shown in the right panel. ***Indicates $P < .001$ when comparing Y-27632-treated cells with no treated cells. All experiments were carried out 4 times, and the error bars represent the mean ± standard deviation. A *t* test was used for statistical analysis; *P* value as indicated with "*"

human primary melanocytes (HEM) (Figure 3D). Finally, we tested another BRAF-mutant melanoma cell M14, and, again, we found that Y-27632 could enhance cell migration (Figure S1). Interestingly, we did not observe a significant difference in ROCK activity between BRAF wild-type and mutant cells at normal culture conditions (Figure S2). In summary, these data suggest that the effect of Y-27632 on melanoma cell growth and migration depends on the mutant status of BRAF gene.

3.4 | ROCK inhibitor enhanced BRAF-mutant melanoma cell growth and migration through the activation of AKT and ERK

To understand the underlying molecular mechanisms of the Y-27632 effect, we analyzed 2 essential pathways: PI3K/AKT/mTOR pathway and RAF/MEK/ERK pathway, in both B16F1 and UACC257 cells in the presence of Y-27632 by the immunoblotting analysis. In the B16F1 cells, Y-27632 had no significant effect on the ATK/mTOR pathway but it did reduce the activation of ERK (red arrowheads, Figure 4A). In contrast, the activation of ATK, in UACC257 cells as indicated by phosphorylation of AKT at Thr 308 and Ser 473, was significantly induced by treatment of Y-27632 (red arrows, Figure 4A); in these studies, there was no reduction of ERK activity (green arrows, Figure 4A). To further confirm the relationship between Y-27632 on ATK and ERK, and the mutation status of BRAF gene, we treated human melanoma cells, MeWo, with a wild-type BRAF gene and UACC62 with a BRAF-mutated gene, and these studies showed similar results (red arrowhead, red arrows, and Figure 4B-C); moreover, we found a moderately increased activation of ERK of UACC62 cells by Y-27632 (green arrows, Figure 4B). This result suggested that Y-27632 inhibits BRAF wild-type melanoma cell growth and migration, probably by blocking ERK activation, and enhances BRAF-mutated melanoma cell proliferation and migration by induced activation of AKT and ERK. To further confirm this conclusion, we grew UACC257 melanoma cells in the presence of both Y-27632 and AKT and RAF/ERK pathway inhibitors. We found that the enhanced growth of melanoma cells was completely abolished by all 3 inhibitors, suggesting that the effect of Y-27632 on BRAF-

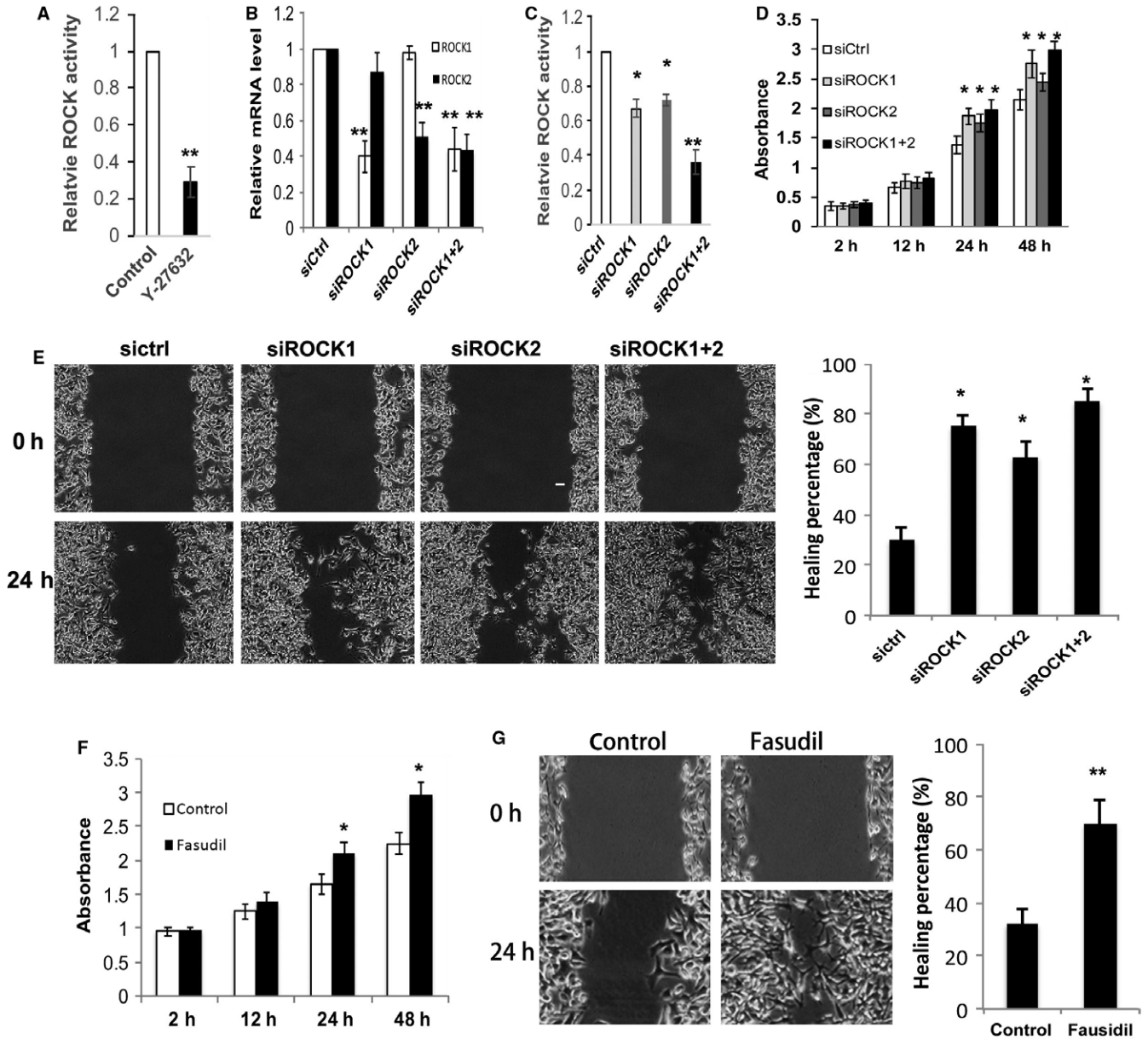


FIGURE 2 Knockdown of ROCK promoted human melanoma cell growth and migration. A, UACC257 melanoma cells were treated with Y-27632 for 24 h, and the cells were lysed for analysis of ROCK activity with the ELISA kit. B, Real-time RT-PCR analysis of ROCK1 and ROCK2 expression at 48 h after transfection with siRNA of ROCK1 (siROCK1), ROCK2 (siROCK2) and both ROCK1 and ROCK2 (siROCK1 + 2); the control cells were transfected with the scrambled siRNA, with 36B4 expression as internal control. C, The cells from (B) were lysed for analysis of ROCK activity by ELISA kit. D, UACC257 cells were collected at different time points as indicated after transfection of siRNA for proliferation assay with CCK8 kit. E, UACC257 cells were transfected with siRNA of ROCK, as indicated, and at 48 h after transfection, cells were scratched; the representative images of cells are shown at 0 and 24 h after scratching. The quantification of the healing percentage of UACC25 cells is shown in the right panel. F, UACC257 cells were collected at different time points as indicated after treatment of Fasudil for proliferation assay. G, The representative image of UACC257 cells at 0 and 24 h after scratch assay; the quantification of healing percentage is shown in the right panel. All experiments were carried out at least 3 times, and the error bars represent the mean ± standard deviation; a *t* test was used for statistical analysis when comparing the treated cells with the corresponding control group. *P* value as indicated with "*": ** $P < .01$, * $P < .05$

mutant melanoma cells depends on both AKT and RAF/ERK pathways (Figure 4C). Furthermore, when the in vitro wound healing assay was carried out with UACC257 cells in the presence of Y-27632 and the other inhibitors, we found that AKT inhibitor partially blocks the enhanced wound healing stimulated by Y-27632. Both BRAF inhibitor PLX4032 and a multi-kinase inhibitor Sorafenib, which can inhibit RAF/ERK pathway, completely blocked wound healing (Figure 4D-E). Taken together, these data demonstrated that Y-27632 promotes BRAF-mutated melanoma cell growth and migration through the activation of both AKT and RAF/ERK pathways.

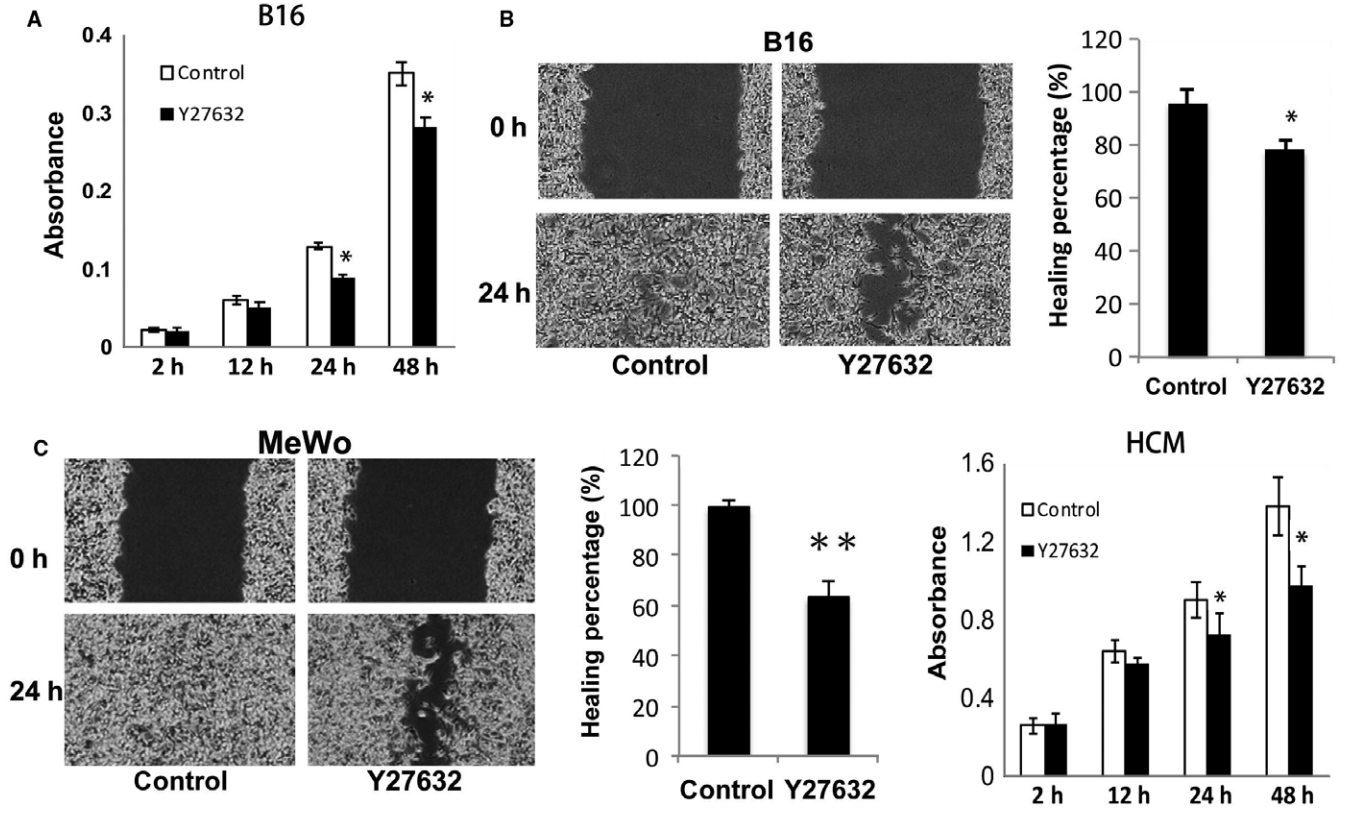


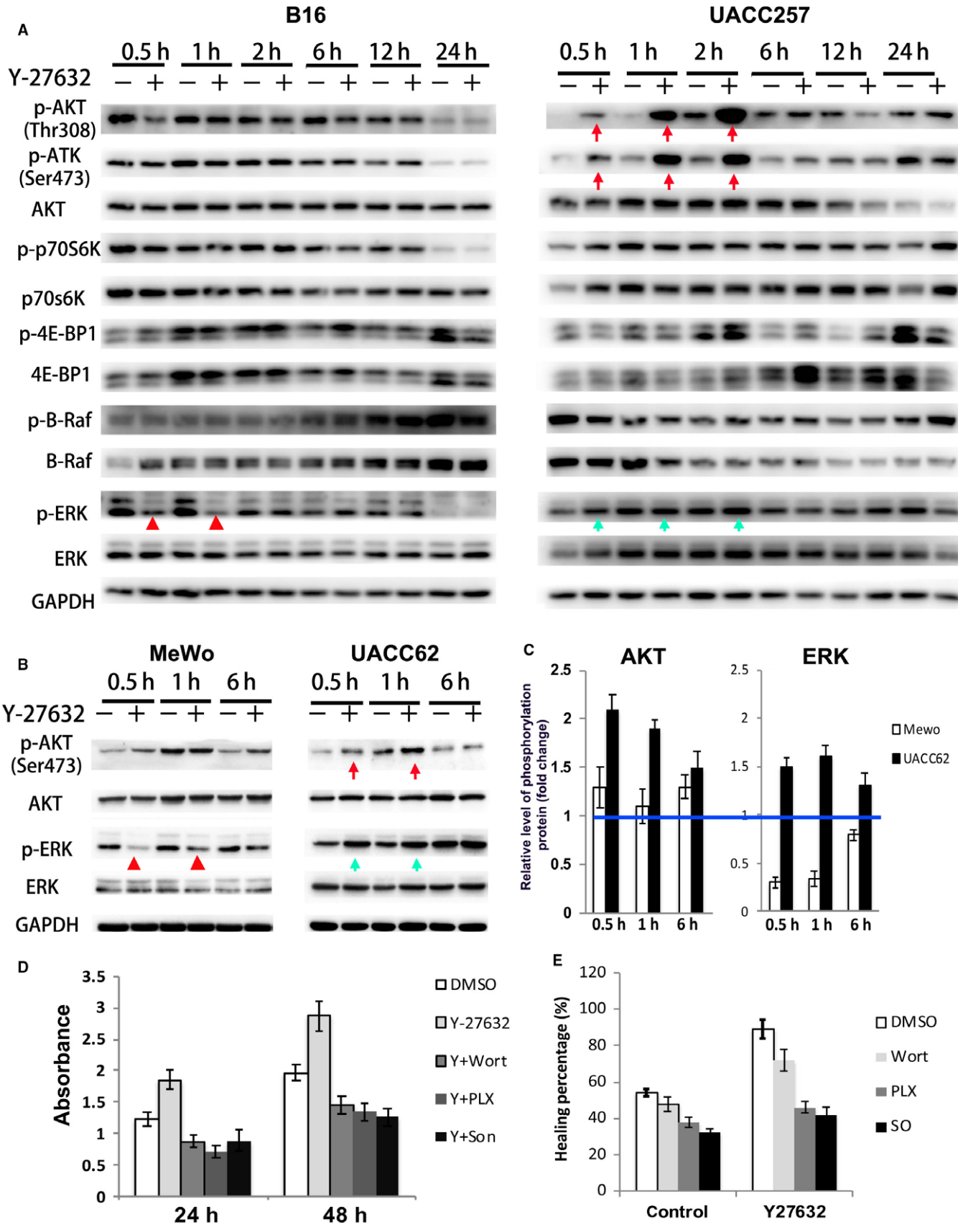
FIGURE 3 ROCK inhibitor promotes BRAF-mutant melanoma cell growth and migration. A, B16F1 cells were collected for proliferation assay at different time points as indicated after treatment of Y-27632. *Indicates $P < .05$ when comparing Y-27632-treated cells with untreated control cells. B, The wounding assay was carried out with B16F1 cells treated with Y-27632 cells. The representative images of B16F1 cells at 0 and 24 h after scratching are shown in the left panel, and the quantification of wound healing percentage is shown in the right panel; * indicates $P < .05$ when comparing Y-27632-treated cells with the untreated cells. C, The in vitro wound healing assay was carried out with MeWo cells in the presence of Y-27632, and the quantification of wound healing percentage is shown in the right panel. **Indicates $P < .001$. D, Y-27632 decreased the proliferation of primary human melanocytes (HCM). Human melanocytes were treated with Y-27632 and collected at different time points as indicated. *Indicated $P < .05$ compared the Y-27632-treated cells with the control cells. All experiments were carried out 4 times, and the error bars represent the mean ± standard deviation. *t* test was used for statistical analysis; *P* value as indicated with "*"

3.5 | In vivo ROCK inhibitor administrated increases the formation of BRAF-mutated melanoma

The previous study showed that Y-27632 reduced murine B16F1 tumor cell growth in vivo.[17] To verify that Y-27632 can promote BRAF-mutated melanoma cell growth in vivo, we subcutaneously injected UACC257 cells into the back skin of nude/nude mouse; 6 injections were delivered into each mouse, 1 million UACC257 cells per injection, followed by i.p. injections of Y-27632 twice a week. Three weeks later, we collected and weighed the induced tumors. The tumors from Y-27632-treated mice were much bigger than those from the control mice (Figure 5A); the average weight of tumors treated with Y-27632 was significantly heavier than in the control group (Figure 5B). Moreover, we observed that nearly 100% of injections could form tumors in the mice treated with Y-27632; around 80% of injections generated tumors in the control group. These data suggested that ROCK inhibitor could increase BRAF-mutated melanoma cell growth in vivo.

4 | DISCUSSION

The ROCK signaling pathway plays an important role in regulating proliferation and migration in various tumor cell lines.[1,2] Previous reports have demonstrated that ROCK inhibitor Y-27632 negatively controls melanoma cell growth and migration and suggested that ROCK inhibitors could be used for melanoma treatment.[17,24] However, in contrast to the previous reports, we found that Y-27632 actually enhances the growth and migration of UACC257 melanoma cells. To validate this unexpected finding, we took 4 approaches. First, we tested another human melanoma cell UACC62 and observed the same effect of Y-27632 on UACC257. Second, we tested another ROCK inhibitor, Fasudil, and found that it also enhanced UACC257 proliferation and migration. Third, we tested whether the difference was due to our culture system or to the particular human cells we used. When we tested the same mouse melanoma B16F1 cells, used in the previous studies, with Y-27632, we also found a negative effect of Y-27632, as previously reported. Finally, to test the mechanism that the positive effect


A
B16
UACC257
0.5 h 1 h 2 h 6 h 12 h 24 h
Y-27632 − + − + − + − + − + − +
p-AKT (Thr308)
p-ATK (Ser473)
AKT
p-p70S6K
p70s6K
p-4E-BP1
4E-BP1
p-B-Raf
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DMSO
Y-27632
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Y+PLX
Y+Son
E
Healing percentage (%)
120
100
80
60
40
20
0
Control
Y27632
DMSO
Wort
PLX
SO

FIGURE 4 The effect of ROCK inhibitor on melanoma cells depends on the activation of AKT and ERK pathway. A, Both B16F10 and UACC257 cells were treated with 10 μmol/L Y-27632 and were collected at different time points for immunoblotting of phosphorylation and total form of components (as indicated) of both AKT/mTOR and RAF-ERK pathways. GAPDH is a housekeeping gene for loading control. The red arrowhead indicates the lower density of the phosphorylation form of ERK bands in B16 cells compared with the control group without treatment of Y-27632, the red arrows indicate the stronger bands of the phosphorylation form of AKT in UACC257 cells, and the green arrows indicates no reduction in band density of the phosphorylation form of ERK in UACC27 cells treated with Y-27632. B, Both MeWo and UACC62 cells were treated with Y-27632 and were collected at .5, 1 and 6.0 h after treatment for immunoblotting analysis of activation (phosphorylation form) of both AKT and ERK. The indication of the red arrowhead and red and green arrows is same as in (A). C, The quantification of the relative level of phosphorylation protein (p-ATK or p-ERK) in cells treated with Y-27632 (+shown in B), which was relative fold change to that of the control cells (–, in B), indicated by a blue line as 1, and each phosphorylation protein was first normalized by its corresponding total protein (AKT or ERK). *Indicates $P < .05$ when comparing Y-27632-treated cells with the control group. D, UACC257 melanoma cells were treated with different conditions: DMSO (control), Y-27632, Y-27632 + wortmannin (Y + Wort), Y-27632 + PLX4032 (Y + PLX), Y-27632 + Sorafenib (Y + So). Cells were collected at 24 and 48 h after treatment for proliferation assay by CCK8 kit. *Indicates $P < .005$ when comparing with Y-27632-treated group. E, The in vitro wound healing assay was carried out with UACC257 melanoma cells in the presence of Y-27632 and combined with inhibitors wortmannin (Wort), PLX4032 (PLX) and Sorafenib (So), with DMSO as control. The relative healing percentage is shown. *Indicates $P < .05$ when comparing healing percentage of 2 groups as labeled. All experiments were carried out 3 times, and the error bars represent the mean ± standard deviation. A *t* test was used for statistical analysis; *P* value as indicated with "*"

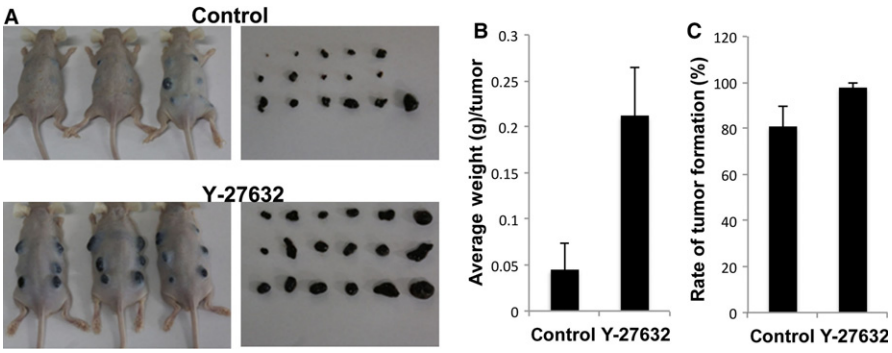

FIGURE 5 Y-27632 promotes BRAF-mutant melanoma cell tumor formation and growth. A, The representative of images of mice at 3 wk after subcutaneously injection of either PBS (control) or Y-27632, and each tumor was collected shown in the right panel. B, The average weight of each tumor was quantified. **Indicates $P < .001$ when comparing Y-27632 with the control group. C, The percentage of tumor formation is shown, and * indicates $P < .05$. The mouse experiment was performed 3 times; 6 mice (3 for Y-27632-treated group, 3 for the control group) were used each time, and a total of 18 mice were used

on human melanoma cell growth was not due to off targeting of Y-27632, we confirmed that Y-27632 treatment blocked the ROCK activity of melanoma cells; we further inhibited ROCK pathway by siRNA-mediated knockdown of ROCK expression, either by knocking down of ROCK1 or ROCK2 alone or by a double knockdown of both ROCK1/ROCK2. We found that knockdown of ROCK expression enhanced the growth of UACC257 melanoma cells. Taken together, our results indicate that ROCK inhibitor Y-27632 enhances melanoma cell proliferation and migration and that in the face of the previous report indicates that ROCK function in melanoma is cell type, cell-context or microenvironment-dependent. This finding agrees with findings from previous work with other cancer cell types. For instance, Y-27632 decreased the invasion of SW620 colon cancer cells but increased the migration of SW480 colon cancer cells.[7,9] Moreover, RND3, an internal ROCK inhibitor, has been shown to enhance melanoma cell migration,[25] a finding corroborating our studies here.

In examining the difference effect of Y-27632 on different melanoma cells, we noticed that both UACC257 and UACC62 carry BRAF gene mutations, while B16F1 contains the wild-type BRAF gene. To test if the difference is due to the mutation status of BRAF gene, we assessed the effect of Y-27632 on BRAF wild-type MeWo melanoma cells, on primary human melanocytes, and on BRAF-mutated M14 melanoma cells. The results of this study support the conclusion that Y-27632 enhances the proliferation and migration of BRAF-mutated melanoma, but not BRAF wild-type melanoma or primary melanocytes (Figures 3, S1).

To further test how Y-27632 positively regulates BRAF-mutant melanoma cells, we analyzed the changes of AKT/mTOR and RAF/ERK pathways, because both are reported to play crucial roles in melanoma cell growth and migration,[26-28] induced by treatment of Y-27632. Interestingly, we found that Y-27632 could dramatically activate AKT and slightly increased ERK activity of BRAF-mutant UACC257 and UACC62 melanoma cells, while there was no clear activation of AKT and, notably, reduced activity of ERK in wild-type B16 cells. In a previous study, it was found that a ROCK inhibitor could promote tumor cell proliferation by downregulating the tumor

suppressor gene PTEN, leading to the upregulation of ATK activity.[29,30] AKT has been shown to phosphorylate RAF, inhibit the RAF-MEK-ERK pathway, and cause reduced ERK activation in normal cells,[31-33] and the downregulation of ERK pathway possibly further reduces the AKT activation as a feedback mechanism.[33] We speculated that the feedback inhibition of ERK on AKT activity is deregulated in the BRAF-mutant melanoma and that the deregulation is significantly enhanced in the presence of Y-27632. Therefore, we concluded that Y-27632 could block PTEN activation and, thus, lead to the activation of ATK in BRAF-mutant melanoma cells; moreover, the activation of AKT cannot sufficiently inhibit RAF-MEK-ERK pathway in the BRAF-mutant melanoma cells in the presence of Y-27632. Therefore, the ATK inhibitor wortmannin could completely block the enhanced growth of melanoma cell by Y-27632 (Figure 4D). The latter supports the notion that Y-27632 enhanced BRAF melanoma cell growth through the activation of AKT. We noted that AKT inhibitor did not completely block the induced migration by Y-27632, but BRAF or ERK inhibitor could completely blocked both growth and migration of melanoma cells induced Y-27632 (Figure 4E). These findings suggest that Y-27632 blocked BRAF wild-type B16 cell growth and migration by inhibiting ERK activation, while there was no significant inhibition of ERK in the BRAF-mutant melanoma cells. Although we could show that Y-27632 increased the activation of ATK or ERK in BRAF-mutant melanoma cells, in this study, we still have not confidently defined how Y-27632 activates AKT or ERK, because we did not see significant change in both mTOR targets and RAF/MEK in the presence of Y-27632 (Figure 4A).

Finally, we demonstrated that Y-27623 could enhance tumor formation and promote melanoma cell growth in a mouse model; the in vivo data support our in vitro findings.

In summary, the present study shows that Y-27632 enhances BRAF-mutant melanoma cell proliferation and migration in vitro and promotes tumor cell growth in vivo. Combined with the previous report, we conclude that the effect of ROCK inhibitor on melanoma cells is largely dependent on cell type and cell context, suggesting that the role of ROCK inhibitors as a therapeutic tool may be tumor-specific or patient-specific.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Chang F, Zhang Y, Mi J, et al. ROCK inhibitor enhances the growth and migration of BRAF-mutant skin melanoma cells. *Cancer Sci*. 2018;109:3428–3437. https://doi.org/10.1111/cas.13786