BRD4 induces cell migration and invasion in HCC cells through MMP-2 and MMP-9 activation mediated by the Sonic hedgehog signaling pathway

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Abstract. Hepatocellular carcinoma (HCC) is a highly aggressive form of carcinoma with poor prognosis, and HCC-associated mortality primarily occurs due to migration and invasion of HCC cells. The manipulation of epigenetic proteins, such as BRD4, has recently emerged as an alternative therapeutic strategy. The present study aimed to investigate the novel mechanism of BRD4 involvement in the migration and invasion of HCC cells. Reverse transcription-quantitative polymerase chain reaction was used to assess BRD4 mRNA expression levels in HCC cell lines. This analysis demonstrated that BRD4 was significantly overexpressed in HCC cell lines compared with a human immortalized normal liver cell line. A short hairpin RNA (shRNA) was then used to suppress BRD4 expression in HCC cells, and resulted in impaired HCC cell proliferation, migration and invasion. When the HepG2 HCC cell line was treated with recombinant human sonic hedgehog (SHH) peptide, the migration and invasion capabilities of HepG2 cells that were inhibited by BRD4 silencing were restored. BRD4 induced cell migration and invasion in HepG2 cells through the activation of matrix metalloproteinase (MMP)-2 and MMP-9, mediated by the SHH signaling pathway. Taken together, the results of the present study demonstrated the importance of BRD4 in HCC cell proliferation and metastasis. Thus, BRD4 is a potential novel target for the development of therapeutic approaches against HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer-associated mortality in the USA, with an estimated annual diagnosis rate of 33,190 people and an annual mortality rate of 23,000 cases in 2014 (1). Despite significant advances in the diagnosis and treatment of HCC, its prognosis is poor. Migration and invasion are 2 fundamental properties determining the prognosis of HCC patients (2). Therefore, determining the molecular mechanisms underlying HCC migration and invasion may aid in the identification of novel therapeutic targets and consequently lead to improved prognosis in the future.

The sonic hedgehog (SHH) signaling pathway is a highly conserved system; it is involved in proliferation and control of the migration and invasion of HCC (3-6). A number of SHH signaling pathway molecular inhibitors, such as cyclopamine, have demonstrated clinical efficacy against basal cell carcinoma, medulloblastoma and pancreatic cancer (7-9). However, primary and secondary resistance have been encountered (10,11). Furthermore, the exact regulatory mechanisms of SHH signaling in the migration and invasion of HCC remain unclear. Thus, further investigation of the SHH signaling pathway mechanism in tumor migration and invasion is required.

A previous study demonstrated that BRD4 regulates the SHH signaling pathway (12). Therefore, the SHH signaling pathway may be controlled by BRD4 to promote cell migration and invasion. BRD4 is an epigenome reader and a member of the BET family of proteins, which consist of two bromodomains in tandem and an extra terminal domain. Previous studies have demonstrated that BRD4 promotes cell cycle progression and regulates cell growth and transcription (13). Subsequent studies have demonstrated that BRD4 serves a critical role in tumor proliferation and growth in melanoma (14), neuroblastoma (15), glioblastoma (16), malignant peripheral nerve sheath tumors (17), lymphoblastic leukemia (18) and lung adenocarcinoma (19). However, to the best of our knowledge, the involvement of BRD4 in migration and invasion has not been reported in HCC.

The present study aimed to investigate the role of BRD4 expression in HCC proliferation, migration and invasion *in vitro*. The potential mechanisms through which BRD4 enhances the migration and invasion of HCC were also investigated, including the SHH signaling pathway and matrix metalloproteinase (MMP)-2 and MMP-9 activation.

Materials and methods

Cell culture. The human HCC cell lines (HepG2, Hep3B, SMMC-7721, Bel-7402 and Huh7) and an immortalized normal liver cell line (L02) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Beijing, China). These cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Gibco Life Technologies, Grand Island, NY, USA) in a humidified incubator at 37˚C with 5% carbon dioxide. In certain experiments, recombinant human SHH peptide (rhSHH) (R&D Systems, Inc., Minneapolis, MN, USA) was added to the culture medium.

Establishment of cell lines stably expressing BRD4 short hairpin RNA (shRNA). shRNA plasmids for BRD4, which were designed against the BRD4 gene and constructed in Phblv-u6-puro vectors, were purchased from Han Heng Biotechnology Co., Ltd. (Shanghai, China). A non-target scrambled oligonucleotide served as the negative control (shcontrol). All plasmids were verified through sequencing. To generate stable BRD4-silenced cell lines, HepG2 cells were cultured in 6-well plates until they reached 40% confluence. The medium was then replaced with 1 ml fresh FBS-free culture medium supplemented with 50 μl viral supernatant ($1x10^8$ UT/ml) and 6 μg/ml polybrene (Han Heng Biotechnology Co., Ltd.) for 24 h. Cells were cultured and screened in medium containing 3 μg/ml puromycin (Han Heng Biotechnology Co., Ltd.). Individual puromycin-resistant colonies were isolated during drug screening. The knockdown efficiency was verified through western blot analysis. The shRNA sequences used in the present study were as follows: shBRD4, 5'-GATCCGCCTGGAGATGAC ATAGTCTTATTCAAGAGATAAGACTATGTCATCTCC AGGTTTTTTc-3' and shcontrol, 5'-GATCCTTCTCCGAAC GTGTCACGTAATTCAAGAGATTACGTGACACGTTCG GAGAATTTTTTg-3'.

Cell proliferation assay. Cell proliferation was measured with a CCK-8 assay (Beyotime Institute of Biotechnology). Briefly, shBRD4 and shcontrol HepG2 cells were plated onto 96-well plates at a density of 3,000 cells/well. Following culture for the indicated time periods (0, 24, 48 and 72 h), 10 μl CCK-8 solution was added into each well and incubated at 37˚C. After 3 h, the absorbance of each well was measured using a Multiskan MK3 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a wavelength of 450 nm.

Colony formation assay. shcontrol- or shBRD4-expressing HepG2 cells were seeded at 200 cells/well in 6-well plates. Following 1 week of culture, the colonies were stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China), images were captured under a CKX41 light microscope (Olympus Corporation, Tokyo, Japan) and counted.

Cell apoptosis analysis. The fraction of apoptotic cells was analyzed through the annexin V and propidium iodide staining method according to the manufacturer's instructions (Nanjing KeyGen BioTech Co., Ltd., Nanjing, China). The stained cells were immediately analyzed through flow cytometry (FACS101; Becton Dickinson, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse-transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. RT-PCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). The specificity of amplification was verified using a melting curve and electrophoresis in agarose gel. The following PCR conditions were applied for detecting mRNAs: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The relative expression levels of the target gene mRNA was calculated as the inverse log of ΔΔCT and was normalized to the reference gene β-actin. The primer sequences were as follows: BRD4, F 5'-CATGGACAT GAGCACAATCA-3' and R 5'-TCATGGTCAGGAGGGTTG TA-3'; MMP-2, F 5'-AAGTCTGAA-GAGCGTGAAGTTTGG A-3' and R 5'-TGAGGGTTGGTGGGATTGGAG-3'; MMP-9, F 5'-AGTCCACCCTTGTGCTCTTCCC-3' and R 5'-TCT GCCACCCGAGTGTAACCAT-3'; GLI1, F 5'-AGGGCTGCA GTAAAGCCTTCA-3' and R 5'-CTTGACATGTTTTCG CAGCG-3'; SHH, F 5'-CCCAATTACAACCCCGACATC-3' and R 5'-TCACCCGCAGTTTCACTCCT-3'; and β-actin, F 5'-GATCATTGCTCCTCCTGAGC-3' and R 5'-ACTCCT GCTTGCTGATCCAC-3'.

Western blot analysis. Equal amounts of proteins (30 μg) from the lysates of the HepG2 cells were subjected to electrophoresis through 10% SDS-PAGE (Beyotime Institute of Biotechnology) at 80 V for 30 min then 100 V for 1.5 h, and were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Following blocking in 5% skimmed milk, the membranes were then incubated with the following diluted primary antibodies: Rabbit polyclonal BRD4 (1:200; ab75898; Abcam, Cambridge, MA, USA), goat polyclonal SHH (1:200; sc1194; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal GLI1 (1:200; sc20687; Santa Cruz Biotechnology, Inc.), rabbit monoclonal MMP-2 (1:400; D8N9Y; Cell Signaling Technology, Inc., Beverly, MA, USA), and rabbit polyclonal MMP-9 (1:400; ab38898; Abcam) or mouse monoclonal β-actin (1:800; AA128; Beyotime Institute of Biotechnology) overnight at 4˚C, and then with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Specific bands were visualized with enhanced chemiluminescence reagent (Nanjing KeyGen Biotech Co., Ltd.) on an autoradiographic film.

Cell migration and invasion assay. Cell migration and invasion were assessed by Boyden chamber assay. For the invasion

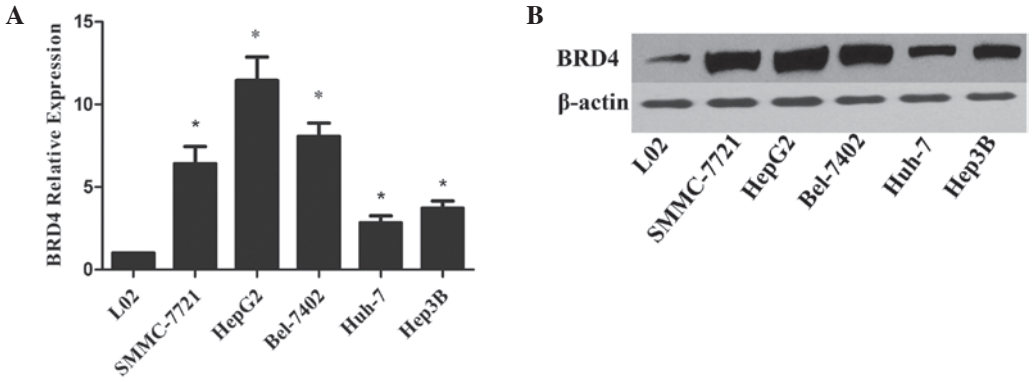

Figure 1. Increased expression of BRD4 in HCC cells. (A) Expression levels of BRD4 mRNA in the human HCC cell lines HepG2, Hep3B, SMMC-7721, Bel-7402, and Huh-7 cells, as assessed through reverse transcription-quantitative polymerase chain reaction. Human immortalized normal liver cells, L02, served as the control. *P<0.05 vs. L02 cells. (B) Increased protein expression levels of BRD4 in HCC cell lines compared with L02 as assessed through western blot analysis. HCC, hepatocellular carcinoma.

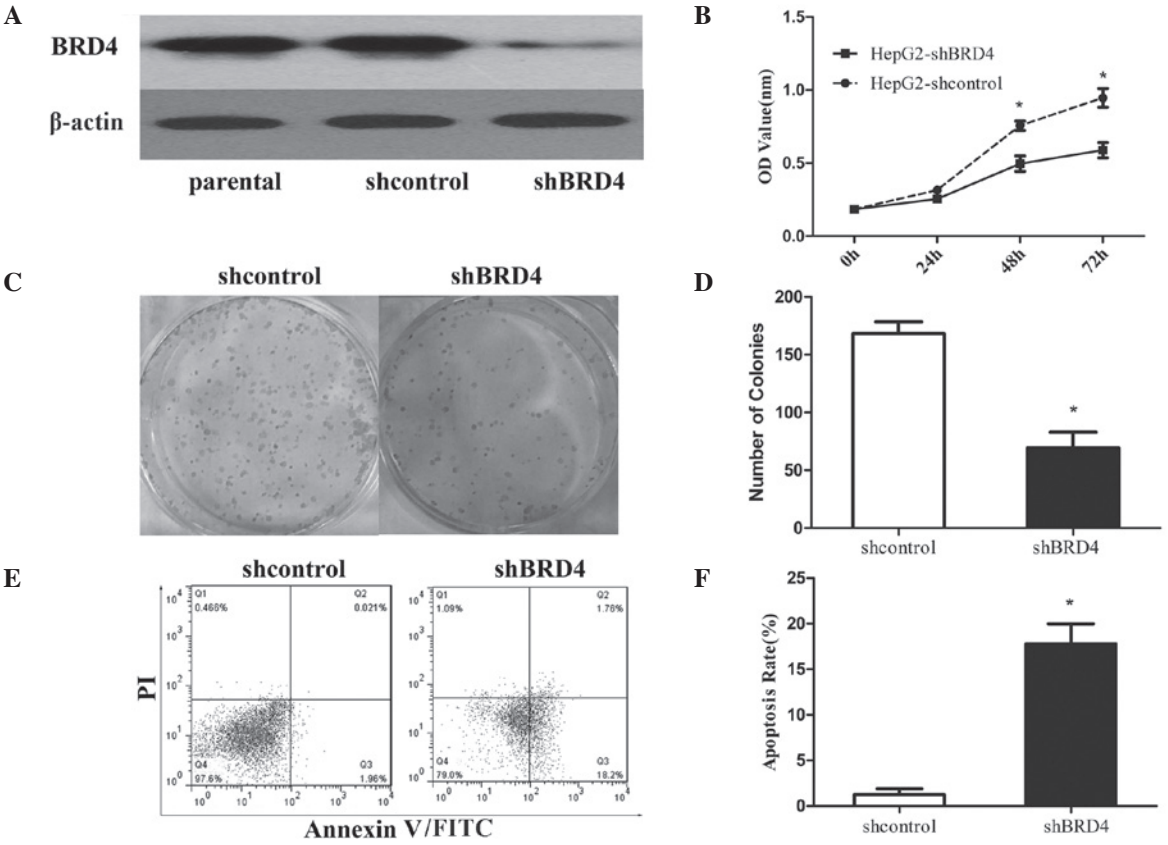

Figure 2. BRD4 expression promotes proliferation and inhibits apoptosis in HCC cells. (A) BRD4 protein expression in shcontrol- or shBRD4-treated HepG2 cells was assessed through western blot analysis. (B) Cell proliferation, determined by CCK-8 assay, revealed that BRD4 knockdown markedly inhibited HepG2 proliferation. (C) BRD4 silencing in HepG2 cell reduced the colony-forming efficiency. (D) Quantification of colony forming efficiency. (E) Annexin V-FITC/PI-stained cells transfected with shBRD4 exhibited a higher rate of apoptosis, as evaluated through flow cytometry. (F) Quantification of apoptosis rates. *P<0.05 vs. shcontrol. HCC, hepatocellular carcinoma; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide; OD, optical density.

assay, the upper sides of the filters were coated with 50 μl Matrigel (BD Biosciences, Bedford, MA, USA). shcontrol or shBRD4-expressing HepG2 cells were resuspended in FBS-free medium with 0.5% bovine serum albumin (Santa Cruz Biotechnology, Inc.) with or without rhSHH added to the upper side of the chamber. Medium containing 5% FBS was added to the lower chamber. Following incubation at 37˚C with 5% CO_2 for 8 h (migration) or 24 h (invasion), cells on the lower filter were fixed with methanol, stained with crystal violet, and then counted under a light microscope (CKX41; Olympus Corporation).

Statistical analysis. All experiments were performed in triplicate. Unless otherwise indicated, experimental values are expressed as the mean ± standard error. Statistical significance was determined through unpaired Student's t-tests using

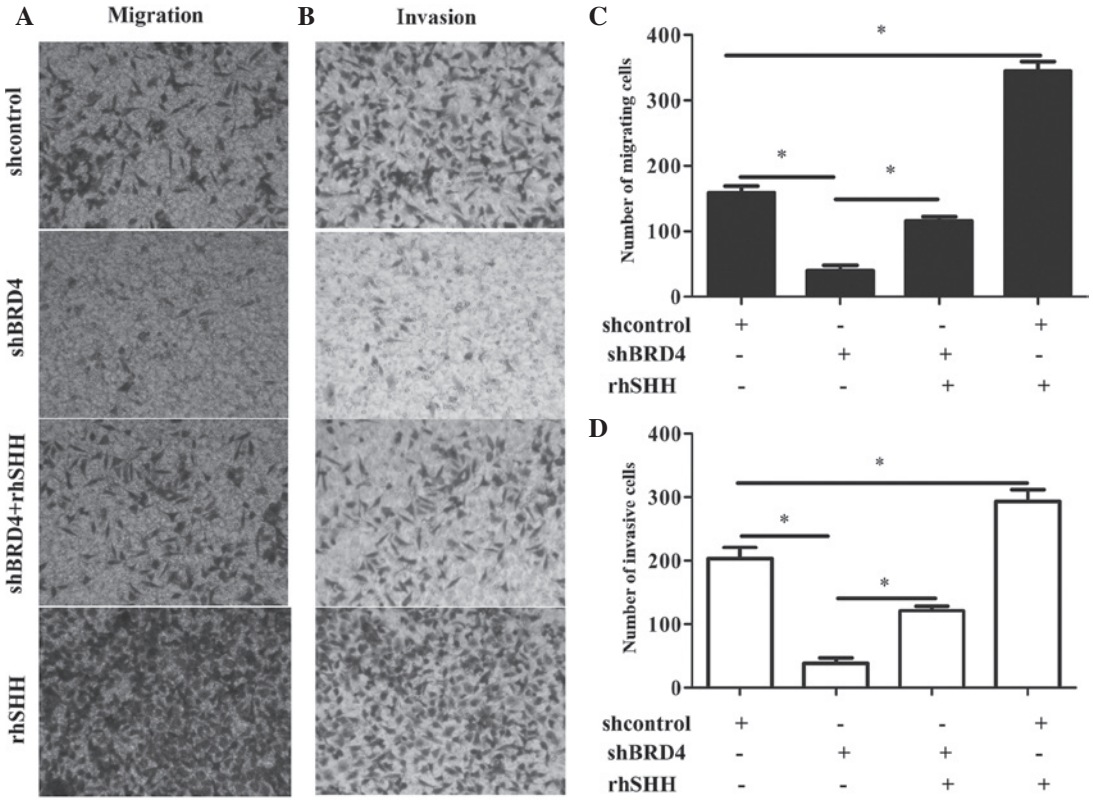

Figure 3. BRD4 silencing inhibited the migration and invasion of HepG2 cells, while rhSHH reversed the migration and invasion of HepG2 cells reduced by BRD4 silencing. (A) shcontrol- or shBRD4-expressing HepG2 cells were added to the upper Transwell chambers and incubated for 8 h with or without 0.5 μg/ml rhSHH. Migrant cells were fixed, stained and counted and images were captured (magnification, x100). (B) shcontrol- or shBRD4-expressing HepG2 cells were added to the upper Matrigel-coated filter of the Transwell chambers for 24 h with or without of 0.5 μg/ml rhSHH. Invading cells were stained with crystal violet and counted and then images were captured (magnification, x100). (C) Quantification of migrating HepG2 cells in the lower chamber. (D) Quantification of invading HepG2 cells in the lower chamber. *P<0.05, comparison indicated by brackets. rhSHH, recombinant human sonic hedgehog peptide.

SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

BRD4 was specifically overexpressed in HCC cells. To investigate the role of BRD4 in HCC, the expression levels of BRD4 mRNA and protein were measured in various HCC cell lines (HepG2, Hep3B, SMMC-7721, Bel-7402 and Huh-7), and the immortalized normal liver cell line, L02. The results demonstrated that the expression levels of BRD4 mRNA were significantly increased in all five HCC cell lines compared with the normal liver L02 cell line (P<0.05; Fig. 1A). The protein levels were also increased (Fig. 1B), and the highest expression level was detected in the HepG2 cell line. Therefore, subsequent experiments were performed using this cell line.

BRD4 promotes the proliferation and inhibits the apoptosis in HCC cells. To illustrate the role of BRD4 in HCC, BRD4-knockdown lentiviral constructs were produced. HepG2 cells were transfected with BRD4 knockdown lentiviral vector. The post-transfection knockdown of BRD4 was confirmed using western blot analysis (Fig. 2A). BRD4 knockdown significantly suppressed the proliferation of the HepG2 cells, as demonstrated by the CCK-8 and colony formation assays (P<0.05; Fig. 2B-D). In addition, BRD4 silencing markedly increased the apoptosis rate of the cells (Fig. 2E and F).

BRD4 is critical for the migration and invasion potential of HCC cells. Cell migration and invasion are critical events in tumor metastasis. Thus, the effects of BRD4 expression on the migration and invasion of HCC cells was explored *in vitro*. HepG2 cell migration and invasion were investigated by Boyden chamber assay. The results indicated that BRD4 silencing significantly inhibited the cell migration and invasion (P<0.05; Fig. 3A-D).

BRD4 induced cell migration and invasion through SHH signaling pathway-mediated expression of MMP-2 and MMP-9 in HCC cells. MMPs, particularly MMP-2 and MMP-9, have been implicated in tumor invasion and metastasis. A previous study demonstrated that BRD4 directly regulates MMP-9 in osteoclasts (20). Therefore, to explore the mechanism through which BRD4 induces migration and invasion in HCC cells, BRD4 expression was knocked down in HepG2 cells and the effects on the expression of MMP-2 and MMP-9 were assessed. The expression levels of MMP-2 and MMP-9 mRNA in HepG2 cells stably transfected with shBRD4 were significantly reduced compared with shcontrol cells, as measured through RT-qPCR (P<0.05; Fig. 4A). Protein levels were also markedly reduced, as indicated by

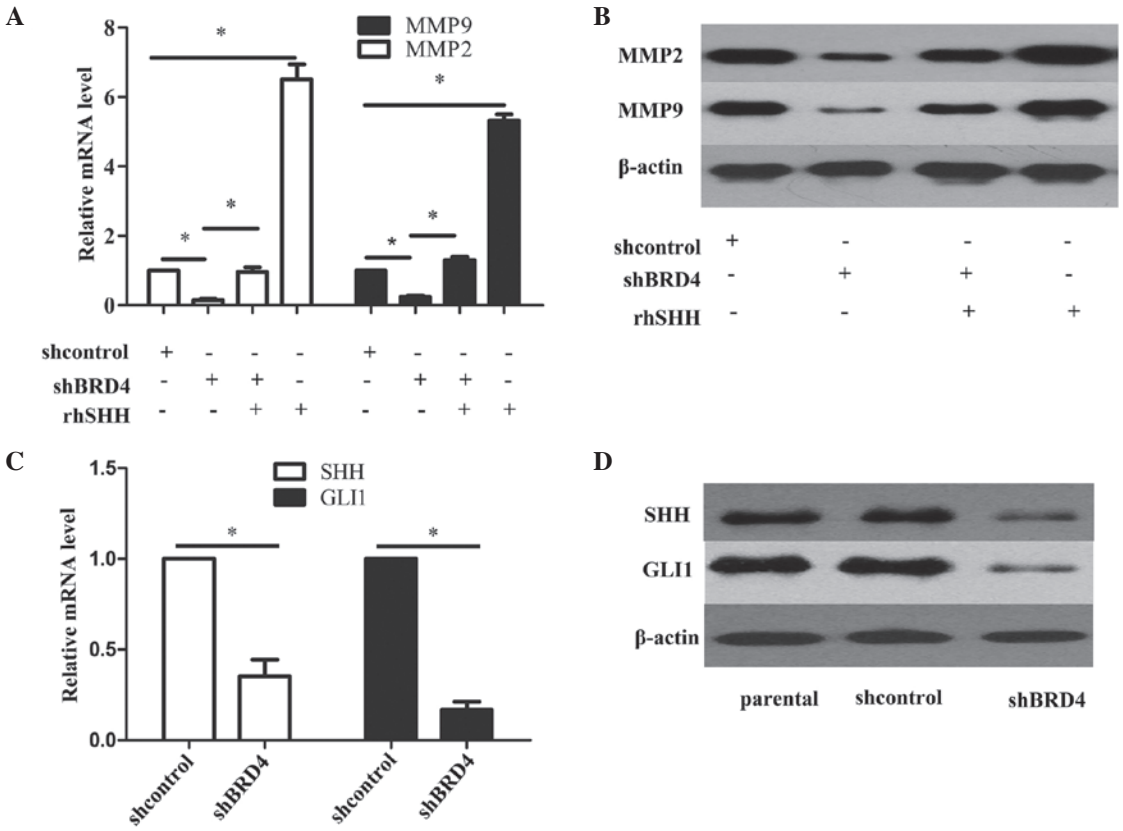

Figure 4. BRD4 induced cell migration and invasion through the SHH signaling pathway via increasing the expression levels of MMP-2 and MMP-9 in HepG2 cells. BRD4 silencing downregulated expression of MMP-2 and MMP-9, while rhSHH reversed this reduction. Shcontrol- or shBRD4-expressing HepG2 cells were cultured with or without 0.5 μg/ml rhSHH for 24 h, and MMP-2 and MMP-9 expression levels were detected by (A) RT-qPCR and (B) western blot analyses. BRD4 upregulated the SHH signaling pathway. Expression levels of SHH and GLI1 were downregulated in shBRD4-treated HepG2 cells compared with shcontrol-treated HepG2 cells, as evaluated by (C) RT-qPCR and (D) western blot analyses. *P<0.05, comparison indicated by brackets. SHH, sonic hedgehog; MMP, matrix metalloproteinase; rhSHH, recombinant human SHH peptide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

western blot analysis (Fig. 4B). These results indicate that the reduction in cell migration and invasion when BRD4 is silenced may be attributed to the downregulation of MMP-2 and MMP-9. Previous studies have demonstrated that the SHH signaling pathway serves a critical function in the migration and invasion of HCC by regulating MMP-2 and MMP-9 (5). Therefore, it was important to determine whether the SHH signaling pathway was involved in the BRD4-induced cell migration and invasion, and MMP-2 and MMP-9 expression in HepG2 cells. The potential effect of BRD4 on the SHH signaling pathway in HCC cells was examined. The results demonstrated that BRD4 knockdown significantly reduced the downstream target genes shh and gli1 a in HepG2 cells (P<0.05; Fig. 4C). Protein levels were also markedly reduced (Fig. 4D).

Effects of rhSHH treatment. It was also determined whether BRD4 silencing reduces the invasion and migration of HepG2 cells through the SHH signaling pathway. rhSHH was used in the invasion and migration assays with BRD4 knockdown of HepG2 cells (Fig. 3A-D). The results demonstrated that treatment with rhSHH markedly increased the migration and invasion capabilities of HepG2 cells that were previously reduced by BRD4 silencing. Furthermore, the association of the activation of the SHH signaling pathway and the BRD4-mediated upregulation of the MMP-2 and MMP-9 expression was investigated: The results of this analysis demonstrated that rhSHH significantly increased the expression levels of MMP-2 and MMP-9, which were previously reduced by BRD4 silencing in HepG2 cells (Fig. 4A and B).

Discussion

BRD4 has been demonstrated to serve a critical role in carcinogenesis and progression of various types of human cancer (14,16,17). However, the role of BRD4 in cancer metastasis has not been illustrated. The present study focused on the effects of BRD4 on the migration and invasion of liver cancer. BRD4 expression was correlated with migration and invasion in HCC. Furthermore, the BRD4 mechanisms in HCC invasion and metastasis were investigated. Metastasis is a multistep process that initiates when cancer cells disseminate from a primary tumor to distant secondary organs or tissues (21,22). The degradation of the extracellular matrix (ECM) is an essential step in cancer invasion and metastasis (21,22). MMPs are proteolytic enzymes, and their basic mechanism of action is to degrade proteins in the ECM. MMPs, particularly MMP-2 and MMP-9 have long been associated with cancer cell metastasis in HCC (5,23). In the present study, the expression levels of MMP-2 and MMP-9 were significantly downregulated *in vitro*

following BRD4 silencing. These results indicate that BRD4 induces HCC cell migration and invasion through the elevated expression of MMP-2 and MMP-9.

Accumulating evidence has demonstrated that the SHH signaling pathway serves an important role in the development and progression of HCC (8,24-27). The SHH signaling pathway has been demonstrated to contribute to HCC cell metastasis by regulating MMP-2 and MMP-9 (5). Furthermore, a study demonstrated that the SHH signaling pathway is modulated by BRD4 (12). In the present study, to further illustrate the mechanism underlying BRD4 regulation of MMP-2 and MMP-9 expression in HCC, the association between BRD4, the SHH signaling pathway, and MMP-2 and MMP-9 were investigated. The expression levels of SHH and GLI1 were reduced by BRD4 knockdown in HepG2 cells. In addition, the data demonstrated that rhSHH significantly increased the migration and invasion of the HepG2 cells, thus reversing the attenuation achieved by BRD4 silencing. The results illustrated that rhSHH significantly increased the expression of MMP-2 and MMP-9 in HepG2 cells, previously reduced by BRD4 silencing. Thus, BRD4 promotes hepatoma cell migration and invasion through the SHH signaling pathway via inducing expression of MMP-2 and MMP-9. However, other molecular mechanisms underlying the BRD4-induced migration and invasion of HCC cells may exist that were not investigated in the present study.

In summary, the present study demonstrated that BRD4 contributes to the invasion and migration of HCC cells. The results emphasized the potential role of SHH signaling pathway-mediated MMP-2 and MMP-9 expression in BRD4-induced HCC cell migration and invasion. The data also indicated that BRD4 is a novel potential target for the development of alternative approaches to HCC treatment.

References

1. Wang X, Jiang F, Mu J, Ye X, Si L, Ning S, Li Z and Li Y: Arsenic trioxide attenuates the invasion potential of human liver cancer cells through the demethylation-activated microRNA-491. Toxicol Lett 227: 75-83, 2014.
2. Chen XP, Qiu FZ, Wu ZD, Zhang ZW, Huang ZY, Chen YF, Zhang BX, He SQ and Zhang WG: Effects of location and extension of portal vein tumor thrombus on long-term outcomes of surgical treatment for hepatocellular carcinoma. Ann Surg Oncol 13: 940-946, 2006.
3. Sicklick JK, Li YX, Jayaraman A, Kannangai R, Qi Y, Vivekanandan P, Ludlow JW, Owzar K, Chen W, Torbenson MS and Diehl AM: Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis. Carcinogenesis 27: 748-757, 2006.
4. Che L, Yuan YH, Jia J and Ren J: Activation of sonic hedgehog signaling pathway is an independent potential prognosis predictor in human hepatocellular carcinoma patients. Chin J Cancer Res 24: 323-331, 2012.
5. Chen JS, Huang XH, Wang Q, Huang JQ, Zhang LJ, Chen XL, Lei J and Cheng ZX: Sonic hedgehog signaling pathway induces cell migration and invasion through focal adhesion kinase/AKT signaling-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9 in liver cancer. Carcinogenesis 34: 10-19, 2013.
6. Lu JT, Zhao WD, He W and Wei W: Hedgehog signaling pathway mediates invasion and metastasis of hepatocellular carcinoma via ERK pathway. Acta Pharmacol Sin 33: 691-700, 2012.
7. LoRusso PM, Rudin CM, Reddy JC, Tibes R, Weiss GJ, Borad MJ, Hann CL, Brahmer JR, Chang I, Darbonne WC, *et al*: Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. Clin Cancer Res 17: 2502-2511, 2011.
8. Chen JS, Li HS, Huang JQ, Zhang LJ, Chen XL, Wang Q, Lei J, Feng JT, Liu Q and Huang XH: Down-regulation of Gli-1 inhibits hepatocellular carcinoma cell migration and invasion. Mol Cell Biochem 393: 283-291, 2014.
9. Jeng KS, Sheen IS, Jeng WJ, Yu MC, Tsai HH, Chang FY and Su JC: Blockade of the sonic hedgehog pathway effectively inhibits the growth of hepatoma in mice: An in vivo study. Oncol Lett 4: 1158-1162, 2012.
10. Yauch RL, Dijkgraaf GJ, Alicke B, Januario T, Ahn CP, Holcomb T, Pujara K, Stinson J, Callahan CA, Tang T, *et al*: Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science 326: 572-574, 2009.
11. Buonamici S, Williams J, Morrissey M, Wang A, Guo R, Vattay A, Hsiao K, Yuan J, Green J, Ospina B, *et al*: Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. Sci Transl Med 2: 51ra70, 2010.
12. Tang Y, Gholamin S, Schubert S, Willardson MI, Lee A, Bandopadhayay P, Bergthold G, Masoud S, Nguyen B, Vue N, *et al*: Epigenetic targeting of Hedgehog pathway transcriptional output through BET bromodomain inhibition. Nat Med 20: 732-740, 2014.
13. Yang Z, He N and Zhou Q: Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. Mol Cell Biol 28: 967-976, 2008.
14. Segura MF, Fontanals-Cirera B, Gaziel-Sovran A, Guijarro MV, Hanniford D, Zhang G, González-Gomez P, Morante M, Jubierre L, Zhang W, *et al*: BRD4 sustains melanoma proliferation and represents a new target for epigenetic therapy. Cancer Res 73: 6264-6276, 2013.
15. Puissant A, Frumm SM, Alexe G, Bassil CF, Qi J, Chanthery YH, Nekritz EA, Zeid R, Gustafson WC, Greninger P, *et al*: Targeting MYCN in neuroblastoma by BET bromodomain inhibition. Cancer Discov 3: 308-323, 2013.
16. Cheng Z, Gong Y, Ma Y, Lu K, Lu X, Pierce LA, Thompson RC, Muller S, Knapp S and Wang J: Inhibition of BET bromodomain targets genetically diverse glioblastoma. Clin Cancer Res 19: 1748-1759, 2013.
17. Patel AJ, Liao CP, Chen Z, Liu C, Wang Y and Le LQ: BET bromodomain inhibition triggers apoptosis of NF1-associated malignant peripheral nerve sheath tumors through Bim induction. Cell Rep 6: 81-92, 2014.
18. Ott CJ, Kopp N, Bird L, Paranal RM, Qi J, Bowman T, Rodig SJ, Kung AL, Bradner JE and Weinstock DM: BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia. Blood 120: 2843-2852, 2012.
19. Lockwood WW, Zejnullahu K, Bradner JE and Varmus H: Sensitivity of human lung adenocarcinoma cell lines to targeted inhibition of BET epigenetic signaling proteins. Proc Natl Acad Sci USA 109: 19408-19413, 2012.
20. Lamoureux F, Baud'huin M, Rodriguez Calleja L, Jacques C, Berreur M, Rédini F, Lecanda F, Bradner JE, Heymann D and Ory B: Selective inhibition of BET bromodomain epigenetic signalling interferes with the bone-associated tumour vicious cycle. Nat Commun 5: 3511, 2014.
21. Vihinen P, Ala-aho R and Kähäri VM: Matrix metalloproteinases as therapeutic targets in cancer. Curr Cancer Drug Targets 5: 203-220, 2005.
22. Freije JM, Balbín M, Pendás AM, Sánchez LM, Puente XS and López-Otín C: Matrix metalloproteinases and tumor progression. Adv Exp Med Biol 532: 91-107, 2003.
23. Gao J, Ding F, Liu Q and Yao Y: Knockdown of MACC1 expression suppressed hepatocellular carcinoma cell migration and invasion and inhibited expression of MMP2 and MMP9. Mol Cell Biochem 376: 21-32, 2013.
24. Xu Q, Liu X, Zheng X, Yao Y, Wang M and Liu Q: The transcriptional activity of Gli1 is negatively regulated by AMPK through Hedgehog partial agonism in hepatocellular carcinoma. Int J Mol Med 34: 733-741, 2014.
25. Zheng X, Zeng W, Gai X, Xu Q, Li C, Liang Z, Tuo H and Liu Q: Role of the Hedgehog pathway in hepatocellular carcinoma (review). Oncol Rep 30: 2020-2026, 2013.
26. Pinter M, Sieghart W, Schmid M, Dauser B, Prager G, Dienes HP, Trauner M and Peck-Radosavljevic M: Hedgehog inhibition reduces angiogenesis by downregulation of tumoral VEGF-A expression in hepatocellular carcinoma. United European Gastroenterol J 1: 265-275, 2013.
27. Wang Y, Han C, Lu L, Magliato S and Wu T: Hedgehog signaling pathway regulates autophagy in human hepatocellular carcinoma cells. Hepatology 58: 995-1010, 2013.