Targeting ROCK1/2 blocks cell division and induces mitotic catastrophe in hepatocellular carcinoma

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Abstract

Background

Rho-Associated kinases ROCK1 and ROCK2 have been extensively investigated in the pathogenesis of cardiovascular disease. However, their roles are not fully understood in carcinogenesis. In this study, we investigated whether ROCK1 or ROCK2 is required for the survival and growth of hepatocellular carcinoma (HCC) cells and underlying mechanism.

Methods

ROCKs expression was determined in human HCC tissue and cell lines using qRT-PCR, western blotting, and immunohistochemistry (IHC). Cell growth and proliferation were assayed using cell counting kit-8 (CCK-8) and EdU incorporation assay. Cell cycle and apoptosis analysis were performed using flow cytometry. HCC cell division or mitosis was observed using a confocal microscope and a time relapse fluorescence microscope. Inhibitory role of targeting ROCK1/2 on HCC was assayed in both xenograft and primary HCC mouse models.

Results

Both ROCK1 and ROCK2 are over-expressed in human HCC tissues and cell lines. Knockdown of ROCK1 or ROCK2 inhibited HCC cell growth. Pharmacological inactivation of ROCK1/2 with Fasudil further blocked the growth and survival of HCC both in vitro and in vivo. Mechanically, Fasudil induces cell cycle arrest in HCC cells, but not apoptosis. Instead, Fasudil treatment led to mitotic catastrophe in HCC cells, characterized with the multipolar and asymmetric mitosis, and disassociated stress fibers. Knockdown of cofilin restored the cell morphology and division, and reduced the mitotic catastrophe induced by Fasudil.
Conclusions

Both ROCK1 and ROCK2 are required for HCC cell division and growth. Targeting ROCK1 or ROCK2 rather than both can serve as a potential approach for HCC treatment and may reduce the side effects.

Abbreviations

AKT1, AKT serine/threonine kinase 1; CCK-8, cell counting kit-8; FACS, fluorescence activating cell sorter; HCC, hepatocellular carcinoma; LIMK, LIM domain kinase; NRas, neuroblastoma ras oncogene; PI, propidium iodide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROCK1, Rho associated coiled-coil containing protein kinase 1; ROCK2, Rho associated coiled-coil containing protein kinase 2; siRNA, small interfering RNA; shRNA, short hairpin RNA.

Keywords: ROCK; Fasudil; cofilin; mitotic catastrophe; hepatocellular carcinoma

1. Introduction

Rho kinases are protein serine/threonine kinases which exist in two isoforms: ROKα (also called ROCK2) and p160ROCK (also known as ROKβ or ROCK1). Rho kinases can be activated by small GTPase RhoA or RhoC when bound with GTP Rho-kinase. The small GTPase Rho and its downstream effector Rho kinase were initially discovered for their function in regulating smooth muscle contraction by increasing Ca^{2+}-sensitization \cite{[1]}. Further studies revealed that Rho-kinase-mediated Ca^{2+}-sensitization is implicated in diseases such as
hypertension and coronary artery spasm, and Rho-kinase is thus considered as a therapeutic
target for these diseases [2, 3]. Rho and Rho kinase were also demonstrated to be involved in the
Ras activated cellular transformation, suggesting that they play roles in cancer formation [4].
Subsequent studies demonstrated that Rho kinase is connected with cancer by promoting
oncogenic transformation, cell invasion, migration and metastasis [5, 6].

Primary liver cancer is the third leading cause of death from cancer and is responsible for
approximately 781,631 deaths in 2018 [7]. Hepatocellular carcinoma (HCC) accounts for
approximately 80% of primary liver cancers. Current treatment options for HCC remain limited
and generally ineffective [8-10]. Discovery of therapeutic targets and subsequent development of
medicine for HCC is urgently needed. Given that ROCKs has been connected with cancer
growth or metastasis in some cancer types, we sought to explore whether ROCKs play a role in
hepatic carcinogenesis and can serve as treatment target.

Several compounds have been developed to block Rho-kinase, including Y-27632 and the
isoquinoline sulfonyl derivative HA1077, named as Fasudil. Fasudil [1-(5-
isoquinolinesulfonyl)-homopiperazine] has been approved in Japan since 1995 and in other
countries later on for clinical treatment of vascular spasms in the brain [11]. It is metabolized
into the active metabolite hydroxyfasudil, which shows vasodilatory activity, neuroprotective
properties, and cardiovascular protection properties. In our previous study, we reported that
Fasudil can inhibit HCC cell growth and liposomal Fasudil can further enhance this effect in
mice [12]. Here, we investigated the role of ROCK1 and ROCK2 in maintaining HCC cell
growth and dissected underlying mechanism.
2. Materials and methods

2.1. Fasudil and Cell Culture

Fasudil mesylate was a gift of Wuhan Qirui pharmaceutics (Wuhan, China). Human cell lines (HepG2, SMMC7721, Bel7402, Hep3B and Huh7) were purchased from China Type Culture Collection at Wuhan University, China. All cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), 1,000 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C with 5% CO₂.

2.2. Human HCC tissue samples

The HCC samples used in this study were collected from Union Hospital affiliated with Tongji Medical College of Huazhong University of Science and Technology. Collection of HCC samples has been approved by Medical Ethics Committees of Huazhong University of Science and Technology. Written informed consent was obtained from all patients before surgery.

2.3. Constructs, siRNA and Transfection

The hyperactive sleeping beauty construct pCMV/SB, NRas construct pCaggs-NRasV12 and Akt constructs pT3/EF1α-HA-Akt were gifts of Dr Xin Chen of the University of California at San Francisco. Construction of pCaggs-NRasV12 and pT3/EF1α-HA-Akt has been described in previous publication [13]. All plasmids were purified using endotoxin free Maxiprep kit (OMEGA Bio-Tek, Norcross, GA, USA) before injected into mice. siRNAs used in this study were listed in Table 1. shROCK1 and shROCK2 lentivirus were purchased from Santa Cruz
Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 (Invitrogen) was used for transfection of siRNA oligos into Huh7 and Hep3B cells according to the manufacturer’s instructions.

<table>
<thead>
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<th>siRNAs</th>
<th>Sequences</th>
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<tr>
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<td>anti-sense: 5'-CUUCAUGUCGUUGAACACCTT-3'</td>
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<tr>
<td>ROCK1 siRNA</td>
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<td></td>
<td>anti-sense: 5'-UGAUCUUGUAGCUCCCAGCAUCUGUC-3'</td>
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<td></td>
<td>anti-sense: 5'-UUUAUGUCAGGUUCUAACTT-3'</td>
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</table>

2.4. RNA isolation and qRT-PCR

Total RNA was isolated from the cultured cells using Trizol (Invitrogen) according to the manufacturer’s instructions. The cDNA was synthesized with 1 μg of total RNA and cDNA synthesis mix (Thermo Fisher Scientific, Bremen, Germany). The primers used in this study are listed in Table 2. Gene expression was measured by real-time PCR assay on an ABI Prism H7300 (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR core reagents (Bio-Rad Laboratories, Berkeley, CA, USA).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
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<tr>
<td>18s rRNA</td>
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<tr>
<td></td>
<td>R 5'-GCTGGAATTACCGGCT-3'</td>
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<tr>
<td>ROCK2</td>
<td>F 5'-CAACTGTGAGGCTGTATGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>R 5'-TGCAAGGTGCTATAATCTCCTC-3'</td>
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ROCK1 F 5’-AACATGCTGCTGGATAAATCTGG-3’
R 5’-TGTATCACATCGTACCATGCCT-3’

2.5. *Protein extraction and Western blot analysis*

Protein extracts of cell lines or tissues were prepared by using M-PER mammalian protein extraction reagent (Thermo Fisher) plus protein inhibitor cocktail (Roche, Indianapolis, IN, USA), PMSF and phosphatase inhibitors. Protein concentrations of the lysate were quantified using the BCA protein assay (Beyotime, Beijing, China). Western blotting procedures were performed as usual. All antibodies used are listed in Table 3.

Table 3. Antibodies used in this study.

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<th>Dilution</th>
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<td>Mouse IgG</td>
<td>Proteintech</td>
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ROCK1  1:1000  Rabbit IgG  Abcam

**For immunohistochemistry**

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<td>Cell Signaling Technology</td>
</tr>
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<tr>
<td>ROCK2</td>
<td>1:200</td>
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<td>Cell Signaling Technology</td>
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**For immunofluorescence**

<table>
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<th>IgG Type</th>
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<tr>
<td>Cyclin B1</td>
<td>1:200</td>
<td>Rabbit IgG</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>α-tubulin</td>
<td>1:200</td>
<td>Rabbit IgG</td>
<td>Proteintech</td>
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<tr>
<td>Anti-rabbit IgG (Alexa Fluor 596-)</td>
<td>1:100</td>
<td>Goat IgG</td>
<td>Proteintech</td>
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</table>

2.6. **Immunofluorescence and immunohistochemistry**

Cells were fixed by 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and pre-treated with 0.5% goat serum. They were then incubated with monoclonal anti-cyclin B1 (Cell Signaling, MA, USA) 1:200 or α-tubulin (Proteintech, Chicago, IL, USA) 1:200 for 1.5 h. Cells were incubated with Alexa Fluor 596-conjugated Affinipure Goat Anti-rabbit IgG (Proteintech) 1:100 for 45 min. Finally, the coverslips with cells were mounted on glass slides after staining by DAPI. Cells after fixing and permeabilizing were stained by Actin-stain 488 Fluorescent Phalloidin for 15 min to label the actin filaments. Tissue samples were fixed in 4% cold paraformaldehyde overnight and embedded in paraffin for hematoxylin and eosin (H&E) staining and other immunohistochemistry. Immunohistochemistry was performed as reported
previously. Briefly, paraffin slides were stained with primary antibody at 4°C overnight followed by the avidin-biotin-peroxidase protocol. Antibodies used for immunostaining were listed in Table 3.

2.7. Microscopy

Bright field microscopy and some fluorescence microscopy of cells or tissue section slides were performed with an Olympus fluorescence microscope equipped with a digital camera and connected to a PC running MagnaFire 2.0 camera software (Optronics, Goleta, CA, USA). Pictures were taken at equal exposure times for each sample. Time relapse fluorescence microscopy was performed with an Andor Dragonfly spinning disk confocal system (objective: Nikon CFI Apo Lambda S 60X/1.4 Oil, Camera: Zyla Plus 4.2 Megapixel) equipped with weather chamber which can maintain the cells being imaged in humidified CO₂ and 37°C.

2.8. Cell Proliferation Assay

Cell proliferation was assayed using CCK-8 kits and 5-ethynyl-20-deoxyuridine (EdU) labeling. For the CCK-8 assay, 5,000 cells were seeded into 96-well plates and then treated with 40 uM Fasudil for 24 h. Next, the cells were incubated with 10 μl of CCK-8 (Dojindo, Japan) for 2 h at 37 °C. Optical density of each was measured using a microplate reader at the wavelength of 450 nm. For EdU labeling, a 1:1,000 dilution of EdU reaction solution was added to the culture medium during the last 2 h of cell culture and the staining was performed according to the manufacture’s manual (Beyotime Biotech, Beijing, China). Microscopic examination of cells or tissue section slides was performed with an Olympus SZX12 fluorescence microscope equipped with digital camera and connected to a PC running
MagnaFire 2.0 camera software from Optronics (Goleta, CA, USA). Pictures were taken with the same exposure for each sample.

2.9. Cell Cycle and Apoptosis Analysis by Flow Cytometry

For cell cycle analysis, cells were seeded into 6-well plates at $2 \times 10^5$ cells per well and synchronized at the G0/G1 phase by culturing in serum-free medium for 24 h. After treatment for 24 h, cells were then trypsinized, and fixed in 70% ethanol overnight at 4°C. Fixed cells were incubated with 0.5 mg/mL RNase and 0.025 mg/mL propidium iodide (PI) for 30 min. For each sample, $2 \times 10^4$ cells were detected by flow cytometry (CytomicsTM FC 500, Beckman Coulter, CA, USA).

For apoptosis analysis, $2 \times 10^5$ cells per well were seeded into 6-well plates. After treatment for 24 h, cells were harvested by trypsinization (EDTA free), washed and resuspended in 300 μl PBS buffer by the amount of cells $5 \times 10^5$/ml. The cell suspension was then mixed with 5 μl Annexin V-FITC and 5 μl PI, and incubated at 4°C for 20 min in the dark. For each sample, $2 \times 10^4$ cells were detected by flow cytometry (CytomicsTM FC 500). Both cell cycle and apoptosis assay data were analyzed using software FlowJo7.6.

2.10. Xenograft and Primary HCC Mouse Model

Both female Balb/C nude and FVB/N mice were purchased from Huafukang Technology Corporation (Beijing, China). Mice were kept in filter-topped cages with standard rodent chow and water available ad libitum and a 12 h light/dark cycle. Mice were fed with standard rodent chow. The experiments were performed according to the Regulations for the Administration of
Affairs Concerning Experimental Animals in China and approved by the Animal Experiments Ethical Committee of Huazhong University of Science and Technology.

For xenograft tumor model, $5 \times 10^6$ Hep3B cells infected with/without shRNA lentivirus were inoculated subcutaneously at the right flank in female Balb/C nude mice (4 – 6 weeks of age, 16 – 18 g). Treatment was initiated when the tumor reached a volume around 100 mm$^3$ and mice were randomly divided into three groups ($n = 5$). Saline (0.9% NaCl) or Fasudil (30 mg/kg) was injected intraperitoneally into the mice every day, for a total of 28 times. The length (L) and width (W) of the tumor and body weight were monitored every other day, and the tumor volume was calculated at $L \times W^3/2$. At the end of the experiment, all the mice were sacrificed. The collected tumor tissues were analyzed by hematoxylin-eosin (H&E) staining assay and immunohistochemistry analysis against cyclin B1, cyclin D1 and Ki67 antibodies, or frozen for further analysis.

To generate primary HCC in mice, the hydrodynamic injection was performed as described $^{[14]}$. In brief, plasmids (pT3/EF1α-Akt : pCaggs-NRasv12 : pCMV-SB = 5 μg : 5 μg : 1 μg per mouse) were diluted in 2 ml saline, filtered through a 0.22-μm filter, and injected into the lateral tail vein of 6-week-old FVB/N mice in 5 to 7 seconds. The mice only injected with saline were used as normal control. Two weeks after injection, the mice were injected with saline or Fasudil (30 or 60 mg/kg) every day for 4 weeks. Body weights of mice were weighed and recorded every 3 days. After 27 days, mice were euthanized with CO$_2$ inhalation and mouse livers were weighed and collected for further analysis.
2.11. Statistical Analysis

All experiments were performed at least three times, and data were expressed as the mean ± SD. Means of two groups were compared using Student’s t-test (SPSS Software, Chicago, IL, USA). Means of multiple groups were compared by one-way ANOVA and Dunnett’s post-tests. Values of $p < 0.01$ were considered to be very significant while $p < 0.05$ were considered to be significant.

3. Results

3.1. ROCK1/2 are overexpressed in HCC

We first analyzed the expression level of ROCK1 and ROCK2 in our freshly isolated HCC tissues. Western blot showed that ROCK1 was upregulated in 7 of 10 samples (except for #2, #4 and #5), and ROCK2 was increased in 8 samples (except for #2 and #3) (Figure 1A). qRT-PCR showed similar results in those samples (Figure 1B). Immunohistochemical staining confirmed increased levels of both ROCK1 and ROCK2 in HCC cell cytoplasm but not in adjacent liver tissues (Figure 1C). Gene Expression Omnibus (GEO) data analysis (accession number GSE62044) showed that ROCK1 and ROCK2 are highly expressed in two-thirds of HCC tissues in comparison with adjacent liver tissues (Figure 1D). These results indicate that ROCK1 and ROCK2 are all upregulated in HCC tissues.

“We noticed that ROCK1 or ROCK2 bands appear in doublets in some samples. This suggests that phosphorylation or other modification may happen to ROCK1 or ROCK2 in those samples. Indeed, ROCK activity is not dependent on auto-phosphorylation like other protein kinases such as Akt $^{[15]}$. The crystal structures of the ROCK catalytic domains indicate that
phosphorylation of the activation loop of ROCK is not required for its catalytic activity [16, 17]. However, phosphorylation for both ROCK1 and ROCK2 has been described. Auto-phosphorylation of ROCK1 at Ser1333 and of ROCK2 at Ser1366 have been reported reflecting the activation status of these kinases [18, 19]. In addition, Polo-like kinase-1 was shown to phosphorylate ROCK2 at Thr-967, Ser-1099, Ser-1133, or Ser-1374, thereby activating ROCK2 together with RhoA [20]. Together with these observations, those doublets indicate that there was possibly phosphorylation with ROCK1/2, although phosphorylation is not required for their activation.”

3.2. Knockdown of ROCK1 or ROCK2 inhibits HCC cell growth but not DNA replication

We then asked whether ROCK1 or ROCK2 is essential for the survival and growth of HCC. We compared the ROCK1 and ROCK2 levels among Hep3B, Huh7, HepG2, SMMC7721 and Bel7402 cells and found that Huh7 and Hep3B have the highest ROCK1 and ROCK2 level (Figure 2A). We knocked down ROCK1 and ROCK2 with siRNA and found both ROCK1, ROCK2, and their downstream targets p-LIMK and p-Cofilin were reduced in both Huh7 and Hep3B cells (Figure 2B). Double knockdown with ROCK1+ROCK2 siRNAs or treatment with ROCK1/2 pan-inhibitor Fasudil (40 μM, ~ IC₅₀) led to further reduced p-LIMK and p-cofilin levels, suggesting that ROCK1 and ROCK2 isoforms are not redundant in regulating downstream signaling. Knockdown of ROCK1 or ROCK2 with siRNA inhibited survival of Huh7 or Hep3B cells (Figure 2C). Double knockdown or Fasudil treatment showed more robust inhibitory effects than single siRNA treatments, confirmed again that ROCK1 and ROCK2 are not functionally redundant. Treatment with ROCK1 and/or ROCK2 siRNAs or Fasudil did not inhibit incorporation of EdU into chromosomes of Hep3B and Huh7 cells,
indicating that DNA replication was not impaired by ROCK1/2 blockade (Figure 2D). Together, these results suggest that genetic ablation or pharmacological inactivation of ROCK1/2 inhibits HCC cell survival, but does not block their DNA synthesis. Moreover, these data suggest that ROCK1 and ROCK2 are not redundant in promoting HCC cell growth. Therefore, we investigated their roles using double knockdown or Fasudil in successive experiments.

3.3. Knockdown or inactivation of ROCK1/2 blocks HCC cell cycle progression

ROCKs were reportedly essential for cell cycle progression in many cancer types, thus we examined whether inhibition of ROCK1/2 would block cell-cycle progression in HCC cells. Knockdown of ROCK1/2 or Fasudil treatment (40 μM) resulted in the accumulation of Hep3B and Huh7 cells in G2/M phase (Figure 3A). Western blot results showed cyclin D1 was decreased while cyclins B1 and A2 were increased following Fasudil treatment or ROCK1/2 knockdown, in line with the FACS analysis (Figure 3B). Immunostaining also demonstrated that cyclin B1 was increased after Fasudil treatment or ROCK1/2 knockdown (Figure 3C). Collectively, these results indicate that knockdown or inactivation of ROCK1/2 inhibits cell cycle progression in HCC cells.

3.4. Blockade of ROCK1/2 does not induce apoptosis in HCC cells

Cell cycle blockade generally leads to apoptosis in mammalian cells. Thus, we examined apoptosis in HCC cells upon ROCK1/2 knockdown or Fasudil treatment. Chemotherapeutic drug Topotecan led to evident apoptosis, while Fasudil or ROCK1/2 siRNAs treatment did not induce significant apoptosis in Hep3B or Huh7 cells (Figure 4A). PI and FITC-annexin V staining showed no apoptosis induced in Hep3B and Huh7 cells as well (Figure 4B). Likewise,
apoptotic bodies were found in Topotecan treated cells but not in Fasudil treated cells (Figure 4C). In accordance, anti-apoptotic Bcl-2 was reduced, and pro-apoptotic Bax and cleaved caspase-3 were increased in Topotecan treated Hep3B or Huh7 cells (Figure 4D). However, no decreased Bcl-2 or increased Bax and cleaved caspase-3 were found in Fasudil treated cells. These data indicates that blockade of ROCK1/2 does not induce apoptosis in HCC cells.

3.5. Inactivation of ROCK1/2 inhibits HCC growth in mice

We then investigated the effect of Fasudil on HCC growth in vivo using both xenograft and primary HCC mouse models. For HCC xenografts, we either treated mice via tail vein with Fasudil at 30 mg/kg when tumor grew to ~100 mm³ or knocked down ROCK1/2 in Hep3B cells with shRNA lentivirus before inoculation. Fasudil at 30 mg/kg inhibited tumor growth by ~67%, and double knockdown suppressed the tumor growth ~75% (Figure 5A-5C). Lower body weights also indicate less tumor burden in mice treated with Fasudil or ROCK1/2 knockdown (Figure 5D). Elevated cyclin B1 or decreased cyclin E2 indicated a cell cycle arrest in the G2/M phase, in line with the observation in vitro (Figure 5E). Bax and cleaved caspase3 were slightly increased. Intriguingly, Ki67 staining results showed that the proliferation rate of HCC cells in xenograft was not affected by Fasudil treatment, consistent with the result of EdU assay in vitro (Figure 5F). Likewise, cyclin B1 was increased but cyclin D1 was reduced after Fasudil treatment or ROCK1/2 knockdown, indicating that cell cycle of those cells was arrested in G2/M phase. These results indicate that ROCK1/2 knockdown or inactivation inhibits HCC growth via induction of cell cycle arrest in vivo as well.

We then explored the effects of Fasudil on HCC growth in primary mouse HCC. To mimic HCC pathology in human patients, an Akt/Ras induced primary HCC mouse model was
generated by hydrodynamic injection of plasmids carrying these two genes into mice as described before [13]. Two weeks after Akt/Ras injection, tumor formation was confirmed via palpation in mice and then Fasudil was administrated through tail vein injection. Saline treated mouse livers were pale, ballooned and spread with tumor nodules all over the liver surfaces (Figure 6A). In comparison, 30 mg/kg Fasudil treated mice showed restrained tumor growth. Treatment with 60 mg/kg Fasudil further inhibited the tumor growth and only sporadic nodular lesions were observed on the surface of livers. The average body weight of 30 mg/kg Fasudil treated mice were significantly lower than that of saline treated mice. Average body weights and liver/body weight ratio of mice treated with 60 mg/kg Fasudil were reduced to the level of normal mice, suggesting a complete recession of tumor nodules after Fasudil treatment (Figure 6B and 6C). Taken together, these results demonstrated the therapeutic effects of Fasudil or ROCK1/2 knockdown in vivo.

3.6. ROCK1/2 inactivation induces mitotic catastrophe in HCC cells

We then asked how ROCK1/2 inactivation leads to cell death in HCC cells. In the HCC cells treated with Fasudil, we noticed the existence of multiple nuclei, a typical trait of “mitotic catastrophe” (Figure 7A) [21]. Cell division was significantly delayed or retarded upon Fasudil treatment (Figure 7B). Moreover, Fasudil treated cells underwent asymmetrical division, featuring with asymmetrically distributed tubulin fibers and asymmetrical chromosome segregations (Figure 7B and 7C). In quiescent Hep3B or Huh7 cells, Fasudil treatment reduced assembly of stress fibers and the amount of focal adhesions (Figure 7D). Cell morphology shifted from stretched spear or triangle shapes to round or half-moon-like, indicating the dissembled stress fibers. These morphological alterations including aneuploidy, asymmetrical
chromosomal segregation and disassembled tubulin fibers refer to “mitotic catastrophe” induced by Fasudil.

3.7. *Fasudil inhibits phosphorylation of coflin via p-LIMK*

We then investigated how Fasudil induces mitotic catastrophe in HCC cells. Cell division and maintenance of specialized structures in proliferating cells depend directly on the well-regulated dynamics of the actin cytoskeleton \(^{[22]}\). The turnover and assembly of actin filaments are highly dependent on coflin, a ubiquitous actin-binding protein \(^{[23, 24]}\). Cofilin stimulates actin filament disassembly and is critical for rapid turnover of actin assembly and disassembly. Cofilin is a physiological substrate of LIM-kinase 1 that induces stabilization of F-actin structures \(^{[25, 26]}\). LIM-kinase 1 phosphorylates coflin, leading to inactivation of coflin and subsequent accumulation of actin filaments.

To further illustrate how inhibition of ROCK2 leads to mitotic catastrophe, we examined the role of coflin in regulating actin dynamic. We knocked down coflin in Fasudil treated Huh7 and Hep3B cells and found that silencing coflin restored cell growth blocked by Fasudil (Figure 8A). Reduced coflin reversed the accumulation of cyclin A2 and cyclin B1 and increased the expression of cyclin D1 in Fasudil treated cells, suggesting that suppression of coflin relieved the Fasudil induced cell cycle arrest (Figure 8B). Upon coflin knockdown, Fasudil failed to induce depolymerization of actin and disarrangement of stress fiber (Figure 8C). Importantly, Hep3B or Huh7 cells treated with Fasudil showed multiple nuclei and aberrant spindle structure, featured with spindle fibers paralleled with the metaphase plates rather than crossed with them (Figure 8D). In contrast, coflin knockdown resulted in symmetrical spindles and segregation of chromosomes in the cells treated with Fasudil. These
results suggest that ROCK1/2 inactivation leads to HCC cell mitotic catastrophe via inhibiting the phosphorylation of LIMK and coflin. Accumulation of non-phosphorylated coflin promotes the disassembly of F-actin and thus perturbs the chromosome segregation and cell division.

4. Discussion

ROCK1 and ROCK2 are highly expressed in many types of cancers, but their roles in HCC development are not fully understood. In this study, we demonstrated that ROCK1 and ROCK2 are essential for HCC cell proliferation and inhibition of ROCK1/2 by Fasudil leads to mitotic catastrophe in HCC cells. Cellular and molecular mechanistic analysis revealed that Fasudil induces mitotic catastrophe by blocking phosphorylation of LIMK and coflin, ultimately leading to the disassembly of actin filaments and failure of mitosis. Our study, therefore, uncovered the critical role of ROCK1/2 in the mitosis of cancer cells and demonstrated the potential of ROCKs as targets for HCC treatment. Moreover, our study also illustrates the pivotal role of coflin in the induction of mitotic catastrophe.

This study highlights the essential role of ROCK1/2 in maintaining HCC cell growth. Both ROCK1 and ROCK2 are upregulated in human HCC tissues and cell lines. Inhibition of ROCK1 or ROCK2 inhibited HCC cell growth in vitro. Simultaneous inactivation or knockdown of ROCK1/2 by Fasudil or siRNA inhibited the growth of HCC in vivo. Our observation on the fundamental role of ROCKs in HCC is in concert with the results conducted in pancreatic adenocarcinoma [27, 28], breast cancer [6, 29], lung cancer [5] and colon cancer. Although Fasudil has been demonstrated having inhibitory role in HCC cells [30], our study
provides more solid evidence using HCC mouse models and provides deeper insights into molecular mechanism ROCK1/2 in maintaining HCC growth.

Our study uncovered critical and essential role of ROCKs in maintaining cell division. Previous studies on ROCKs were generally focusing on their roles in regulating cancer cell migration and metastasis. It is well-documented that Fasudil inhibits human tumor cell migration and metastasis by inhibiting ROCKs [6, 31, 32]. However, the impact of ROCKs on cancer cell growth is not fully illustrated. In this study, we noticed that knockdown or inactivation of ROCK1 or ROCK2 induced cell cycle arrest in HCC cells, but no apoptosis was induced. This observation is in disagreement with a previous study by Takeba et al, who reported that inhibition of ROCKs by Fasudil leads to apoptosis in HepG2 and Huh7 cells [30]. Indeed, activated caspase 3 is involved in mitotic catastrophe as well [21, 33]. Mitotic catastrophe can induce both caspase-dependent and caspase-independent cell death. However, they failed to provide cell morphology or confirm the apoptosis using other methods including TUNEL for fragmented genomic DNA or annexin V based FACS for outer leaflet of the plasma membrane. Instead, our FAC results showed that there was no apoptotic cell after Fasudil or ROCK1/2 siRNA treatment.

Instead, our experiments demonstrated that ROCKs siRNA or inhibitor kills HCC cells by inducing mitotic catastrophe, featuring with multinuclei daughter cells or asymmetrical cell division. Compared with the studies by Takeba et al and other groups, our study provides an alternative mechanism illustrating how increased ROCKs promote cancer cell growth. Of note, both ROCK1 and ROCK2 govern the F-actin assembly and segregation of chromosomes since knockdown of either one will block cell division and cause the cell cycle arrest. This suggests
that ROCK1 and ROCK2 play similar role in maintaining HCC cell division. Targeting either ROCK1 or ROCK2 will inhibit HCC growth. This is different with their distinct roles in cardiovascular diseases, in which ROCK1 is responsible for cardiac fibrosis while ROCK2 plays a role in cardiac hypertrophy.\textsuperscript{[15]}

Moreover, our results indicate that ROCK/LIMK/cofilin/F-actin pathway plays an essential role in maintaining cell division. As the ROCK1/2 was inhibited by Fasudil or siRNA, the assembly of tubulin and F-actin was blocked. Aborted assembly of stress fibers led to aborted or aberrant cell division. Increased unphosphorylated cofilin is responsible for this outcome and knockdown of cofilin restores the assembly of F-actin and tubulin, and subsequent cell division. The role of cofilin and LIMK in spindle formation and regulation of actin dynamic has been reported previously.\textsuperscript{[26, 34]} However, increased ROCKs stabilize F-actin via LIMK/cofilin has not been reported in a cancer context. Hence, our study sheds light on the critical role of ROCK/LIMK/cofilin/F-actin pathway on modulating stress fiber assembly and formation of the mitotic spindle in cancer cells. These results also prompt us to explore the feasibility to target cofilin for cancer treatment.

**CRediT authorship contribution statement**

**Chuanrui Xu:** Conceptualization, Methodology, Writing - Original Draft, Writing - Review and Editing, Funding acquisition. **Lei Li:** Investigation, Funding acquisition. **Hua Wu:** Investigation, Validation. **Yuyuan Chen:** Investigation, Data Curation. **Bin Li:** Investigation, Data Curation. **Chun Li:** Investigation. **Jun Guo:** Investigation. **Jia You:** Investigation. **Xian Hu:** Investigation. **Dong Kuang:** Resources. **Shibo Qi:** Investigation. **Pin Liu:** Investigation, Funding acquisition.
Conflict of interest

No conflicts of interest exist.

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References


Figure 1. **ROCK1 and ROCK2 are upregulated in HCC**. (A and B) Protein and mRNA levels of ROCK1 and ROCK2 in human HCC samples. Tubulin and rRNA were used as loading control, respectively. (C) Immunohistological staining of ROCK1 and ROCK2 in two human HCC tissues. (D) mRNA levels of ROCK1 and ROCK2 in HCC samples analyzed using the data from PubMed database (accession number GSE62044). Data are expressed as mean ± SD (n = 3 technical repeat). Para-tumor refers to 5 mm thick layer of adjacent tissues surrounding the tumor nodules.
Figure 2. **Inactivation or knockdown of ROCK1 and ROCK2 suppressed HCC cell growth but not DNA replication.** (A) ROCK1 and ROCK2 levels in HCC cell lines. LO2 is a fetal liver cell line and was used as the normal cell control. (B) p-LIMK and p-cofilin levels in Hep3B and Huh7 cells treated with siRNA or Fasudil for 48 and 24 h, respectively. (C) Viabilities of HCC cells treated with siRNA or Fasudil (40 μM) for 48 and 24 h, respectively. Cell viability was determined using CCK-8 assay after treatment. (D) Proliferation of Hep3B and Huh7 cells detected with EdU staining. Nuclei were stained with DAPI. Scale bar = 50 μm. Data are expressed as the mean ± SD (n = 3).
Figure 3. **Fasudil or ROCK1/2 siRNA induces cell cycle arrest in HCC cells.** Hep3B and Huh7 cells were treated with Fasudil (40 μM) or ROCK1/2 siRNA for 24 and 48 h, respectively. (A) Cell-cycle analysis with FACS in Hep3B and Huh7 cells. (B) Levels of cyclins in Hep3B and Huh7 cells. (C) Immunofluorescence of cyclin B1 in Hep3B and Huh7 cells. Cyclin B1 was stained as red and cell nuclei were counterstained as blue with DAPI. Scale bar indicates 50 μm.
Figure 4. **ROCK1/2 knockdown or inhibition does not induce apoptosis in Hep3B and Huh7 cells.** Hep3B and Huh7 cells were treated with siRNA (48 h), Fasudil (40 μM, 24 h) or topotecan (50 nM, 24 h). (A) FACS analysis of apoptosis in Hep3B and Huh7 cells. (B) PI (red) and FITC-annexin V (green) staining of Hep3B and Huh7 cells. (C) Morphology of Hep3B and Huh7 cells after treatment. (D) Bcl2, Bax and cleaved caspase-3 levels in Hep3B and Huh7 cells.
Figure 5. **ROC1/2 knockdown or inactivation inhibited growth of HCC xenografts in mice.**

HCC xenografts were established by injecting $5 \times 10^6$ Hep3B cells with or without shRNA infection into Balb/C nude mice subcutaneously. (A and B) Gross images and weights of HCC xenografts after treatment for 30 days. (C and D) Growth curve of tumor volumes and body weights of mice. (E) Levels of cyclin B1, cyclin E2, Bax and cleaved caspase-3 in tumor tissues.
(F) Immunohistochemical analysis of Ki67, cyclin B1 and cyclin D1 in xenograft tissues. Scale bar = 50 μm. Data are shown as mean ± SD (n = 5). *: P < 0.05.

Figure 6. Fasudil inhibited primary HCC growth in mice. Primary HCC was induced by simultaneously hydrodynamic injection of Akt and Ras plasmids together with sleeping beauty transposase into mouse livers. (A) Gross images of livers from Akt/Ras HCC mice with Fasudil treatment for 3 weeks. (B) Body weights of tumor-bearing mice. (C) Liver to body weight ratio of mice treated with Fasudil. Data are shown as mean ± SD (n = 5). **: P < 0.01, ***: P < 0.001.
Figure 7. **Fasudil induced mitotic catastrophe in Hep3B and Huh7 cells.** Hep3B and Huh7 cells were treated with Fasudil (40 μM) for 24 h. (A) Fasudil led to aneuploidy and polyploidy in treated Hep3B and Huh7 cells. (B) Time relapsed photograph of cell division in Huh7 cells. (C) Immunostaining of tubulin and nuclear in Fasudil treated Hep3B and Huh7 cells. White arrows indicate the aberrant chromosome segregations leading to multipolar mitosis. (D) Immunostaining of α-tubulin and F-actin in Fasudil treated Hep3B and Huh7 cells. Note the round cells due to defective microtubule attachment.
Figure 8. **Fasudil induces mitotic catastrophe in HCC cell via inhibiting phosphorylation of coflin.** Hep3B and Huh7 cells treated with Fasudil (40 µM) with or without coflin siRNA for 48 h. (A) Morphology and survival of Hep3B and Huh7 cells after treatment. (B) Levels of cyclin A2, cyclin B1 and cyclin D1 detected with Western blotting. (C) Immunostaining of α-tubulin (red) and F-actin (green) in Hep3B and Huh7 cell after treatment. Nuclei were counterstained with DAPI (blue). (D) Immunostaining of α-tubulin and nuclear staining to
detect the multiple nuclei. Data are shown as mean ± SD. n = 8 for figure 8A and n = 3 for figure 8B-8D. *: P < 0.05.