Cancer Letters 444 (2019) 82–93

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Pin1 inhibition reverses the acquired resistance of human hepatocellular carcinoma cells to Regorafenib via the Gli1/Snail/E-cadherin pathway

Jichuang Wang[a,1], Nan Zhang[a,1], Qi Han[a], Wenxian Lu[a], Long Wang[a], Dayun Yang[a], Min Zheng[a], Zhenzhen Zhang[a], Hekun Liu[a], Tae Ho Lee[a], Xiao Zhen Zhou[b], Kun Ping Lu[a,b,*]

[a] Fujian Key Laboratory for Translational Research in Cancer and Neurodegenerative Diseases, Institute for Translational Medicine, The School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian, 350108, China

[b] Division of Translational Therapeutics, Department of Medicine and Cancer Research Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA; Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

ARTICLE INFO

Keywords:
HCC
Regorafenib resistance
Metastasis
Pin1
EMT

ABSTRACT

Hepatocellular carcinoma (HCC) is the second leading cancer death because of its high metastasis and drug resistance. Regorafenib was newly approved by FDA for HCC treatment, but its resistance is not understood. The unique isomerase Pin1 is critical for HCC development, but its role in metastasis and drug resistance is unknown. Here we generated Regorafenib-resistant HCC cells and found that they exhibited enhanced tumor invasion and metastasis *in vitro* and *in vivo*, and elevated Pin1 levels. Furthermore, Pin1 was highly overexpressed and closely related to the EMT in human HCC tissues. Depletion or overexpression of Pin1 correspondingly inhibited or promoted HCC cell migration and invasion, with altered expression of EMT-related molecules, E-cadherin and Snail. Significantly, Pin1 interacted with Gli1, a regulator of the EMT, and silencing Gli1 partly blocked Pin1-induced EMT in HCC cells. Moreover, genetic or chemical Pin1 inhibition reversed Regorafenib resistance of HCC with reducing EMT, migration, invasion and metastasis *in vitro* and *in vivo*. These results reveal a novel molecular mechanism underlying Regorafenib resistance in HCC, and also provide first evidence that Pin1 inhibitors offer an attractive strategy for treating Regorafenib-resistant HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 70–90% of primary liver cancer. Liver cancer is the sixth largest cancer in the world, but it has become the second leading cause of cancer-related deaths [1]. HCC has a high degree of malignancy, developmental concealment, and rapid progression. Most patients have entered the middle and late stages of diagnosis. At the same time, HCC has the potential for invasion and metastasis, and the prognosis is often very poor, with the vast majority of HCC patients dead within 9–12 months after diagnosis [2]. With the resistance of the first-line targeted drug sorafenib, in 2017, Regorafenib (Stivarga) has been approved by FDA for second-line treatment of patients with unresectable hepatocellular carcinoma [3,4]. However, the two drugs belong to the same class of targeted drugs, and Regorafenib also faces the risk of drug resistance and subsequent progression of liver cancer after resistance [5]. Therefore, elucidation of the mechanisms underlying metastasis and therapy resistance is fundamental for the development of new therapeutic treatments for HCC.

The epithelial-mesenchymal transition (EMT) plays critical roles in the development, tumor metastasis and drug resistance in many types of cancer [6,7]. Differentiated epithelial cells lose cell polarity and acquire fibroblast-like mesenchymal traits during the EMT, which is characterized by loss of E-cadherin and gain of N-cadherin [8]. Several studies have implied an association between EMT and sorafenib resistance [9]. Long-term exposure of liver cancer cells to sorafenib induces resistance and the EMT phenotype in HCC [10]. Recent studies have shown that cancer stem cells (CSCs) are a major cause of malignant tumorigenesis, recurrence, metastasis and drug resistance [11,12] and that EMT is one of important reasons for cancer stem cells evading drug treatment [13]. It is hoped that the targeted prevention and treatment of the EMT and its related mechanisms will overcome the shortcomings of existing treatments and improve the therapeutic effect and prognosis of patients with liver cancer. Our recent results have

* Corresponding author. Fujian Key Laboratory for Translational Research in Cancer and Neurodegenerative Diseases, Institute for Translational Medicine, The School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian, 350108, China.
E-mail address: klu@bidmc.harvard.edu (K.P. Lu).
[1] These authors equally contributed to the work.

https://doi.org/10.1016/j.canlet.2018.12.010
Received 13 September 2018; Received in revised form 29 November 2018; Accepted 11 December 2018

shown a critical role for Pin1 in regulating the EMT in breast cancer [14,15].

Pin1 is a unique phosphorylation-specific peptidyl-prolyl cis/trans isomerase (PPIase), and is a common regulator of multiple oncogenic signaling networks [16,17]. Pin1 regulates the activity, stability and subcellular localization of a series of proteins by specifically binding and catalyzing the *cis-trans* isomerization of phosphorylated Ser/Thr-Pro motifs [18–20]. Pin1 is found to be upregulated in a variety of cancer types including HCC [21,22]. Pin1 activates numerous oncogenes and growth enhancers, and inactivates numerous tumor suppressors and growth inhibitors [17]. Moreover, many of Pin1's downstream target proteins are mostly carcinogenic transcription factors and are difficult to make medicine. And $Pin1^{-/-}$ mice develop normally and do not exhibit obvious defects for about half of lifespan [23]. Therefore, Pin1 is an ideal target for cancer treatment [24]. All-trans retinoic acid (ATRA) has recently been identified to bind Pin1, inhibit its activity and degrade active Pin1 specifically in cancer cells [16].

Regarding the role of Pin1 in liver cancer, we and others have shown that Pin1 is overexpressed in most human HCC patients and that genetic and chemical Pin1 inhibition effectively inhibits the growth of HCC cells [25–27] and sensitizes HCC response to sorafenib treatment [28]. However, nothing is known regarding whether Pin1 is involved in resistance to Regorafenib and the EMT properties of HCC. Here, we demonstrate for the first time that Pin1 genetic and chemical inhibition of Pin1 reverses the acquired resistance of human HCC to Regorafenib via the Gli1/Snail/E-cadherin pathway. These results not only reveal a novel molecular mechanism underlying Regorafenib resistance in HCC, but also provide first evidence that Pin1 inhibitors offer an attractive strategy for treating aggressive and Regorafenib-resistant HCC.

2. Materials and methods

2.1. Cell culture

The human HCC cell lines (SMMC-7721, Huh7 and MHCC-97H), normal non-malignant liver cells (THLE3) and human kidney 293 T cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured as our previously described [34]. All cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Generation of drug-resistant cells

Using the low concentration of Regorafenib (0.5 μM) and intermittent gradient induction method, the Regorafenib resistant cell line was selected for 6 months. After resistant cells and its parental cells treated with 0.5–100 μM of Regorafenib for 48 h, the cell viability was determined by CCK-8 assay. The resistance index (RI) was calculated according to the following formula: RI = the IC50 of the resistant cell line/the IC50 of the parental cell line.

2.3. CCK-8 assay

Cells were seeded in 96-well plates (5000 cells per well). Twenty-four hours after seeding, indicated concentrations of anti-cancer drugs were added to cells. Cells were then incubated for 24 h or 48 h with indicated anti-cancer drugs and cell viability was measured using Cell Counting Kit-8 assay (Beyotime, China) according to the manufacturer's instructions. Relative survival was normalized to the untreated controls after background subtraction.

2.4. Colony formation assay

Hepatocellular carcinoma cells were suspended in culture media in 6-well plates at a density of 1000 cells/well, and there are three duplicate holes in each group. After 10–15 days, the number of colonies (≥50 cells) within 5 microscope fields per well were counted and photographed. Each experiment was independently repeated at least three times.

2.5. Establishment of knockdown and overexpression cell lines

For overexpression, Pin1 CDS were subcloned into the pBybe lentiviral vector. Specific point mutations were introduced using the Quickchange Kit (TransGen Biotech, China) and sequences were verified. The Pin1 lentiviral shRNA constructs were provided by Dr. K.P. Lu, Harvard Medical School, Boston, MA. For Gli1 knockdown, short hairpin RNA (shRNA) sequences targeting Gli1 (sh-Gli1-1: 5′-CCGGCC TGATTATCTTCCTTCAGAACTCGAGTTCTGAAGGAAGATAATCAGGTT TTT-3'; sh-Gli1-2: 5′- CCGGGCTCAGCTTGTGTGTAATTATCTCGAGAT AATTACACACAAGCTGAGCTTTTT-3′) were cloned into pLKO.1-puro vectors (Sigma, St. Louis, MO, USA). The sequences were confirmed by DNA sequencing (Sangon, Shanghai, China). Lentivirus was packaged in HEK293T cells using Turbofect (Thermo Scientific, MA, USA) and the viral DNA was transduced into HCC cell lines. Cells were selected with medium containing 1.0 μg/mL puromycin (Sigma) for 24 h after 48 h of infection.

2.6. Wound healing assay

This assay followed the manufacturer's recommendations, using 2.0×10^5 cells per well in triplicate for 24 h in a 12-well plate. After incubating for 2 h in serum-free medium containing mitomycin C, a perpendicular scratch wound was generated by scratching with a 10-μL pipette tip. Images of wound fields were acquired at 0, 48 or 72 h after removing inserts. Migration rates were calculated as: migration distance/initial width of wound field. The experiment was repeated three times.

2.7. Transwell migration and invasion assay

Cells were seeded into Boyden chambers containing 24-well Transwell plates with a pore size of 8 μm (BD Bioscience). The upper chamber was either left uncoated for the migration assay or precoated with 50 μL of 1:8 diluted Matrigel (BD Bioscience) for the invasion assay. For the migration assay, cells ($2–4 \times 10^4$) were seeded into the upper chamber; for the invasion assay, $4–8 \times 10^4$ cells were seeded into the upper chamber. The upper chamber was filled with 200 μL serum-free specified medium whereas the lower chamber was filled with specified medium containing 10% FBS. Following incubation for 24 h or 48 h or 72 h at 37 °C, cells that had invaded into the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet for 1 h at room temperature and counted in five randomly-selected microscopic fields. All these experiments were performed in triplicate.

2.8. RNA extraction and qRT-PCR

Total RNA isolation, qRT-PCR, and the quantification of target gene expression were performed as previously described [25]. Briefly, total RNA was extracted from cells using Trizol reagent (Invitrogen) and reversely transcribed using the PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology). The real-time PCR was subsequently performed according to the manufacturer's instructions (TaKaRa Biotechnology). The expression levels were normalized against the internal reference gene β-actin, and the relative expression levels were displayed using the $2^{-\Delta\Delta Ct}$ method. The primers used to amplify the indicated genes are shown in Supplementary Table S1.

2.9. Western blotting

Western blotting was performed as previously described [34]. Cells were collected and lysed by RIPA buffer (150 mM NaCl, 0.5% EDTA, 50 mM Tris, 0.5% NP40) and centrifuged for 20 min at 14,000 × *g* and 4 °C. Twenty or ten micrograms of harvested total protein was loaded and separated on the 8, 10 or 12% SDS-polyacrylamide gradient gel. The proteins were then transferred onto polyvinylidene difluoride membranes and blocked with 5% non-fat milk for 2 h at room temperature. The membranes were incubated with primary antibody overnight at 4 °C, followed by HRP-conjugated secondary antibodies for 1 h at room temperature. After washing three times in TBST, protein bands were visualized using an ECL chemiluminescence system (Bio-rad). Primary antibodies used were as follows: Pin1 (Abcam, ab192036, 1:1000), Snail (CST, 3879, 1:1000), E-cadherin (CST, 3195S, 1:1000), Vimentin (CST, 3932, 1:1000), ABCG2 (CST, 42078, 1:1000), Gli1 (CST, 3538/2643, 1:1000) and β-actin (CST, 3700, 1:2000).

2.10. Cycloheximide chase experiment

SMMC-7721/Rego cells were transfected with control or Pin1 shRNA lentivirus. At 48 h after transfection, cells were treated with 50 ng/mL of cycloheximide (CHX) for the indicated timepoints. Proteins were collected at 0, 2, 4 and 8 h, and the expression of Gli1, Pin1 and β-actin were detected by western blotting.

2.11. Immunoprecipitation assay

Cells were lysed with immunoprecipitation (IP) lysis buffer (20 mM HEPES, pH7.9; 1.5 mM $MgCl_2$; 0.4 M NaCl; 0.5 mM EDTA; 1% NP-40; 10% Glycerol; 1 mM DTT; 1 mM PMSF; 1X protease inhibitor cocktail). Cell lysates were pre-cleared with protein A/G agarose beads, followed by immunoprecipitation with the appropriate antibodies at 4 °C overnight in the presence of 20 μL of protein A/G agarose beads. The beads were washed three times with lysis buffer, resuspended in 20 μL of 2X loading buffer and then subjected to immunoblot analysis.

2.12. GST-pulldown assay

Agarose beads containing GST and GST-Pin1 were incubated with HEK293T or SMMC-7721/Rego cell lysates, followed by rotation at 4 °C for overnight. Binding of the Gli1 to GTS-Pin1 was detected by immunoblotting with anti-Gli1 antibody.

2.13. Immunofluorescence

Cells seeded in confocal dishes or freezing tissue slices were fixed with 4% paraformaldehyde and then kept stable in 0.2% Triton X-100 for 10 min to rupture the cell membranes. Following three washings with PBS, non-specific antigen binding sites were blocked by 2% BSA for 30 min. Cells or slices were then incubated with anti-Pin1 (Abcam, ab192036, 1:200), anti-Snail (CST, 3879, 1; 100) and anti-E-cadherin (CST, 3195S, 1:100) antibodies overnight at 4 °C. After washing, cells or slices were incubated with secondary antibody (Beyotime, China) for 2 h and the nuclei were stained with DAPI (Beyotime, China) for 10 min, which was washed with PBS later. Cells or slices were protected from light before observation with a fluorescence microscope.

2.14. Animal experiments

All animal experiments were performed in accordance with protocols approved by the Research Animal Resource Center of Fujian Medical University. All animals were obtained from Shanghai Lingchang Biological Technology Co., Ltd. (Shanghai, China). A lung metastasis model was used. Indicated cells were harvested and washed twice with PBS. Approximately 1–4 × 10^6 cells resuspended in 150 μL PBS were injected into the tail veins of five-week-old male athymic mice. All mice were sacrificed 6 weeks after the injection, and the lungs were harvested. The metastatic nodules in each lung were counted. After photographing the lung tissues, part of the lung tissues were taken for OCT embedding for immunofluorescence detection.

2.15. Statistical analysis

Data were analyzed using SPSS software version 19.0 (SPSS Inc.). Each experiment was repeated at least three times. Values are expressed as the means ± SD. A paired *t*-test and one-way analysis of variance (ANOVA) were used to determine statistical significance between different groups. Differences were considered significant when the p-values were $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Generation and characterization of Regorafenib-resistant HCC cells

Long-term exposure of human HCC SMMC-7721 cells to low concentrations of Regorafenib resulted in Regorafenib-resistant cells SMMC-7721/Rego. As compared with parent SMMC-7721 cells, Regorafenib-resistant SMMC-7721/Rego cells became larger and the shape was much plump and the cell connection became loose (Fig. 1A). When SMMC-7721 and SMMC-7721/Rego cells were treated with different concentrations of Regorafenib for 48 h, SMMC-7721/Rego cells were less sentitive to Regorafenib, as compared with the parent cells as shown by assaying cell viability using CCK-8 method, with the drug resistance index being 2.67 (Fig. 1B). Clonal formation experiments and cell count statistics revealed that the proliferation ability of drug-resistant cells was higher than that of parental cells (Fig. 1C and E). When the HCC cell was resistant to Regorafenib, the migration and invasion abilities of SMMC-7721/Rego were significantly enhanced (Fig. 1F and I). From the mRNA and protein levels, it was found that Pin1 was significantly up-regulated in SMMC-7721/Rego cells, accompanied by up-regulation of Snail, Vimentin, Gli1, ABCG2 and down-regulation of E-cadherin (Fig. 1J and K). In addition, we also obtained Regorafenib-resistant Huh7/Rego cells, with a resistance index of 2.38 (Supplementary Fig. S1A). In the Huh7/Rego cells, Pin1 was up-regulated and accompanied by EMT, and the migration and invasion ability was enhanced (Supplementary Figs. S1B–1F). Moreover, Pin1 knockdown was also able to reduce the above phenotypes (Supplementary Figs. S2A–2E). These results suggest that Regorafenib-resistant cells display the EMT and might have increased tumor metastasis.

We mainly used SMMC-7721/Rego cells with higher drug resistance index for subsequent experiments. To examine this possibility, we injected SMMC-7721 cells and SMMC-7721/Rego cells into tail vein of mice and compare their lung metastasis *in vivo*. Whereas SMMC-7721 cells showed almost no metastasis, SMMC-7721/Rego cells showed prominent lung metastasis, with multiple pulmonary nodules being observed in the lungs after 6 weeks of experimentation, (Fig. 2A and B), although there was almost no difference in the body weight of the mice (Fig. 2D). H&E staining further confirmed tumor metastasis in the lung (Fig. 2E). In immunofluorescence experiments, Pin1 and Snail were up-regulated, and E-cadherin was down-regulated in lung tissues of SMMC-7721/Rego group (Fig. 2E), consistent with their *in vitro* phenotypes (Fig. 1K). Taken together, these results indicate that the HCC SMMC-7721 cells after Regorafenib resistance display the EMT as well as elevated ability in tumor invasion and metastasis *in vitro* and *in vivo*.

3.2. Pin1 and EMT have correlation in hepatocellular carcinoma

The above findings that Regorafenib resistant SMMC-7721/Rego cells expressed elevated Pin1 and our previous findings that Pin1 promotes the EMT in breast cancer [14] led us to speculate that Pin1 might

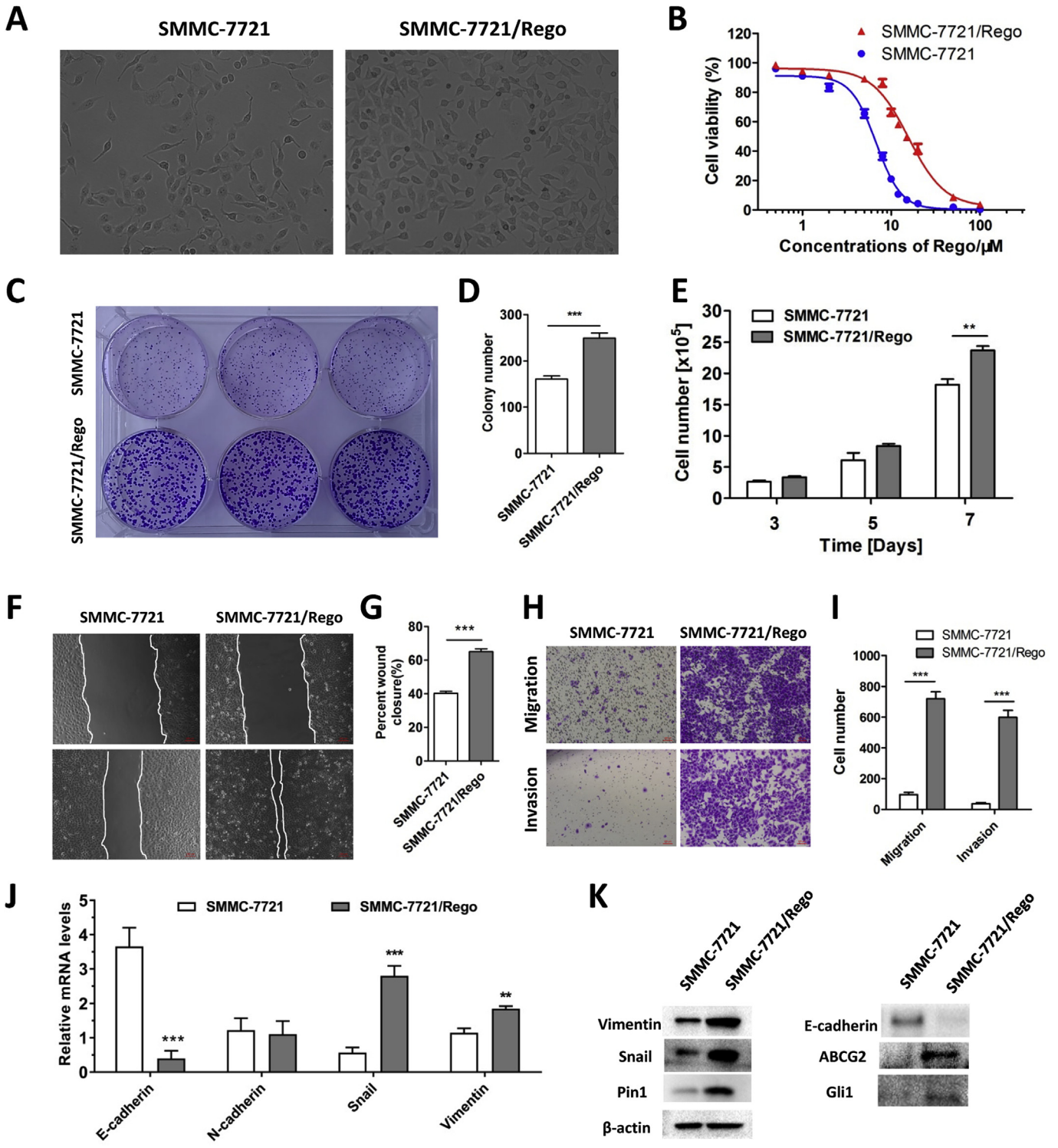

Fig. 1. In-vitro characterization of Regorafenib resistant cells. After SMMC-7721 was long-term treated by Regorafenib for 6 months to prepare SMMC-7721/Rego, SMMC-7721/Rego showed a fibroblast-like, mesenchymal morphology (A) and exhibited resistance to Regorafenib (B). Compared with the parental cell SMMC-7721, SMMC-7721/Rego showed stronger colony forming ability (C, D) and proliferation ability (E), higher migration capabilities by wound-healing assay, and very stronger migration and invasion capabilities by transwell experiments. RT-PCR and western blot were used to detect changes in mRNA and protein levels of EMT-related molecules, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$.

play an important role in the above Regorafenib resistance phenotype. To examine this possibility, we first used expression correlations between Pin1 and EMT phenotypes in human HCC specimens, and used Pin1 knockdown and overexpression to examine the role of Pin1 in EMT and metastasis and elucidate its underlying mechanisms. In order to detect the relationship between the expression levels of Pin1 and EMT-related proteins in clinical samples of hepatocellular carcinoma, we performed protein extraction and made frozen sections by OCT embedding. Western blot and immunofluorescence experiments were used to evaluate the relationship between EMT-related protein and Pin1 expression. The relationships between Pin1 and E-cadherin, Pin1 and Snail in 10 HCC tumor samples were analyzed by western blot and

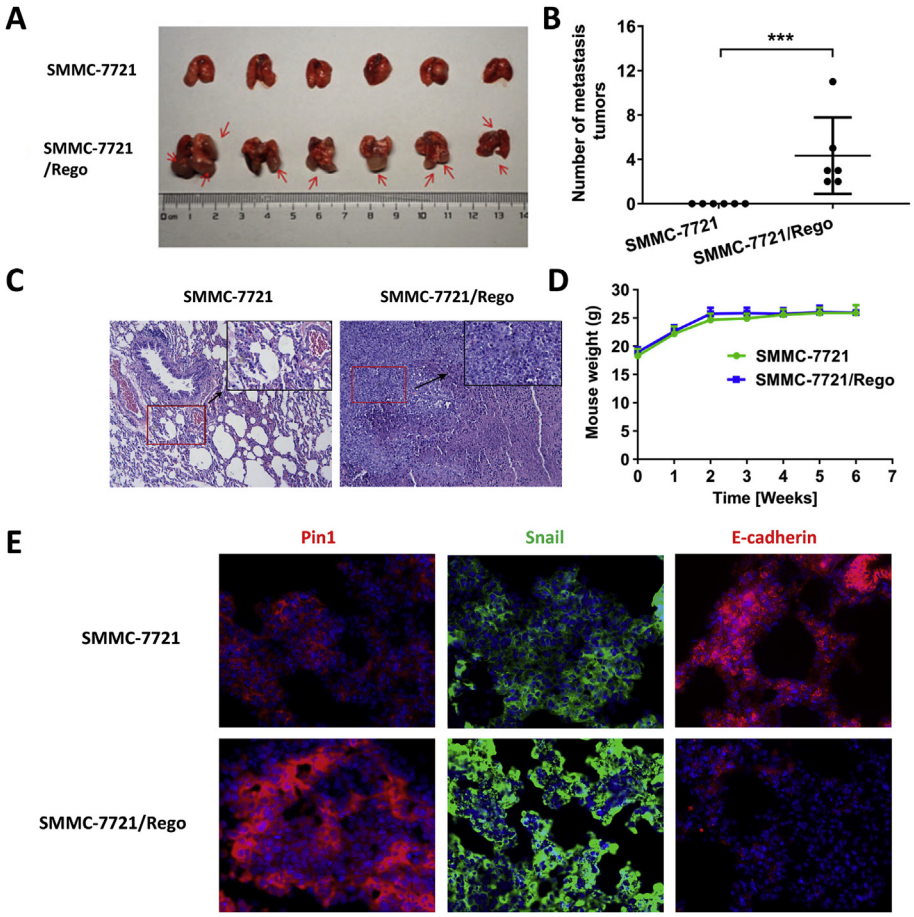

Fig. 2. In-vivo metastasis ability of Regorafenib resistant cells. Nude mice (n = 6/group) were injected with Regorafenib resistant cell SMMC-7721/Rego and its parental cells into the tail vein, respectively. After 6 weeks, the lung tissues were dissected and photographed (A), and the number of nodules was counted (B, red arrow). C, H&E staining analysis of lung tissue. D, The weight change curve of mice within 6 weeks. E, Immunofluorescence analysis of the differential expression of Pin1, Snail, and E-cadherin proteins in pulmonary nodules. ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fitted within scatter diagram (Fig. 3A and B), suggesting a correlation between Pin1 and EMT markers. It was found that samples with high expression of Pin1, transcription factor Snail was also in high expression, while E-cadherin was in low expression. On the contrary, the expression of Snail was decreased and the expression of E-cadherin was significantly decreased in samples with low expression of Pin1 (Fig. 3C and D). These results show that Pin1 overexpression is correlated with EMT phenotypes including upregulated Snail and downregulated E-cadherin in human HCC specimens.

3.3. Pin1 knockdown reduces migration and invasion of HCC cells

Pin1 is prevalently overexpressed in human cancers including ~70% of HCC, and promotes tumorigenesis by activating multiple cancer-driving pathways [21]. However, its role in liver cancer metastasis is poorly understood. Therefore, we explored whether knockdown of Pin1 affected the migration and invasion of HCC cells. After Pin1 was stably silenced in Huh7 and MHCC-97H cell lines, which had high Pin1 expression, via lentivirus-mediated transduction, cells showed the epithelial-like phenotype (Fig. 4A). The wound-healing and transwell assays indicated that Pin1 knockdown dramatically inhibited the migration (Fig. 4B–E) and invasion ability of HCC cells (Fig. 4B and D). Pin1 knockdown decreased EMT-associated transcription factor Snail and increased E-cadherin expression (Fig. 4F). Similar results were observed by RT-PCR and immunofluorescence experiments (Fig. 4G and I). Taken together, these data indicate that Pin1 knockdown reversed the EMT in HCC cells with downregulated Snail and upregulated E-cadherin.

3.4. Pin1 overexpression promotes cell migration and invasion

To further confirm the role of Pin1 in the EMT of hepatocytes and HCC cells, we overexpressed Pin1 expression in human normal hepatocyte cell line THLE3 … In normal hepatocytes, the wound-healing and transwell migration assays showed that Pin1 overexpression increased cell migration (Supplementary Figs. S3A–3D). In liver cancer cells, SMMC-7721, MHCC-97H and Huh7 cells all expressed higher Pin1 than that of normal hepatocytes. Among the above three cells, SMMC-7721 has a relatively low Pin1 expression, so we chose SMMC-7721 for Pin1 overexpression. Pin1-overexpressing SMMC-7721 cells developed a fibroblast-like appearance, suggesting the transition to a mesenchymal phenotype (Fig. 5A). Neither W34A (the substrate binding-deficient mutant [16]) nor K63A (the catalytically inactive mutant [16]) Pin1 point mutant induced such morphologic changes. To confirm that Pin1-overexpressing cells had undergone the EMT, we analyzed epithelial and mesenchymal markers using qRT-PCR and western blot analysis. Indeed, Pin1 overexpression drastically downregulated mRNA levels of epithelial markers, such as E-cadherin, but upregulated expression of mesenchymal markers, such as N-cadherin and Snail (Fig. 5C). These results were further confirmed by the findings that Pin1-overexpressed cells increased protein level of Snail and Gli1, whereas W34A or K63A mutants had no effect (Fig. 5B). Moreover, ectopic Flag-Pin1, but not W34A or K63A mutant expression caused an increase in cell migration and invasion, the property associated with EMT, as measured by wound healing assay and transwell assay (Fig. 5D and I).

3.5. Pin1 promotes metastasis of HCC cells partly by upregulating Gli1

Gli1 is a key factor in the Hedgehog pathway that contributes to the amplification of Hedgehog signals [29] and has been shown to play a

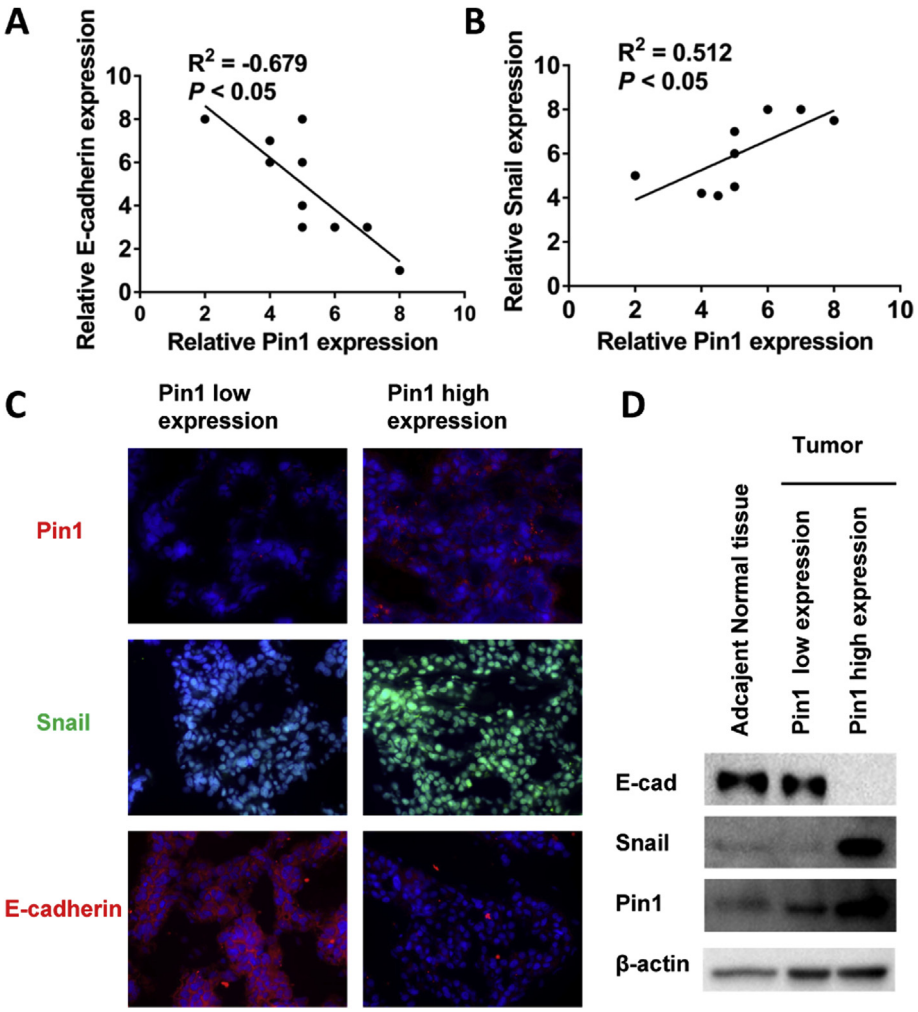

Fig. 3. Pin1 protein expression correlates with EMT-related protein expression in clinical liver cancer samples. Correlation analysis between Pin1 and E-cadherin, Pin1 and Snail were shown in A and B, respectively. C, Immunofluorescence detection of Pin1 and EMT-related protein expression in clinical samples of hepatocellular carcinoma tissues; D, Western blot detection of Pin1 and EMT-related protein expression in above samples.

role in the EMT [30]. After the study of the correlation between the expression of Pin1 and EMT molecules, we further analyzed liver cancer tissues and found that Pin1 and Gli1 expression also had a positive correlation (Fig. 6A). To demonstrate whether Gli1 plays a role in Pin1-regulated EMT, we used Gli1 inhibitor GANT61 to treat Huh7 and SMMC-7721/Rego cells for 48 h. GANT61 effectively inhibited Gli1 expression and down-regulated Vimentin, Snail (Fig. 6B and C). These results suggest that Gli1 might regulate the migration and invasion of HCC cells by regulating EMT-related proteins such as Snail and Vimentin. Though Gli1 inhibition by inhibitor GANT61 resulted in a decrease of Pin1 expression,Gli1 knockdown did not affect Pin1 expression in SMMC-7721/Rego and Pin1-SMMC-7721 cells (Fig. 6D and G). In co-immunoprecipitation assay, endogenous Pin1 co-immunoprecipitated with endogenous Gli1 in SMMC-7721/Rego cells (Fig. 6E). In GST-pulldown assay, GST-Pin1 but not GST pulled down Gli1 from cell lysates of SMMC-7721/Rego (Fig. 6F). Treatment of cell lysates with the calf intestinal alkaline phosphatase (CIP) greatly reduced Pin1/GST-Pin1-associated Gli1 (Fig. 6E and F), indicating that Pin1 binds to phosphorylated Gli1. Since Pin1 has been shown to regulate proteins stability of many Pin1 substrate transcriptional factors, as described [31,32], we examined whether Pin1 modulates Gli1 protein stability using CHX chase experiments in SMMC-7721/Rego cells. The metabolic stability of Gli1 was significantly decreased in Pin1 knockdown cells. The half-life of Gli1 was significantly affected by Pin1 KD, especially at the early time points (Fig. 6I). It is likely due to increased protein turnover. To examine the significance of Gli1 in mediating Pin1 function, we downregulated Gli1 in SMMC-7721-Pin1 (Pin1 overexpression) cells using shRNAs. Gli1 protein and mRNA expression were successfully down-regulated by the shRNAs (Fig. 6G and H). Importantly, knockdown of Gli1 inhibited the migration and invasion capacity of SMMC-7721-Pin1 cells (Fig. 6J–O). Collectively, these results further support that overexpression of Pin1 promoted migration and invasion in hepatocellular carcinoma partly by upregulating Gli1 expression.

3.6. Pin1 regulates migration, invasion and metastasis of Regorafenib-resistant HCC cells in vitro and in vivo

The above results indicate that Pin1 is overexpressed in Regorafenib-resistant HCC cells and that Pin1 overexpression promotes the EMT, migration, invasion and metastasis of HCC cells, suggest that Pin1 inhibition might reverse resistance to Regorafenib. To examine this possibility, we used lentivirus-mediated genetic knockdown and a Pin1 inhibitor ATRA to study their effects on SMMC-7721/Rego, respectively. It was found that Pin1 knockdown did not significantly change the proliferation and clonal formation of SMMC-7721/Rego cells (Fig. 7A and B). However, Pin1 knockdown could effectively inhibit its migration and invasion abilities (Fig. 7C and F), down-regulate Gli1, Vimentin, Snail, and up-regulate E-cadherin (Fig. 7G). In the lung metastasis experiment, Pin1 knockdown reduced the number of

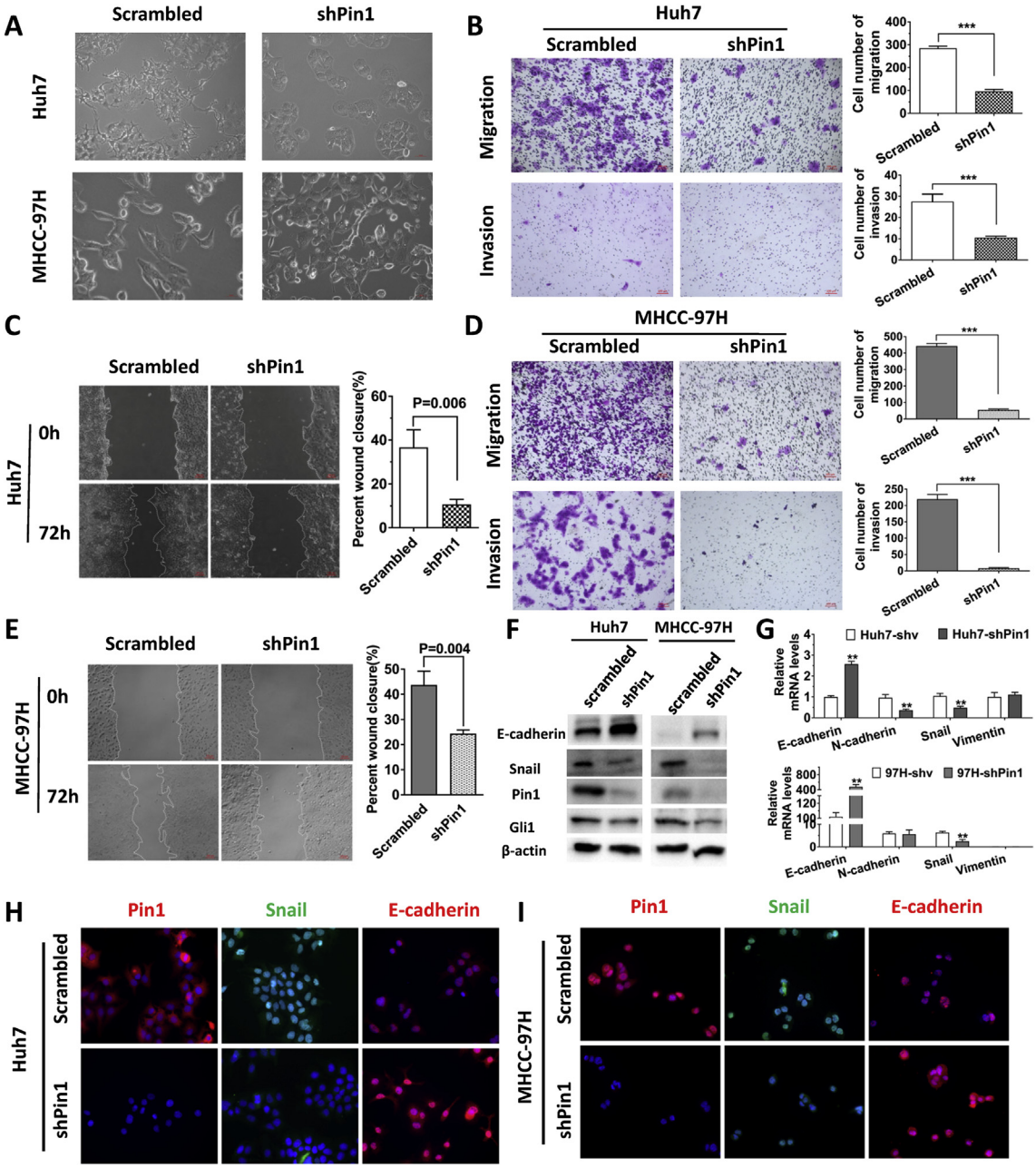

Fig. 4. Pin1 knockdown inhibits HCC cell migration, invasion and interferes with expression of EMT-related molecules. Huh7 and MHCC-97H cells were transfected with lentiviruses containing a shRNA-Pin1 vector or empty control vector. Pin1 knockdown of Huh7 and MHCC-97H cells showed epithelial morphology (A). Pin1 knockdown inhibited cell migration capacity of Huh7 (C) and MHCC-97H (E), as determined by wound-healing migration assay. B and D, Qualitative and quantitative analysis of transwell migration and invasion assays. Magnification, × 200 (n = 3). F and G, Western blot and RT-PCR confirming the significantly different expression of EMT-related molecules for scrambled and shPin1 in Huh7 and MHCC-97H cells. H and I, their corresponding differential expression were detected by immunofluorescence experiments. Values are mean ± SD, **P < 0.01, ***P < 0.001.

metastatic nodules in lung tissue (Fig. 7H and J). Immunofluorescence analyses of lung tissue showed that Pin1 was down-regulated, along with up-regulation of E-cadherin and down-regulation of Snail (Fig. 7K). The above results indicate that Pin1 inhibitors might reverse Regorafenib-resistance. We used the Pin1 inhibitor ATRA to pre-treat SMMC-7721/Rego cells at 0, 20, 30 and 40 μM for 72 h, then further treatment for 48 h. We found that although ATRA did not significantly change cell proliferation (Supplementary Fig. S4), it effectively inhibited the migration and invasion ability of SMMC-7721/Rego cells in a concentration-dependent manner (Fig. 8A and F). Similar results were observed in in-vivo experiments, the number of nodules in the lungs was reduced after treatment with ATRA (Fig. 8G and I). ATRA down-regulated Pin1 and Snail, and up-regulated E-cadherin (Fig. 8J), suggesting that Pin1 inhibitor ATRA might effectively alleviate Regorafenib resistance-induced metastasis, possibly mainly via reversing the EMT.

4. Discussion

Regorafenib has recently been approved by the US FDA for second-line treatment of patients with unresectable hepatocellular carcinoma that had progressed following first-line sorafenib [4]. Despite The patient have received good survival benefit of multikinase inhibitor Regorafenib [3]. Unfortunately, Regorafenib faces the risk of drug

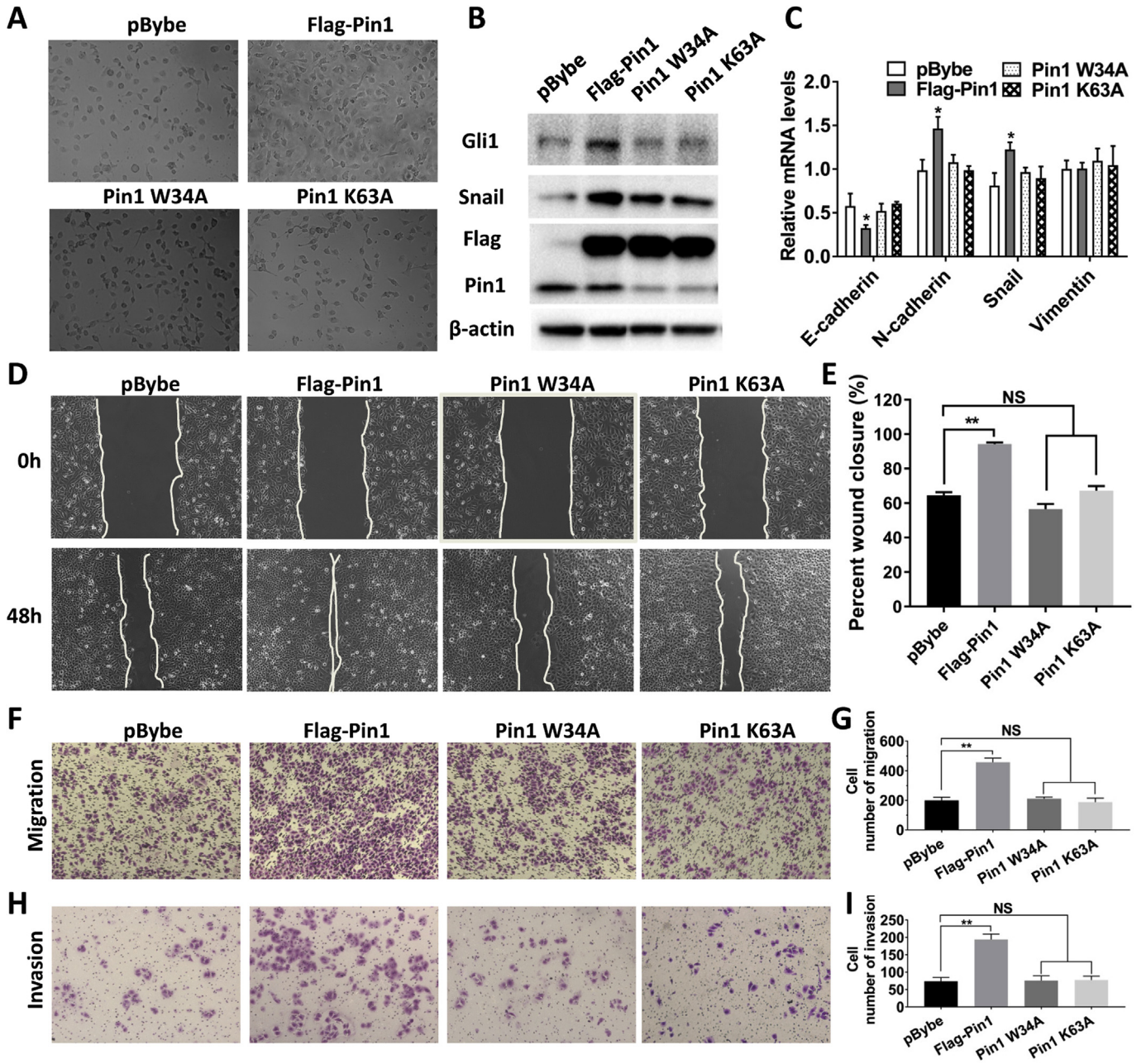

Fig. 5. Pin1 overexpression potently promotes EMT in SMMC-7721 cells. A, SMMC-7721 cells overexpressing Pin1, but not its mutant, showed a fibroblast-like, mesenchymal morphology. B, Pin1 overexpression, but not its mutants, upregulated Snail and Gli1 expression. C, Pin1 overexpression induced the downregulation of E-cadherin mRNA and upregulation of N-cadherin, Snail and Vimentin mRNA, determined by real-time RT-PCR. β-actin expression was used to normalize the variability in template loading. D-E, The wound-healing assay. F and H, Pin1 overexpression, but not its mutants, increased cell migration and invasion in the transwell assay. Quantified were the numbers of cells that transversed the transwell membranes (G and I). In all panels, bar graphs present mean SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

resistance in the treatment of colon carcinoma [33]. However, little is known about underlying mechanisms of Regorafenib resistance in HCC. In present study, we screened the Regorafenib-resistant HCC cell line for the first time and it showed very high metastatic potential *in vitro* and *in vivo*. It is very interesting that Pin1 was abnormally highly expressed in the HCC cell line SMMC-7721 after resistant to Regorafenib. Furthermore, Pin1 was highly expressed in HCC tissues and was associated with EMT and Pin1 promotes the migration and invasion of HCC cells through EMT. Moreover, Pin1 genetic and chemical inhibition potently reverses Regorafenib resistance by inhibiting the EMT partly via the Gli1/Snail/E-cadherin pathway. These results not only reveal for the first time molecular mechanisms underlying Regorafenib resistance in HCC, but also provide first evidence that Pin1 inhibitors offer an attractive strategy for treating aggressive and Regorafenib-resistant HCC.

In cancer, Pin1 is widely overexpressed and/or over-activated, and a high level of overexpression and/or overactivation correlates with poor clinical prognosis [34]. The essence of Pin1 is the catalytic molecule that acts on a series of oncogene signaling pathways that lead to tumorigenesis [17,35]. Both in-vivo and in-vitro experiments show that knockdown or knockout of the Pin1 gene inhibits tumor growth and tumorigenesis [14,36]. Our previous data show that Pin1 was high expression, which is consistent with the literature. These results mainly reflect the role of Pin1 in tumor growth and tumorigenesis. However,

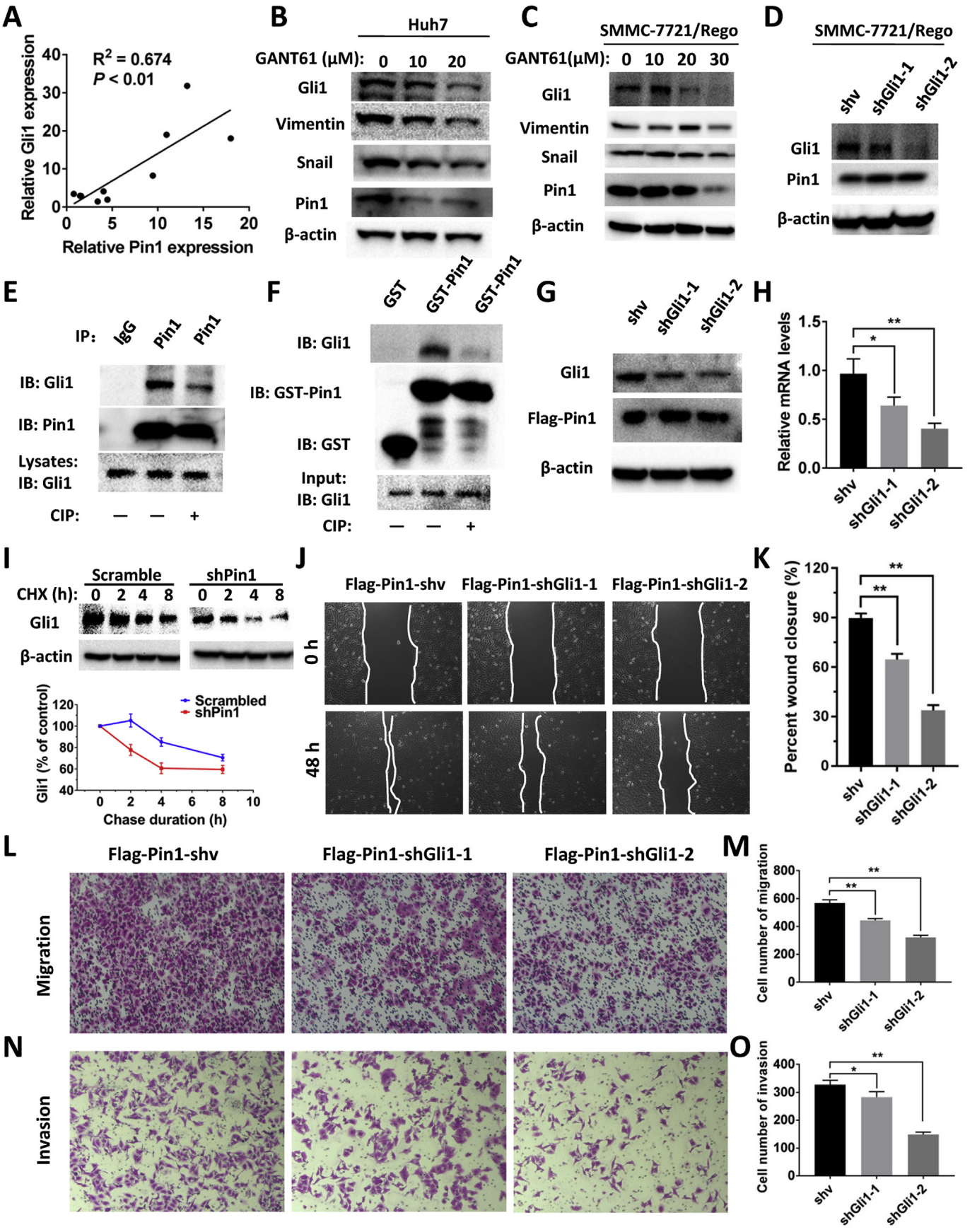

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Fig. 6. Gli1 interacts with Pin1 and Gli1 mediates Pin1-induced migration and invasion of SMMC-7721 cells. A, Correlation analysis between Pin1 and Gli1 in clinical liver cancer samples. B and C, Effect of Gli1 inhibitor GANT61 on Pin1 and EMT-related pathway proteins in Huh7 and SMMC-7721/Rego cells. D, SMMC-7721/Rego cells were transfected with Gli1 shRNAs, western blot of Gli1 and Pin1 protein expression. E, Endogenous Pin1 was immunoprecipitated from SMMC-7721/Rego cells and immunoblotted for associated Gli1. IgG was used as a control. F, GST pulldown with glutathione beads of bacteria-purified GST-tagged Pin1 in the presence of a protein extract derived from SMMC-7721/Rego cells. SMMC-7721/Rego cell lysates were pretreated with CIP for 1 h, followed by GST pull-down assay. SMMC-7721-Flag-Pin1 cells were transfected with Gli1 shRNAs or a control shRNA and assessed 48 h later. G, Western blot of Gli1 protein expression. H, Quantitative PCR analysis of Gli1 mRNA expression. I, SMMC-7721/Rego cells transfected with control or Pin1 shRNA were treated with cycloheximide (CHX) for the indicated time points and immunoblotted for the expression of Gli1, Pin1 and β-actin. J-O, qualitative and quantitative analysis of wound-healing, migration and invasion assays for shGli1 in SMMC-7721-Flag-Pin1 cells. *P < 0.05, **P < 0.01.

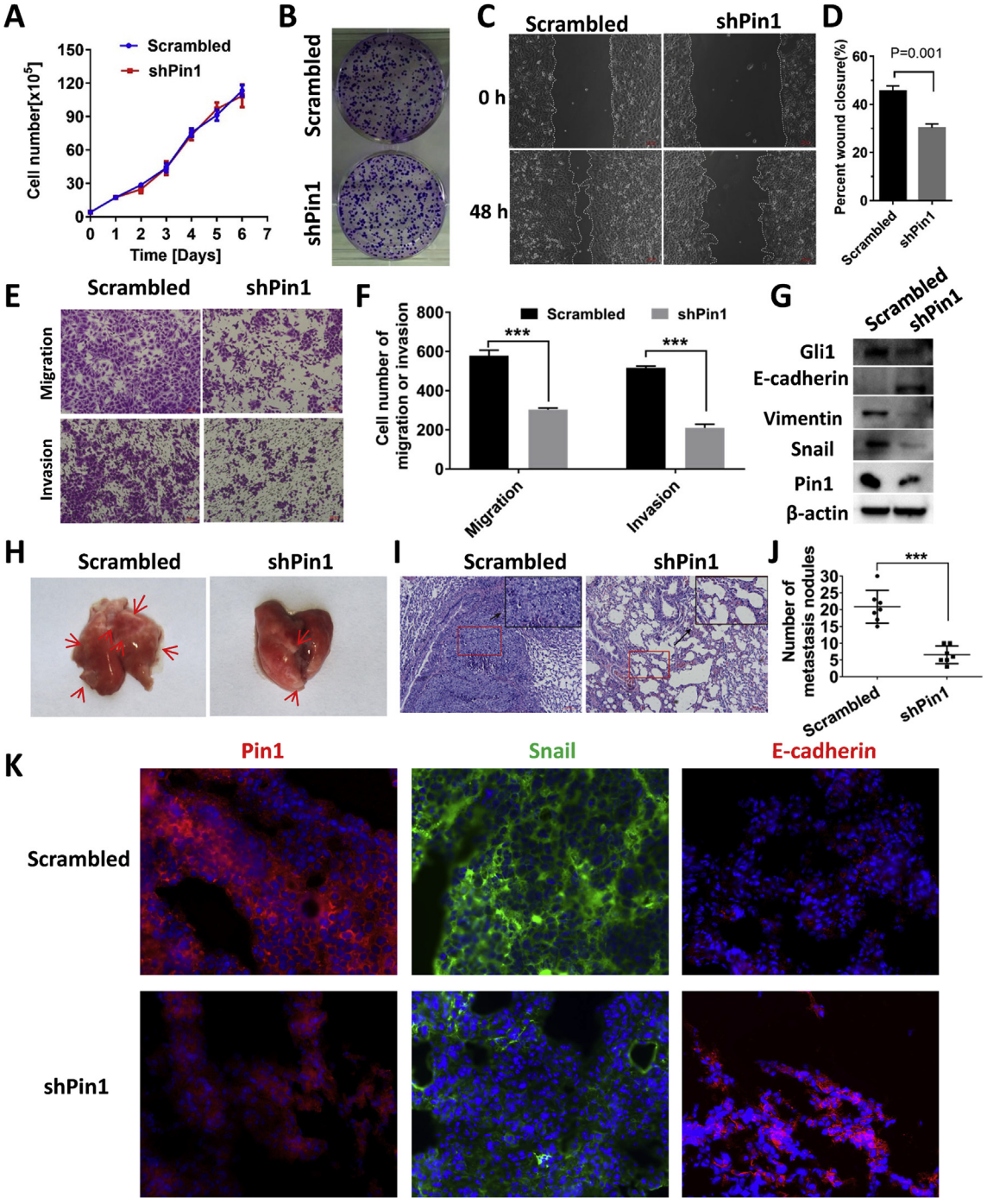

Fig. 7. Effect of Pin1 knockdown on the proliferation, migration, invasion and metastasis of SMMC-7721/Rego. SMMC-7721/Rego cells were infected with lentiviruses expressing scrambled or Pin1 shRNA. A, SMMC-7721/Rego cells with Pin1 knockdown were plotted over time, based on the cell numbers counted daily. B, Qualitative analysis of colony formation. C and D, Pin1 knockdown of SMMC-7721/Rego reduced cell migration in wound-healing assay. E and F, Transwell migration and invasion assays. G, Pin1 knockdown interfered with the expression of EMT-related molecules. In the mouse tail vein metastasis model, Pin1 knockdown reduced lung metastasis of SMMC-7721/Rego (H–J), and immunohistochemical analysis of Pin1, Snail, E-cadherin expression (K). ***P < 0.001.

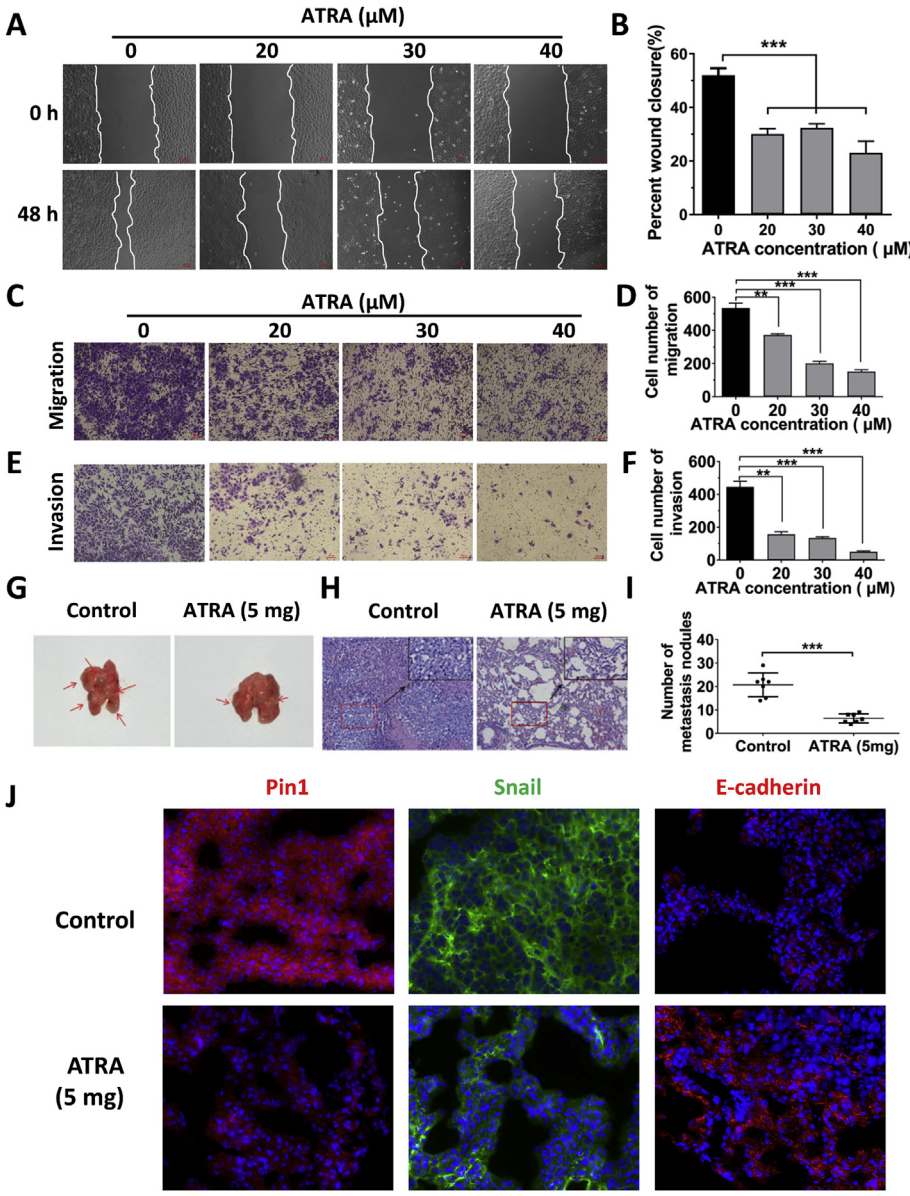

Fig. 8. Pin1 inhibitor ATRA restrains the migration, invasion and metastasis of SMMC-7721/Rego. A and B, Effect of different concentrations of ATRA for 5 days on migration of SMMC-7721/Rego cells by wound-healing assay (Bar = 100 μm). C-F, Transwell migration and invasion assays. G-I, SMMC-7721/Rego cells (2×10^6 cells) were injected into the tail vein of 6-week-old male nude mice (n = 6/group). The next day, the neck was embedding 5 mg ATRA controlled release tablets. Quantification of lung metastasis at 5 weeks after injection. J, ATRA reduced Pin1, Snail, E-cadherin expression by immunohistochemical analysis. **P < 0.01, ***P < 0.001.

the role of Pin1 in invasion and metastasis has rarely been reported, especially in liver cancer. Preliminary analysis of Pin1 expression in HCC tissues had a certain correlation with EMT molecular expression of E-cadherin and Snail which suggests that Pin1 may regulate the EMT of liver cancer cells.

The epithelial-mesenchymal transition has been studied in normal mammalian development for decades, and it has been proposed as a critical mechanism during metastasis and cancer progression [13]. A large number of studies have shown that acquired resistance is closely related to EMT [6]. E-cadherin is considered to be a suppressor of tumor invasion and metastasis [37], and Snail, Slug and Zeb1 bind to the E-cadherin promoter and function as direct transcriptional repressors of E-cadherin [38]. Here, our results showed that HCC cell line SMMC-7721 was highly metastatic and had an EMT process after resistance to Regorafenib, and knockdown of Pin1 upregulated E-cadherin and downregulated Snail in HCC cells and Regorafenib-resistant cells. Novel anti-EMT therapies have the potential to benefit patients with high risk of recurrence or metastatic development. However, Pin1 may be a target for targeted EMT therapy.

Regorafenib has developed resistance in the treatment of colorectal cancer (CRC) [39]. CRC cells containing FBW7-inactivating mutations are insensitive to clinically used Regorafenib [33]. Since Regorafenib has just been clinically approved for the treatment of hepatocellular carcinoma, there is no clinical Regorafenib resistance. We found the resistance of Regorafenib in HCC cells through low-dose induced screening. Regarding the mechanism study, we detected mutations in FBXW7 before and after Regorafenib resistance in HCC cells, and found that several common mutation sites R465, R479, R505, R658 were not mutated. Sorafenib is believed to antagonize tumor progression by inhibiting TGF-β-induced EMT, and HCC cells exhibit EMT after long-term exposure to sorafenib [9]. Therefore, the resistance of Regorafenib in hepatocellular carcinoma may be closely related to EMT.

Hedgehog (Hh) signaling is involved in the progression of hepatocellular carcinoma (HCC). The expression of Gli1, while not Gli2 or Gli3, is significantly increased in HCC cell lines. And overexpression of Gli1 can promote the migration, invasion, and epithelial mesenchymal transition (EMT) of HCC cells [30]. Our results showed that Gli1 regulated Pin1 induced EMT in SMMC-7721/Rego cells. It is speculated that Pin1 regulates the resistance-induced metastasis of Regorafenib, possibly by targeting Gli1.

Recent mechanism-based FP-HTS drug screens has unexpectedly identified that Pin1 is a major drug target for ATRA in APL and triple-

negative breast cancer. The ability of ATRA to inhibit Pin1 function has been confirmed in breast cancer [40], liver cancer [25,28] even using a different ATRA controlled release formulation [27], and acute myeloid leukemia (AML) [35], as well as in lupus [41] and asthma [42]. Now we have shown that ATRA also effectively reverse the invasion and metastasis caused by Regorafenib resistance. This provides some data support for ATRA treatment of solid tumors.

In summary, we screened Regorafenib-resistant HCC cells for the first time, and resistant cells showed extremely strong metastasis characteristics. Our findings suggest that Pin1 inhibition reversed EMT and reduced metastasis of Regorafenib-resistant HCC cells partly via the Gli1/Snail/E-cadherin pathway. These results not only reveal a novel molecular mechanism underlying Regorafenib resistance in HCC, but also provide first evidence that Pin1 inhibitors offer an attractive strategy for treating aggressive and Regorafenib-resistant HCC.

Conflicts of interest statement

Dr. Lu and Dr. Zhou are inventors of Pin1 technology, which was licensed by BIDMC to Pinteon Therapeutics. Both Dr. Lu and Dr. Zhou own equity in, and consult for, Pinteon. Their interests were reviewed and are managed by BIDMC in accordance with its conflict of interes policy.

Acknowledgements

This research was supported by the Joint Funds for the Innovation of Science and Technology, Fujian province (No. 2016Y9037), the Joint Funds for Health and Education of Fujian Province (No. WKJ2016-2-34), the Natural Science Foundation of Fujian Province (No. 2016J05190), the Nursery Research Fund Project of Fujian Medical University (No. 2015MP035), the Scientific Research Personnel Training Project for Health and Family Planning of Fujian Province (No. 2016-1-34), the Youth Research Project of Fujian Provincial Department of Education (No. JAT160213) and National Institutes of Health grant R01CA205153.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.12.010.

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