

Histidine-rich calcium binding protein promotes growth of hepatocellular carcinoma *in vitro* and *in vivo*

Jingmei Liu,¹ Ping Han,¹ Mengke Li,¹ Wei Yan,¹ Jiqiao Liu,² Jiayi He,¹ Jin Gong,¹ Yunwu Wang¹ and Dean Tian¹

Department of ¹Gastroenterology; ²Ultrasound, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Key words

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Correspondence

Dean Tian, Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
Tel: +0086-27-8366-3595; Fax: +0086-27-8366-3595;
E-mail: datian@tjh.tjmu.edu.cn

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Hepatocellular carcinoma (HCC) is a prevalent malignant tumor and the third leading cause of cancer death worldwide.⁽¹⁾ Although substantial oncogenes and tumor suppressor genes have been associated with HCC, the underlying molecular mechanism of HCC pathogenesis remains far from fully understood. Therefore, novel therapeutic candidates for HCC are urgently needed.

The histidine-rich calcium binding protein (HRC) plays a pivotal role in Ca²⁺-homeostasis.⁽²⁾ Calcium (Ca²⁺) is an essential intracellular signaling molecule involved in the regulation of cancer progression, including cell proliferation, apoptosis, invasion and migration.^(3,4) Recently, multiple calcium-binding proteins have been shown to be implicated in HCC progression.^(5–7) Our previous study showed that HRC was overexpressed in human HCC tissues, and positively correlated with the tumor size and metastasis of HCC.⁽⁸⁾ Nevertheless, the exact role of HRC in HCC tumorigenesis remains to be clarified.

Substantial evidence has elucidated that abnormal cellular proliferation and apoptosis are common events in cancer.⁽⁹⁾ Calcium signals can activate ERK and AKT signal transduction, which have been reported to act as the potent proliferative factors in HCC.^(10–12) Endoplasmic reticulum (ER) stress is associated with several types of human cancers, and it plays a crucial role in regulating cell survival and death.^(13–15) More-

We have recently shown that the histidine-rich calcium binding protein (HRC) promotes the invasion and metastasis of hepatocellular carcinoma (HCC). In the current study, we evaluated whether HRC may also affect the growth of HCC. We found that ectopic expression of HRC obviously enhanced proliferation and colony formation, while suppression of HRC exhibited inhibitory effects. Furthermore, we demonstrated that HRC promoted tumor growth in nude mice. These effects may result from the ability of HRC to upregulate cyclinD1 and cyclin-dependent kinase 2 (CDK2) expressions and promote G1/S transition. Further study showed that MEK/ERK signaling pathway was involved in HRC-induced cell proliferation. Interestingly, overexpression or depletion of HRC revealed its regulation on endoplasmic reticulum stress (ERS) and apoptosis, which was partially dependent on PERK/ATF4/CHOP signaling pathway. In addition, blocking ERS using 4-phenylbutyric acid (4-PBA) not only downregulated the expression of PERK, ATF4 and CHOP, but also significantly decreased apoptosis induced by HRC silence, whereas ERS inducer thapsigargin (TG) exerted the opposite effects. Our study thus demonstrates a role of HRC in promoting HCC growth, besides its role in inducing HCC metastasis, and highlights HRC as a promising intervention target for HCC.

over, recent studies have shown that unbalanced calcium homeostasis can lead to ER stress.^(16,17)

In this study, for the first time, we showed that HRC promoted the growth of the human HCC cells both *in vitro* and *in vivo*. The mechanism may be due to the regulation of proliferation, cell cycle and apoptosis. More importantly, we found that HRC-induced cell growth and apoptosis inhibition were mediated by the MEK/ERK pathway and ER stress, respectively. In conclusion, we propose that HRC is a potential intervention target of HCC.

Materials and Methods

Cell culture, reagents and antibodies. The human HCC cell lines Sk-hep-1 and SMMC-7721 (Institute of Liver Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) were routinely cultured in DMEM supplemented with 10% FBS in a 5% CO₂ atmosphere at 37°C. The ER stress inhibitor 4-phenylbutyric acid (4-PBA) and inducer thapsigargin (TG) were purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibodies are shown in Supplementary Table S1.

RNA extraction and quantitative real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA,

USA) according to the manufacturer's instructions. Reverse-transcribed complementary DNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). Real-time PCR was performed using SYBR Premix ExTaq (TaKaRa) on an ABI StepOne Real-Time PCR System (Applied Biosystem, Carlsbad, CA, USA). The sequences of the primers used for RT-qPCR are listed in Supplementary Table S2.

Plasmid, small interfering RNA and transfection. The human HRC plasmid (pcDNA3.1-HRC) was purchased from Genechem (Guangzhou, China), and small interfering RNA (siRNA) was synthesized and purified by RiboBio (Guangzhou, China). The HRC siRNA sequence used was 5'-CCACAGAGACGAG-GAAGAAAdTdT-3'. Transfection of siRNA and plasmids was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Western blotting analysis. Western blotting was performed as previously described.⁽¹⁸⁾ Briefly, Samples containing 30 μ g of total protein were resolved on 10% polyacrylamide SDS gels, and electrophoretically transferred to polyvinylidene difluoride

(PVDF) membranes. The membranes were blocked with 5% skim milk, incubated with appropriate primary antibodies and HRP-conjugated suitable secondary antibodies, followed by detection with enhanced chemiluminescence reagents (Pierce Chemical, Rockford, IL, USA). GAPDH was used as a loading control.

CCK-8 assay. The cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, the cells were pretreated with or without PI3K/Akt inhibitor LY294002(10 μ m) and MEK inhibitor U0126(10 μ m), and then transfected with plasmids or siRNA. At least five wells were used for each group. After the incubation, 10 μ L of the CCK-8 reagent was added to each well. The absorbance was measured at 450 nm using a BioTek ELX800 microplate reader (BioTek, Vermont, NE, USA).

Cell cycle analysis. Forty-eight hours after transfection, cells were harvested, washed with PBS and fixed in 70% ethanol at 4°C overnight. After fixation, cells were washed twice with

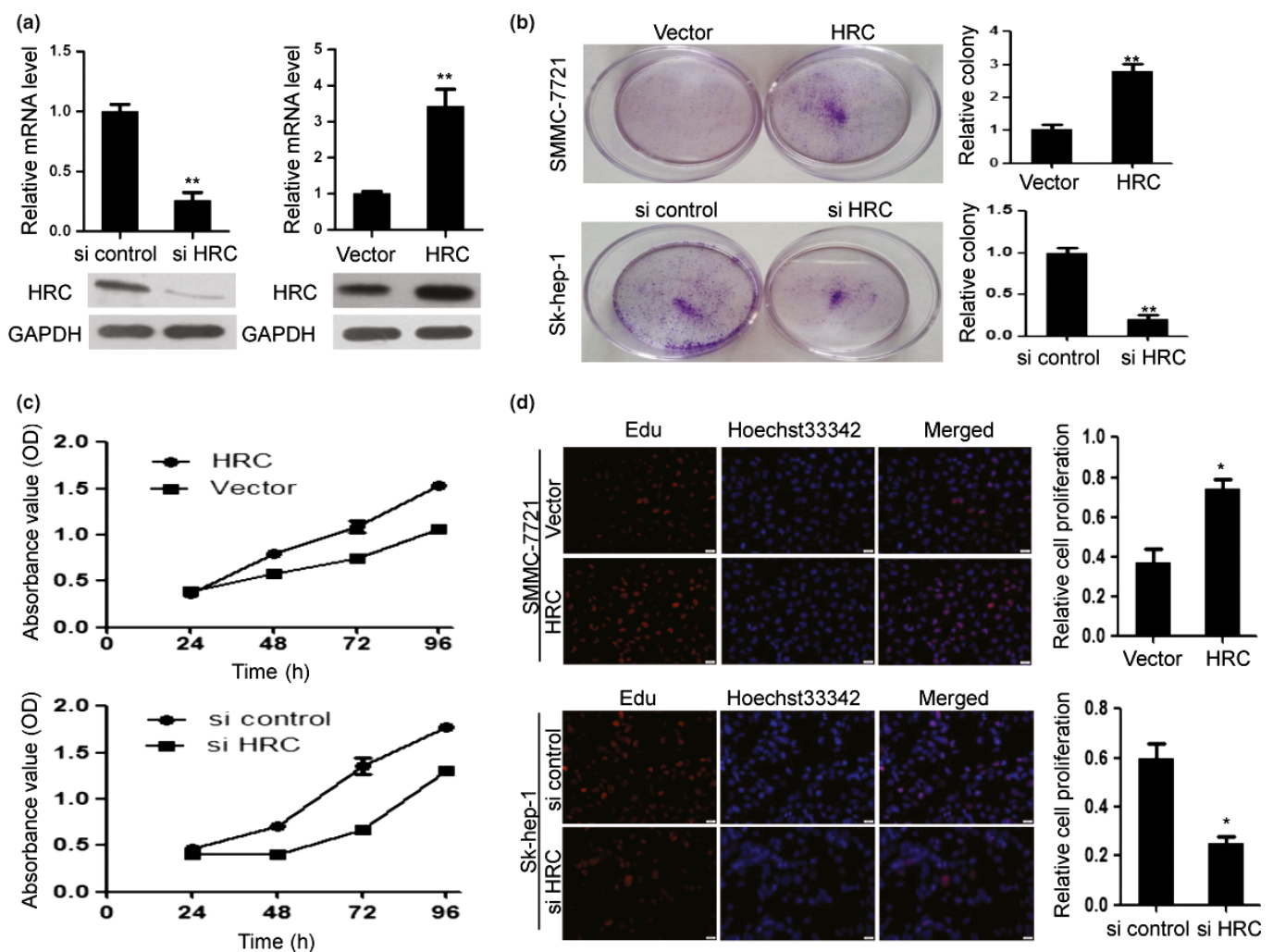


Fig. 1. Histidine-rich calcium binding protein (HRC) promotes hepatocellular carcinoma (HCC) cell proliferation *in vitro*. (a) Relative expression of HRC in SMMC-7721 cells transfected with plasmids (pcDNA3.1-HRC and pcDNA3.1-vector) and Sk-hep-1 cells transfected with siRNA (si control and si HRC) were examined by RT-qPCR and western blotting. (b) Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies in treated SMMC-7721 and Sk-hep-1 cells. (c) Cell Counting Kit-8 (CCK-8) assay revealed that overexpression of HRC in SMMC-7721 cells enhanced cell proliferation, and HRC knockdown in Sk-hep-1 cells suppressed cell proliferation. (d) Representative micrographs (left) and quantification (right) of EdU-labeling cells in treated SMMC-7721 and Sk-hep-1 cells. Each bar represents the mean \pm SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$.

PBS before re-suspension in propidium iodide/RNase A solution (5 µg/mL propidium iodide and 100 mg/mL RNase A). Cells were incubated with propidium iodide at room temperature in the dark for 1 h. Stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA).

Colony formation assay. Cells were harvested 24 h after transfection. Transfected cells were seeded in a fresh six-well plate (500 cells/well) and culture for 12 days. Resistant colonies were fixed with 10% formaldehyde and stained with 1.0% crystal violet.

EdU Assay. Cell proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU) assay using an EdU assay kit (Ribobio) according to the manufacturer's instructions. Briefly, cells were cultured in triplicate at 5×10^3 cells per well in 96-well plates, pretreated with or without LY294002 and U0126, and then transfected with plasmids or siRNA for 48 h. The cells were then exposed to 50 µM of EdU for an additional 1 h at 37°C, fixed with 4% formaldehyde for 15 min at room temperature and treated with 0.5% Triton X-100 for 20 min at room temperature for permeabilization. After washing with PBS, the cells were treated with 100 µL of 1× Apollo reaction cocktail for 30 min. Subsequently, the DNA contents of each well of cells were stained with 100 µL of Hoechst 33342 (5 µg/mL) for 30 min and visualized under a fluorescent microscope (Olympus, Osaka, Japan).

Caspase-3 assays. The HCC cells were cultured at 3×10^5 cells/well in a 24-well plate under serum free conditions, and transfected with siRNA or plasmid. The cells were incubated for 18 h, collected and lysed using the colorimetric

buffers of the Caspase-3 Kit (R&D Systems, Minneapolis, MN, USA). In addition, the concentration of protein in the lysates was measured by Bio-Rad protein assay. The relative activity of caspase-3 to convert their respective substrates was measured by OD (405 nm) per mg of protein lysate.

Apoptosis analysis. To evaluate the percentage of apoptotic cells, we measured the cell surface exposure of phosphatidylserines using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Cells were collected, washed twice in ice-cold PBS and re-suspended in 1× binding buffer. Annexin V and propidium iodide (PI) were added to the cell preparations and incubated for 15 min at 25°C in the dark. After staining, the samples were analyzed by flow cytometer.

Xenograft tumor models. All animal experiments were performed in accordance with protocols approved by the local animal care and use committee. BALB/C nude mice (4–5 weeks, male) were purchased from the animal centre of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Mice were randomly divided into two groups (six per group), which were injected s.c. with 5×10^6 SMMC-7721-HRC or SMMC-7721-vector cells. The length (*L*) and width (*W*) of the tumors were measured with digital vernier calipers. Tumor volume (TV) was determined according to the formula: $TV = (L \times W^2)/2$. After 35 days, the mice were killed and tumors were weighed.

Statistical analysis. All the data are expressed as mean values ± standard deviation (SD). Student's *t*-test was used to

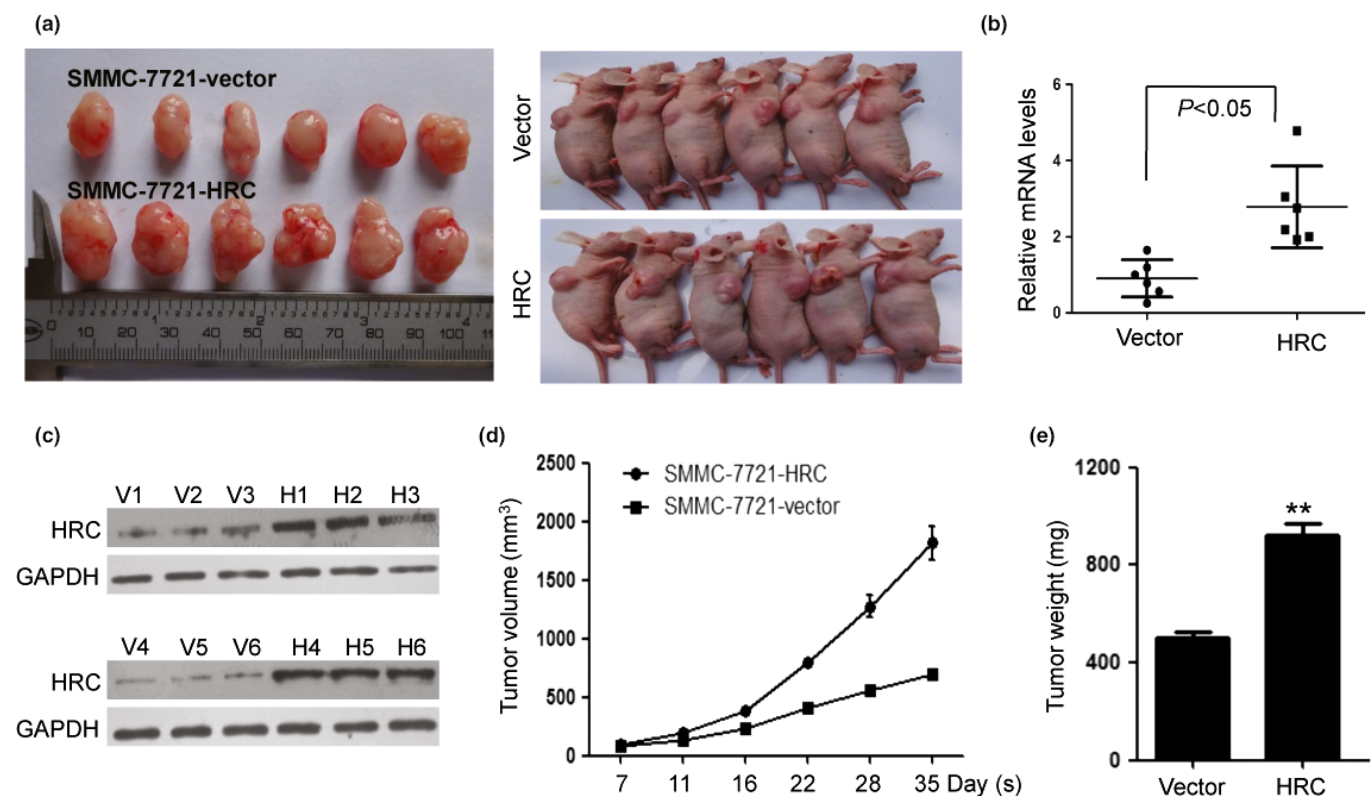


Fig. 2. Histidine-rich calcium binding protein (HRC) promotes tumor growth of hepatocellular carcinoma (HCC) *in vivo*. (a) Images of the tumors from the mice (left) and xenograft model of nude mice (right) in each group (*n* = 6 per group). (b) and (c) RT-qPCR and western blotting showed the level of HRC was actually higher in the SMMC-7721-HRC group than in the SMMC-7721-vector group. V: SMMC-7721-vector, H: SMMC-7721-HRC. (d) Growth curves of tumor resulting from injection of the SMMC-7721-HRC or SMMC-7721-vector cells into nude mice. The tumor volumes were estimated using calipers. (e) Mean tumor weights of nude mice in each group were measured on day 35. Each bar represents the mean ± SD of six mice per group. ***P* < 0.01.

evaluate statistical significance. A P -value < 0.05 was used for statistical significance.

Results

Histidine-rich calcium binding protein contributes to hepatocellular carcinoma cell proliferation *in vitro*. To investigate the functional role of HRC in HCC, both gain-of-function and loss-of-function models were established with references to endogenous HRC expression levels in HCC cell lines. Successful overexpression and knockdown of HRC were illustrated by RT-qPCR and western blotting (Fig. 1a). Our previous study showed the positive correlation between HRC expression and tumor size,⁽⁸⁾ which suggested the importance of HRC in HCC growth. In line with this result, we showed that overexpression of HRC in SMMC-7721 cells remarkably enhanced cell proliferation and clonogenicity, whereas HRC knockdown in Sk-hep-1 cells reduced proliferation and colony-forming ability (Fig. 1b,c), as demonstrated by CCK-8 and colony formation assay. Furthermore, we used the EdU incorporation assay, which is a more sensitive and specific

method to detect cell proliferation. As expected, the number of cells incorporating EdU in SMMC-7721-HRC cells (SMMC-7721 cells stably overexpressing HRC) was increased as compared to the SMMC-7721-vector cells, and HRC knockdown significantly decreased the number of cells incorporating EdU (Fig. 1d). Taken together, these results indicate that HRC enhances cell growth of HCC *in vitro*.

Histidine-rich calcium binding protein promotes tumor growth *in vivo*. Having observed the role of HRC in HCC cell growth *in vitro*, the oncogenic function of HRC in HCC was further confirmed *in vivo* by subcutaneous xenograft models. SMMC-7721-vector and SMMC-7721-HRC cells were subcutaneous injected into nude mice, and tumor growth was monitored weekly. Consistent with our *in vitro* data, ectopic expression of HRC in HCC cells showed significantly higher tumorigenicity, as compared to the control (Fig. 2a). We also confirmed that the expression of HRC was indeed upregulated in the SMMC-7721-HRC group compared to the SMMC-7721-vector group (Fig. 2b,c). Furthermore, the tumor volume and the weight of nude mice in the SMMC-7721-HRC group was larger than that in the SMMC-7721-vector group (Fig. 2d,e).

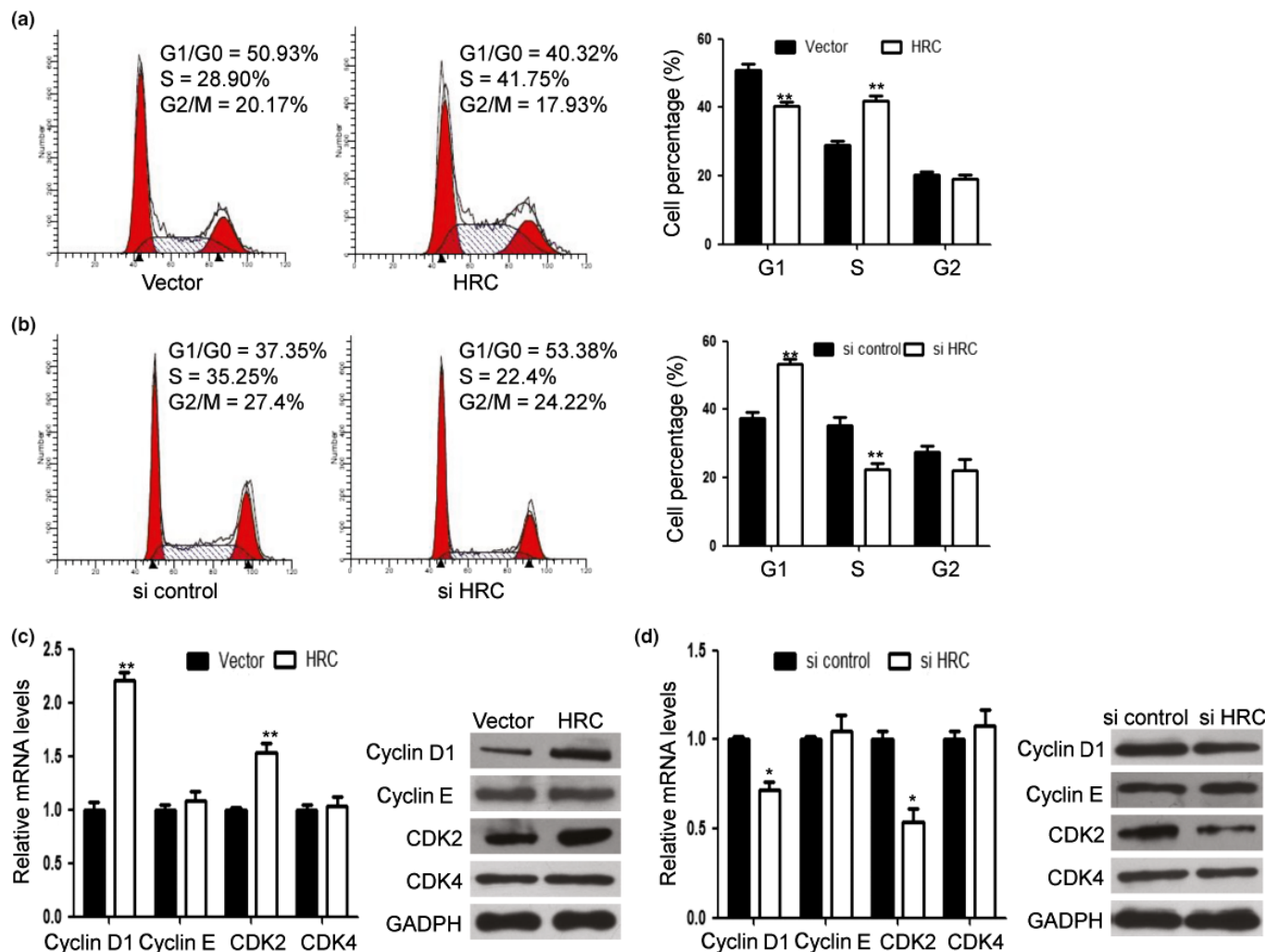


Fig. 3. Histidine-rich calcium binding protein (HRC) contributes to G1/S transition in hepatocellular carcinoma (HCC) cells. (a, b) Cell cycle analysis of treated SMMC-7721 and Sk-hep-1 cells. Left, representative image of flow cytometry analysis. Right, mean \pm SD from three independent experiments of the percentages of cells in each cell cycle phase. (c, d) The levels of cyclinD1, cyclinE, CDK2 and CDK4 in treated SMMC-7721 and Sk-hep-1 cells were assessed by RT-qPCR and western blotting. GAPDH was used as a loading control. * $P < 0.05$, ** $P < 0.01$.

These data further confirmed the biological importance of HRC in HCC development.

Histidine-rich calcium binding protein induces G1/S transition in hepatocellular carcinoma cells. To explore the mechanism of HRC-mediated cell growth, we analyzed the cell cycle of HCC cells. The results showed that ectopic expression of HRC dramatically increased the percentage of cells in the S peak and decreased the percentage of cells in the G1/G0 phase, and HRC silence exerted the opposite effect (Fig. 3a,b). Next, we measured the expression of the commonly utilized proliferation markers, cyclinD1, cyclinE, CDK2 and CDK4. Prominent changes in the levels of G1 phase regulators cyclinD1 and CDK2 were noted in HCC cells, but no differences were shown on the expression of cyclinE and CDK4 (Fig. 3c,d). Collectively, these results suggest that HRC promotes G1/S transition.

Histidine-rich calcium binding protein regulates hepatocellular carcinoma cell proliferation by MEK/ERK signal pathway. Given that MEK/ERK and PI3K/Akt pathways are closely associated with HCC cell proliferation,⁽¹⁰⁾ we analyzed the expression of phosphorylated MEK, ERK and Akt. The results showed that HRC overexpression enhanced while HRC knockdown suppressed the expression of p-MEK and p-ERK, but the phosphorylation of Akt was not changed (Fig. 4a). To clarify whether MEK/ERK pathway or PI3K/Akt pathway or both are involved in HRC-induced cell proliferation, the specific inhibitors of MEK (U0126) and PI3K (LY294002) were used. As expected, CCK-8 and EdU assay both demonstrated that HRC-induced cell proliferation was abolished by U0126, but not by LY294002 (Fig. 4b,c). These data suggest that the pro-growth function of HRC in HCC may be mediated by the MEK/ERK signal pathway.

Histidine-rich calcium binding protein protects cells from endoplasmic reticulum stress-induced apoptosis. To determine whether HRC influences cell apoptosis, flow cytometry was performed. The results demonstrated that HRC overexpression significantly decreased the percentage of apoptotic cells, and HRC silence increased the percentage of apoptotic cells (Fig. 5a). We further assessed the activation of a key apoptosis mediator, caspase-3. Ectopic expression of HRC sup-

pressed whereas HRC knockdown enhanced caspase-3 activation (Fig. 5b,c). Next, we investigated whether the effect of HRC on apoptosis was associated with changes in the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in HCC cells. To our surprise, the levels of Bcl-2 and Bax were not changed (Fig. 5d,e). Interestingly, we found that the PERK/ATF4/CHOP signaling pathway, which is the major pathway of ER stress-mediated apoptosis, was obviously activated following HRC knockdown. In contrast, the expression of PERK, ATF4 and CHOP were obviously suppressed in SMMC-7721-HRC cells as compared with SMMC-7721-vector cells (Fig. 5d,e). From the above results, we conclude that HRC protects HCC cells from ER stress-induced apoptosis.

Endoplasmic reticulum stress is associated with the anti-apoptosis role of histidine-rich calcium binding protein. The role of ER stress in apoptosis has been recognized; thus, it was of great interest to examine whether or not the protective role of HRC in apoptosis is coupled to ER stress. Hence, in the present study we employed an ER stress inducer, thapsigargin (TG), and an ER stress modulator, 4-phenylbutyrate acid (4-PBA). In accordance with our expectation, pretreatment with TG not only upregulated the expression of ER stress molecular indicators (Fig. 6a), but also abolished the anti-apoptosis function of HRC (Fig. 6c), and pretreatment with 4-PBA achieved the opposite effect (Fig. 6b,d). To corroborate these results, the effects of TG and 4-PBA were also examined using caspase-3 activation as the apoptotic endpoint. As expected, HRC overexpression was not able to inhibit caspase-3 activation when cells were pretreated with TG, and the activity of caspase-3 induced by HRC silence was suppressed following 4-PBA pretreatment (Fig. 6e). These results suggested that ER stress is involved in HRC-mediated apoptosis inhibition.

Discussion

Recent studies have revealed that a variety of calcium-binding proteins contribute to the development of cancers.^(19,20) Previously, we showed that the histidine-rich Ca²⁺-binding protein

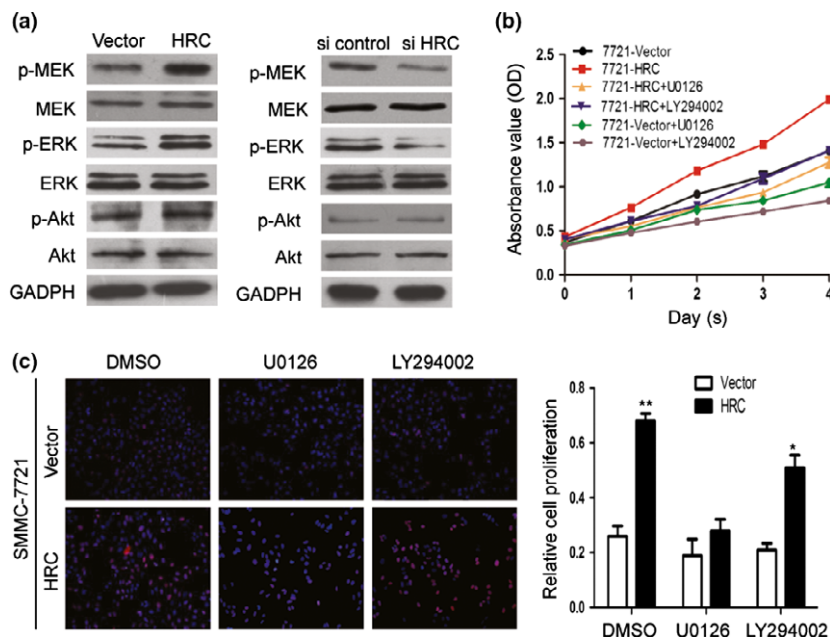


Fig. 4. Histidine-rich calcium binding protein (HRC) enhances cell proliferation by the MEK/ERK pathway. (a) The levels of phospho-ERK, phospho-MEK and phospho-Akt were determined by western blotting analysis. (b, c) The cell proliferation was detected by Cell Counting Kit-8 (CCK-8) (b) and EdU assay (c) after SMMC-7721 cells were pretreated with U0126 (MEK/ERK inhibitor) or LY294002 (PI3K/Akt inhibitor). Each bar represents the mean \pm SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$.

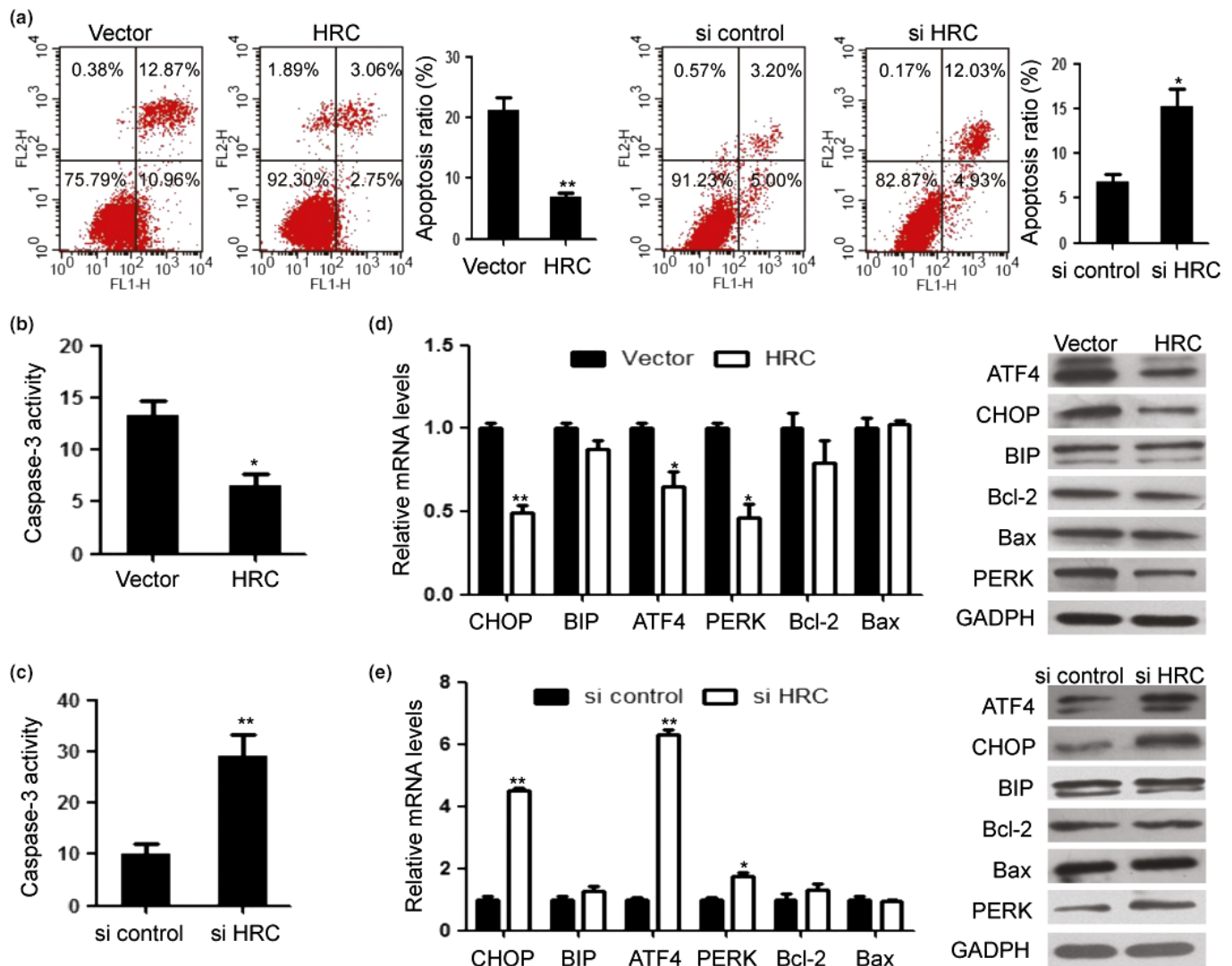


Fig. 5. Effect of Histidine-rich calcium binding protein (HRC) on endoplasmic reticulum (ER) stress-mediated cell apoptosis. (a) FACS analysis of treated SMMC-7721 cells (left) and Sk-hep-1 cells (right) labeled with Annexin-V FITC and propidium iodide (PI) as markers for apoptosis. (b, c) Caspase 3 activity of treated SMMC-7721 and Sk-hep-1 cells. (d, e) The expression of Bax, Bcl-2 and the levels of ER stress-related proteins, including PERK, CHOP, ATF4 and Grp78 (BIP) in treated SMMC-7721 and Sk-hep-1 cells were determined by RT-qPCR and western blotting. Each bar represents the mean \pm SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$.

(HRC) expression significantly correlated with tumor size and metastasis of HCC, and we found that HRC promoted HCC metastasis through $\text{Ca}^{2+}/\text{CaM}$ signal.⁽⁸⁾ However, the role of HRC in the growth of HCC has not been investigated yet. In this study, for the first time, we demonstrate that HRC is involved in the processes of cell proliferation, colony formation, cell cycle progression and apoptosis *in vitro*. The significant pro-growth function of HRC *in vivo* additionally suggests that HRC may be a potential intervention target of HCC.

Recent advances in cancer biology have implicated changes of calcium-binding protein expression as an important mechanism controlling cell proliferation in HCC.^(6,7,21) Wu *et al.*⁽⁶⁾ report that S100A9 promotes the proliferation of HepG2 cells, whereas S100C/A10 is reported to inhibit the growth of hepatoma cells via induction of p21/Waf1.⁽²¹⁾ We demonstrated that exogenous expression of HRC promotes cell growth and enhances the colony formation ability of SMMC-7721 cells, whereas HRC knockdown in Sk-hep-1 cells causes the opposite effects. Loss of cell cycle control is one of the mechanisms that leads to unlimited

growth of cancer cells.^(22,23) To determine whether this mechanism underlies the proliferative promotion effect of HRC, we investigated the effect of HRC on cell cycle regulation. Our results demonstrate that HRC promoted G1/S transition, and the expression of the G1 phase regulators cyclinD1 and CDK2 was upregulated in SMMC-7721-HRC cells. This results support the role of HRC in promoting G1/S transition as a mechanism for enhancement of HCC cells growth.

The MEK/ERK and PI3K/Akt signaling pathways regulate many fundamental cellular functions, such as cell proliferation, survival and motility.^(24,25) Upregulation of these pathways is crucial in the promotion or development of tumor cell growth. We next examined the functional involvement of these two pathways in HRC-induced cell proliferation. Intriguingly, p-MEK and p-ERK, but not p-Akt, were increased following HRC overexpression. Conversely, HRC knockdown inhibited the phosphorylation of MEK and ERK. Furthermore, the proliferation promotion effect of HRC was significantly abolished by the MEK inhibitor U0126. Thus, our data indicate that

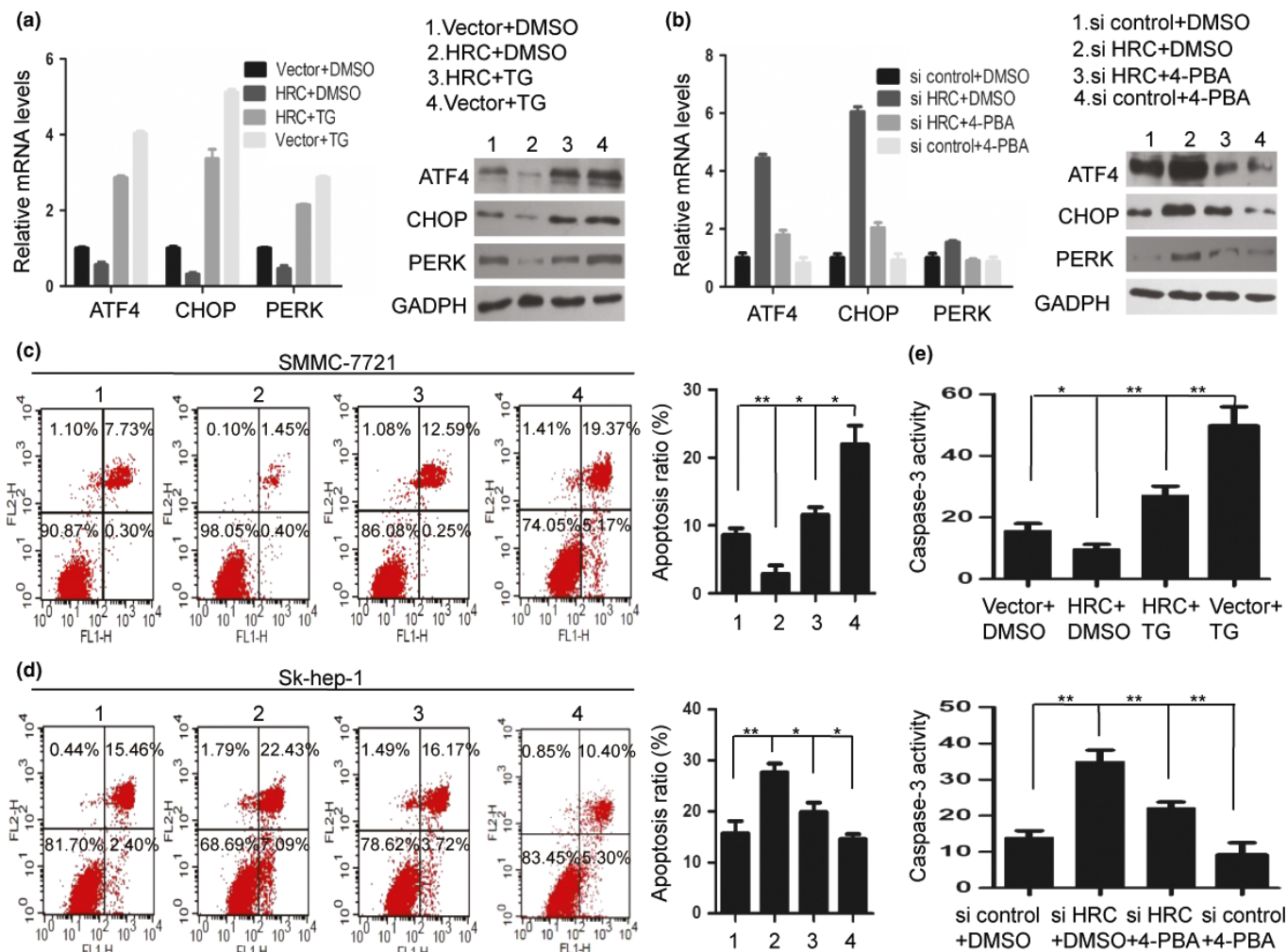


Fig. 6. Endoplasmic reticulum (ER) stress is involved in the induction of histidine-rich calcium binding protein (HRC)-suppressed apoptosis. (a) The expression of PERK, ATF4 and CHOP were assessed by RT-qPCR and western blotting after cells were pretreated with 1 mM thapsigargin (TG). (b) Sk-hep-1 cells were pretreated with 1 mM 4-phenylbutyrate acid (4-PBA). The mRNA and protein levels of PERK, ATF4 and CHOP were measured. (c) After treatment with 1 mM TG, SMMC-7721 cells apoptosis was determined by flow cytometry. 1. Vector+DMSO, 2. HRC+DMSO, 3. HRC+TG, 4. Vector+TG. (d) After pretreatment with 1 mM 4-PBA, Sk-hep-1 cells apoptosis was determined by flow cytometry. 1. si control+DMSO, 2. si HRC+DMSO, 3. si HRC+4-PBA, 4. si control+4-PBA. (e) Caspase 3 activity of hepatocellular carcinoma (HCC) cells pretreated with TG or 4-PBA were assessed. Each bar represents the mean \pm SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$.

activation of MEK/ERK signaling is responsible for HRC-mediated cell proliferation.

Endoplasmic reticulum stress response is often described as one of the mechanisms in liver diseases, including HCC.⁽²⁶⁾ In this study, we provide the first evidence to confirm that HRC can regulate ER stress, as indicated by the change in ER stress-related genes. Mild ER stress is helpful for restoring cellular homeostasis, but the persistent and unalleviated ER stress elicits apoptosis.⁽¹³⁾ The CAAT/enhancer binding protein homologous protein (CHOP) has been reported to be a crucial ER stress responsive factor that executes apoptosis, which can be induced and upregulated by the PERK/ATF4 signaling pathway.^(14,27) This study provides important evidence that HRC suppresses PERK/ATF4/CHOP signaling pathways in HCC cells, and that the induction of ER stress is implicated in HRC-inhibited cell apoptosis. Caspases-3 has been proposed as the specific mediator of ERS-induced apoptosis.⁽²⁸⁾ Our data show that HRC overexpression suppressed while HRC knockdown enhanced the activation of caspase-3. Moreover, blocking ER stress by the ERS inhibitor 4-PBA not only effectively inactivated the PERK

/ATF4/CHOP pathway, but also significantly decreased apoptosis induced by HRC silence. The protective effect of HRC on cell apoptosis could be repressed when TG is applied, which is a well-known ER stress inducer. Certainly, other ER stress-related genes should be studied in future work to elucidate the molecular mechanism of HRC and ER stress.

In summary, we provide the first evidence that HRC is an important oncogene that contributes to HCC growth, which is a consequence of cell proliferation and apoptosis. Further study showed that these changes were partially mediated by the MEK/ERK pathway and ER stress. The reason for the overexpression of HRC in HCC cells requires further research. Our findings have enriched the knowledge on the molecular mechanisms underlying HCC and suggest that HRC is a potential intervention target.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

- Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 448–58.
- Arvanitis DA, Vafiadaki E, Sanoudou D, Kranias EG. Histidine-rich calcium binding protein: the new regulator of sarcoplasmic reticulum calcium cycling. *J Mol Cell Cardiol* 2011; **50**: 43–9.
- Prevorskaya N, Skryma R, Shuba Y. Calcium in tumour metastasis: new roles for known actors. *Nat Rev Cancer* 2011; **11**: 609–18.
- Prevorskaya N, Ouadid-Ahidouch H, Skryma R, Shuba Y. Remodelling of Ca²⁺ transport in cancer: how it contributes to cancer hallmarks? *Philos Trans R Soc Lond B Biol Sci* 2014; **369**: 20130097.
- Pan QZ, Pan K, Weng DS *et al.* Annexin A3 promotes tumorigenesis and resistance to chemotherapy in hepatocellular carcinoma. *Mol Carcinog* 2013; **54**: 598–607.
- Wu R, Duan L, Ye L *et al.* S100A9 promotes the proliferation and invasion of HepG2 hepatocellular carcinoma cells via the activation of the MAPK signaling pathway. *Int J Oncol* 2013; **42**: 1001–10.
- Li Z, Tang M, Ling B *et al.* Increased expression of S100A6 promotes cell proliferation and migration in human hepatocellular carcinoma. *J Mol Med (Berl)* 2014; **92**: 291–303.
- Liu J, Han P, Li M *et al.* The histidine-rich calcium binding protein (HRC) promotes tumor metastasis in hepatocellular carcinoma and is upregulated by SATB1. *Oncotarget* 2015; **6**: 6811–24.
- Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001; **411**: 342–8.
- Matsushima-Nishiwaki R, Adachi S, Yoshioka T *et al.* Suppression by heat shock protein 20 of hepatocellular carcinoma cell proliferation via inhibition of the mitogen-activated protein kinase and AKT pathways. *J Cell Biochem* 2011; **112**: 3430–9.
- Secondo A, Esposito A, Sirabella R *et al.* Involvement of the Na⁺/Ca²⁺ exchanger isoform 1 (NCX1) in neuronal growth factor (NGF)-induced neuronal differentiation through Ca²⁺-dependent Akt phosphorylation. *J Biol Chem* 2015; **290**: 1319–31.
- Li X, Zhao H, Wang Q *et al.* Fucoidan protects ARPE-19 cells from oxidative stress via normalization of reactive oxygen species generation through the Ca²⁺-dependent ERK signaling pathway. *Mol Med Rep* 2015; **11**: 3746–52.
- Yadav RK, Chae SW, Kim HR, Chae HJ. Endoplasmic reticulum stress and cancer. *J Cancer Prev* 2014; **19**: 75–88.
- Verfaillie T, Garg AD, Agostinis P. Targeting ER stress induced apoptosis and inflammation in cancer. *Cancer Lett* 2013; **332**: 249–64.
- Schonthal AH. Targeting endoplasmic reticulum stress for cancer therapy. *Front Biosci (Schol Ed)* 2012; **4**: 412–31.
- Kaufman RJ, Malhotra JD. Calcium trafficking integrates endoplasmic reticulum function with mitochondrial bioenergetics. *Biochim Biophys Acta* 2014; **1843**: 2233–9.
- Bodalia A, Li H, Jackson MF. Loss of endoplasmic reticulum Ca²⁺ homeostasis: contribution to neuronal cell death during cerebral ischemia. *Acta Pharmacol Sin* 2013; **34**: 49–59.
- Han P, Fu Y, Luo M *et al.* BVES inhibition triggers epithelial-mesenchymal transition in human hepatocellular carcinoma. *Dig Dis Sci* 2014; **59**: 992–1000.
- Mussunoor S, Murray GI. The role of annexins in tumour development and progression. *J Pathol* 2008; **216**: 131–40.
- Mishra SK, Siddique HR, Saleem M. S100A4 calcium-binding protein is key player in tumor progression and metastasis: preclinical and clinical evidence. *Cancer Metastasis Rev* 2012; **31**: 163–72.
- Miyazaki M, Sakaguchi M, Akiyama I *et al.* Involvement of interferon regulatory factor 1 and S100C/A11 in growth inhibition by transforming growth factor beta 1 in human hepatocellular carcinoma cells. *Cancer Res* 2004; **64**: 4155–61.
- Kim HS, Lee KS, Bae HJ *et al.* MicroRNA-31 functions as a tumor suppressor by regulating cell cycle and epithelial-mesenchymal transition regulatory proteins in liver cancer. *Oncotarget* 2015; **6**: 8089–102.
- Wang Z, Wei W, Sun CK *et al.* Suppressing the CDC37 cochaperone in hepatocellular carcinoma cells inhibits cell cycle progression and cell growth. *Liver Int* 2015; **35**: 1403–15.
- Dillon LM, Bean JR, Yang W *et al.* P-REX1 creates a positive feedback loop to activate growth factor receptor, PI3K/AKT and MEK/ERK signaling in breast cancer. *Oncogene* 2015; **34**: 3968–76.
- Tyagi N, Bhardwaj A, Singh AP *et al.* p-21 activated kinase 4 promotes proliferation and survival of pancreatic cancer cells through AKT- and ERK-dependent activation of NF-kappaB pathway. *Oncotarget* 2014; **5**: 8778–89.
- Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol* 2011; **54**: 795–809.
- Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 2011; **13**: 184–90.
- Johnson GG, White MC, Grimaldi M. Stressed to death: targeting endoplasmic reticulum stress response induced apoptosis in gliomas. *Curr Pharm Des* 2011; **17**: 284–92.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Primary antibodies for western blot.

Table S2. Primers for RT-qPCR.