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Co-culture of hepatoma cells with hepatocytic precursor (stem-like) cells inhibits tumor cell growth and invasion by downregulating Akt/NF-κB expression

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Abstract. Hepatocytic stem cells (HSCs) have inhibitory effects on hepatocarcinoma cells. The present study investigated the effects of HSC activity in hepatocarcinoma cells *in vitro*. A Transwell co-culture system of hepatocytic precursor (stem-like) WB-F344 cells and hepatoma CBRH-7919 cells was used to assess HSC activity in metastasized hepatoma cells *in vitro*. Nude mouse xenografts were used to assess HSC activity *in vivo*. Co-culture of hepatoma CBRH-7919 cells with WB-F344 cells suppressed the growth and colony formation, tumor cell migration and invasion capacity of CBRH-7919 cells. The nude mouse xenograft assay demonstrated that the xenograft size of CBRH-7919 cells following co-culture with WB-F344 cells was significantly smaller compared with that of control cells. Furthermore, the expression levels of the epithelial markers E-cadherin and β-catenin were downregulated, while the mesenchymal markers α-SMA and vimentin were upregulated. Co-culture of CBRH-7919 cells with WB-F344 cells downregulated NF-κB and phospho-Akt expression. In conclusion, hepatocytic precursor (stem-like) WB-F344 cells inhibited the growth, colony formation and invasion capacity of metastasized hepatoma CBRH-7919 cells *in vitro* and *in vivo* by downregulating Akt/NF-κB signaling.

Introduction

Hepatocellular carcinoma (HCC) is a significant health problem in the world and has a very poor prognosis (1). To date, a treatment strategy does not exist to effectively treat HCC patients (1,2). Although the overall 5-year survival rate of HCC patients receiving conventional chemotherapy after surgical resection or liver transplantation at a sufficiently early stage is increasing (3), the long-term prognosis remains unsatisfactory due to high recurrence rates. Therefore, novel approaches are needed to prevent and treat HCC.

Stem cell research may provide a novel insight into identifying a better strategy to treat HCC. Both embryonic and adult stem cells have self-renewal capacities and have the ability to produce differentiated progenitors. During tumorigenesis, stem cells from normal tissues are able to migrate into tumor lesions and suppress tumor cell growth (4,5). For example, a previous study demonstrated that human bone marrow-derived mesenchymal stem cells could be used to treat gliomas (4). These findings led to other studies that investigated whether modified stem cells can express anticancer molecules as targeted anticancer agents (6-9) and whether normal stem cells have antitumor activity.

In liver tissue, the ability of hepatocytic stem cells to migrate into HCC tissue has been reported previously (10). Thus, stem cells may be used as a vector to deliver therapeutic genes for targeted cancer therapy (11-13). Indeed, the microenvironment of stem cells is considered to serve a role in regulating HCC cell growth (14,15) by downregulating multiple signal transduction pathways, such as the Wnt/β-catenin (14) and TGF-β/Smad (16) pathways. However, the molecular mechanisms of the inhibitory effect of stem cells on tumor cells remain to be defined.

Constitutive activation of Akt and nuclear factor-kappaB (NF-κB) are major cellular abnormalities associated with HCC pathogenesis and progression (17). Inactivation of NF-κB signaling inhibits growth and induces apoptosis of HCC cells (18-21). Similarly, a previous study demonstrated that inhibition of Akt signaling resulted in HCC cell growth, arrest and apoptosis (22,23). Another previous study demonstrated that Akt activates NF-κB transcription by phosphorylating IκBκ (24,25). Thus, inhibiting Akt/NF-κB signaling may be a novel therapeutic approach for the control of HCC (17).

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The present authors previous study demonstrated that hepatocytic precursor (stem-like) cells reduced the tumorigenicity and induced apoptosis of hepatoma cells in a co-culture system (16). In the present study, the effect of co-culturing hepatoma cells with hepatocytic precursor (stem-like) cells on the regulation of HCC phenotypic and gene expression was assessed *in vitro* and in nude mice. The present study aimed to provide useful insight into the development of stem cell-initiated antitumor therapy for HCC patients.

Materials and methods

Cell lines and culture. The diploid rat liver epithelial cell line WB-F344 was obtained from the Shanghai Cell Bank (Shanghai, China). These cells are oval in shape and have the capacity to differentiate into hepatocyte and biliary lineages under suitable conditions (26); thus, these cells are considered to be hepatocytic precursor (stem-like) cells. The rat hepatoma cell line CBRH-7919 was also obtained from the Shanghai Cell Bank.

WB-F344 and CBRH-7919 cells were co-cultured using a Transwell chamber culture system containing 4.0 μm pore polycarbonate membrane inserts (Corning, NY, USA). The WB-F344 to CBRH-7919 cell ratio was 5:1 ($2x10^5$:$4x10^4$ cells/well). CBRH-7919 cells cultured alone in the Transwell chamber culture system were used as a control. All the cells were cultured in serum-free conditioned medium from established cultures at 37˚C with 95% air, 5% CO_2, and 100% humidity for 7 days before the cells were used for subsequent assays. The serum-free conditioned medium was composed of Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20 ng/ml of basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/ml of epidermal growth factor (Sigma-Aldrich), and 20 μl/ml of B27 supplement (Invitrogen; Thermo Fisher Scientific, Inc.).

Nude mouse experiments. The present study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University (Shanghai, China). The mice used in the experiment were maintained under specific pathogen-free conditions and handled in accordance with the procedures and guidelines set by the Institutional Animal Care and Use Committee of The Second Military Medical University (Shanghai, China). Co-cultured WB-F344 and CBRH-7919 cells and single culture CBRH-7919 cells ($1x10^6$ cell/mouse) were subcutaneously inoculated into the axillary fossae of female nude mice (age, 6-8 weeks old). The tumor size was monitored every 3 days by measuring the length and width with calipers. The tumor volume was calculated using the formula: [(L x W^2) x 0.5 mm^3], in which L was the length and W was the width of each tumor. At day 35 post-injection, mice were sacrificed for pathological analysis.

Cell proliferation and clonogenic assays. Cell counting kit-8 (CCK-8) is a sensitive, nonradioactive colorimetric assay that assesses cell proliferation and detects the number of living cells. In the present study, a CCK-8 (Dojindo Molecular Technologies, Inc., Tokyo, Japan) assay was performed to assess the effect of rat WB-F344 stem cells on CBRH-7919 cell proliferation. In brief, after co-culturing these cell lines for 7 days in serum-free conditioned medium, CBRH-7919 cells were trypsinized, counted, and $5x10^4$ cells were seeded in 24-well plates in triplicate and cultured for up to 8 days. At the end of each experiment, the cells were further incubated with an additional equal amount of fresh medium containing 10% CCK-8 at 37˚C for 4 h, and the cell number was then counted. The data are presented as the mean cell number of each count in the curve diagrams.

For the clonogenic assay, CBRH-7919-only cultured cells and CBRH-7919 cells co-cultured with WB-F344 stem cells were seeded in 12-well plates in triplicate at a density of 100 cells/well and grown for 14 days. Subsequently, cell colonies were stained with 0.5% crystal violet and images were captured (EOS 600D Digital SLR; Canon, Inc., Tokyo, Japan) using an Olympus 1X71 inverted microscope (Olympus Corp., Tokyo). The number of colonies was counted 14 days after seeding. A colony was counted only if it contained ≥50 cells. The rate of colony formation was calculated with the following equation: colony formation rate = (number of colonies/number of seeded cells) x100%.

Tumor cell migration and invasion assay. The ability of CBRH-7919-only cultured cells and CBRH-7919 cells co-cultured with WB-F344 stem cells to invade through Matrigel-coated filters was investigated using the 8-μm BD Falcon™ cell culture insert (BD Biosciences, San Jose, CA, USA). Briefly, $1x10^5$ cell were suspended in 500 μl serum-free DMEM/F12 and then seeded into the upper compartments of each chamber. The lower compartments were filled with 1 ml DMEM/F12 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). After 24 h of incubation at 37˚C in 5% CO_2, non-invading cells were removed by scrubbing the upper surface of the membrane. Cells that invaded into the bottom surface of the membrane were fixed with methanol and stained with 0.5% crystal violet. The cells were then reviewed and counted under a microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan) in 5 microscopic fields (x100 magnification).

Wound healing assay. Scratch assays were performed to assess the effect of WB-F344 stem cells on the migration of hepatoma cells. Briefly, cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. When the cells were 90-100% confluent, the monolayer was scratched manually with a plastic pipette tip across each plate. The cells were then washed twice with phosphate buffered saline (PBS) and further incubated for up to 96 h. At the end of each experiment, images of each plate were captured to demonstrate 'wound healing' using a PowerShot G10 camera (Canon, Inc.). The distance of each wound was measured and the mean was calculated for the capacity of tumor cell migration.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed into cDNA using a Two-Step RT-PCR kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR cycling conditions were as

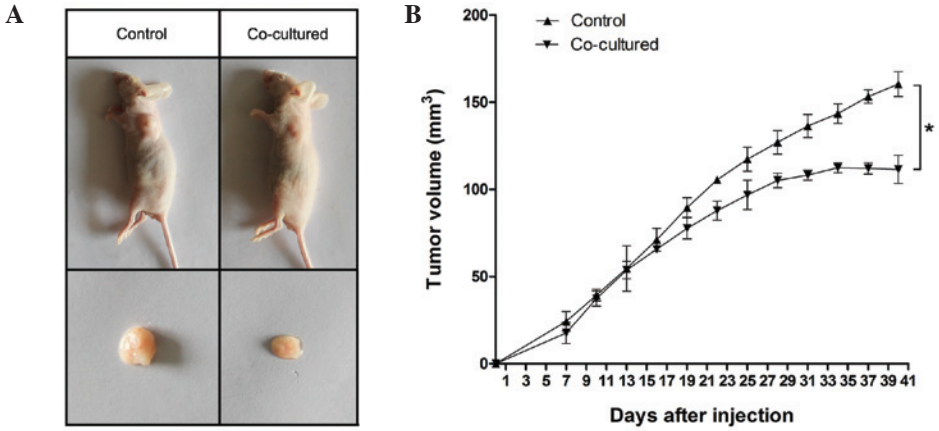

Figure 1. The effects of co-culturing CBRH-7919 and WB-F344 cells on the inhibition of CBRH-7919 cell xenograft growth in nude mice. (A) Example of nude mouse xenograft assay. (B) Quantification of tumor size. *P<0.05.

follows: One cycle at 95˚C for 30 sec, followed by 30 cycles at 95˚C for 5 sec, one cycle at 60˚C for 30 sec, with a final extension step at 72˚C for 1 min, using the SYBR Green Realtime PCR Master Mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The samples were held at 4˚C until required. To determine the levels of E-cadherin, β-catenin, α-SMA and vimentin, qPCR was performed with an ABI 7300 instrument (Thermo Fisher Scientific, Inc.) using a SYBR PrimeScript RT-PCR kit (Takara Suzo, Kyoto, Japan) according to the manufacturer's instructions. The mRNA β-actin level was used as an internal control. The primer sequences used for amplification were as follows: E-cadherin, 5'-TGAGGTCGGTGCCCGTATTG-3' and 5'-GAATGCCCT CGTTGGTCTTGG-3'; β-catenin, 5'-ACTCTAGTGCAG CTTGGTTC-3' and 5'-ATGGCAGGCTCGGTAATGTC-3'; α-SMA, 5'-ATGACCCAGATTATGTTTGAGACC-3' and 5'-CCAGAGTCCAGCACAATACCAG-3'; vimentin, 5'-CGC AGCCTCTATTCCTCGTC-3' and 5'-TCTTGAACTCGG TGTTGATGG-3'; and β-actin, 5'-ACGTTGACATCCGTA AAGAC-3' and 5'-GAAGGTGGACAGTGAGGC-3'. The relative mRNA levels were calculated based on the Ct values and normalized to the level of β-actin mRNA according to the equation: $2^{-\Delta\Delta Ct}$ [ΔCt=Ct (Gene)-Ct (β-actin)]. All of the experiments were performed in triplicate and repeated once.

Protein extraction and western blot analysis. The levels of NF-κB (p65), phospho-Akt, phospho-IKKα/β and phospho-IκBα proteins were assayed in CBRH-7919 cells co-cultured with WB-F344 cells and CBRH-7919-only cultured cells using western blot analysis. Briefly, total cell extracts were prepared using RIPA lysis buffer (50 mM Tris-HCl, 1% NP-40, 2 mM EDTA, 10 mM NaCl, 2 mg/ml aprotinin, 5 mg/ml leupeptin, 2 mg/ml pepstatin, 1 mM DTT, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using the BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein (20 μg) were then separated by SDS-PAGE and transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA) by electroblotting. The membranes were then blocked with 5% non-fat milk in PBS-T (PBS with 0.05% Tween-20) for 1 h and then incubated with specific primary antibodies against rat NF-κB (p65; rabbit monoclonal; dilution, 1:500; catalog no., CST-8242), phospho-Akt (rabbit monoclonal; dilution, 1:1,000; catalog no., CST-12178), phospho-IKKα/β (rabbit monoclonal; dilution, 1:1,000; catalog. no., CST-2697), phospho-IκBα (rabbit monoclonal; dilution, 1:500; catalog no., CST-2859; all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (rabbit polyclonal; dilution, 1:500; catalog no., SC-25778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. The membranes were then washed with PBS-T and incubated with a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (dilution, 1:2,000; catalog no., SC-2004; Santa Cruz Biotechnology, Inc.) for 1 h. The protein bands were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.) and exposed on X-ray films. The level of protein expression was normalized to GAPDH protein.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS version 19 (IBM SPSS, Armonk, NY, USA) was used to analyze the data. Statistical analysis was performed using the two-tailed Student's *t*-test and P<0.05 was considered to indicate a statistically significant difference.

Results

Hepatocytic precursor WB-F344 cells suppressed the growth of hepatoma cell xenografts in nude mice. In the present study, CBRH-7919 cells were co-cultured with WB-F344 cells or were cultured alone for 7 days and then injected into nude mice. On day 22 post-injection, nude mice bearing co-cultured or control CBRH-7919 cells had similar tumor xenograft volumes. From ~4 weeks, mice bearing co-cultured CBRH-7919 cells had a significantly reduced tumor volume compared with mice bearing control xenografts (Fig. 1, P<0.05). The data indicated that WB-F344 cells inhibited the growth of hepatoma CBRH-7919 cell xenografts in nude mice.

Hepatocytic precursor (stem-like) WB-F344 cells suppressed the clonogenic growth of CBRH-7919 cells. After co-culture with hepatocytic precursor (stem-like) WB-F344 cells, hepatoma CBRH-7919 cells exhibited a reduced cell growth and

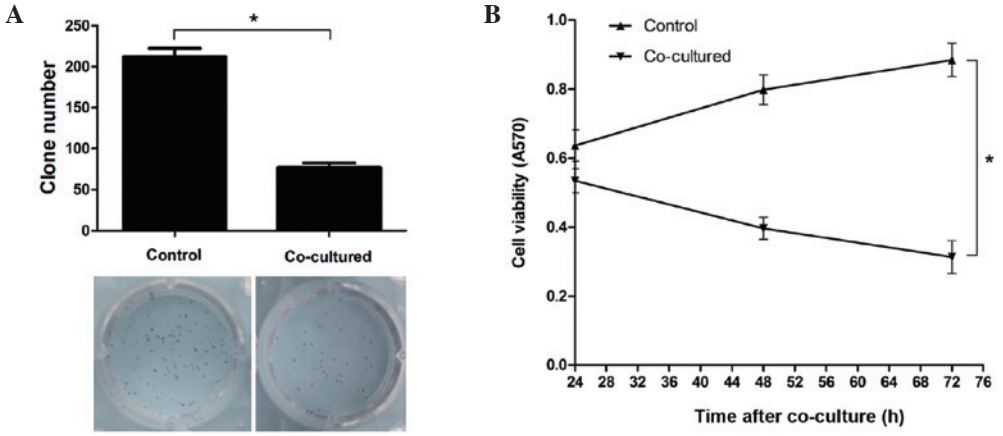

Figure 2. The effects of co-culturing CBRH-7919 cells and WB-F344 cells on the suppression of CBRH-7919 cell proliferation and colony formation. (A) Colony formation assay. The co-cultured CBRH-7919 cells were seeded in 12-well plates and the number of colonies, which contained ≥50 cells, was counted 14 days after seeding. (B) Tumor cell proliferation assay. The cell number was counted every day for 8 days. The mean cell number of each count is presented in the curve diagram. *P<0.05.

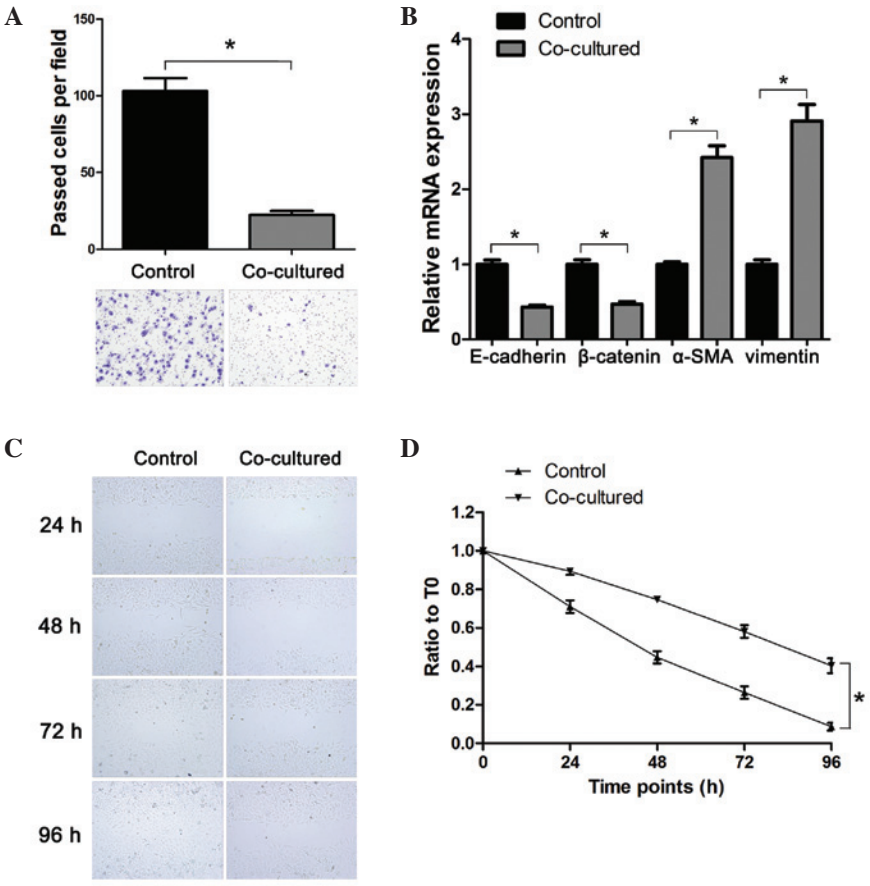

Figure 3. The effects of co-culturing CBRH-7919 cells and WB-F344 cells on the regulation of CBRH-7919 cell migration, invasion capacity and gene expression. (A) Transwell tumor cell invasion assay. (B) Reverse transcription-quantitative polymerase chain reaction demonstrating expression of epithelial mesenchymal transition markers. (C) Representative images of wound healing assay. (D) Quantified data of wound healing assay. *P<0.05.

proliferation rate; there was a significant reduction in the number of CBRH-7919 cells in both monolayer and colony formation (P<0.05, Fig. 2A and B).

Hepatocytic precursor (stem-like) WB-F344 cells suppressed the migration and invasion of CBRH-7919 cells. As presented in Fig. 3A, the Transwell assay demonstrated that co-cultured CBRH-7919 cells demonstrated a reduced invasion capacity compared with that of the control cells (P<0.05).

In addition, epithelial-mesenchymal transition (EMT), a key event in tumor invasion and metastasis (27,28), was also characterized in the present study. The RT-qPCR analysis results demonstrated that the mRNA expression levels of the epithelial markers E-cadherin and β-catenin were

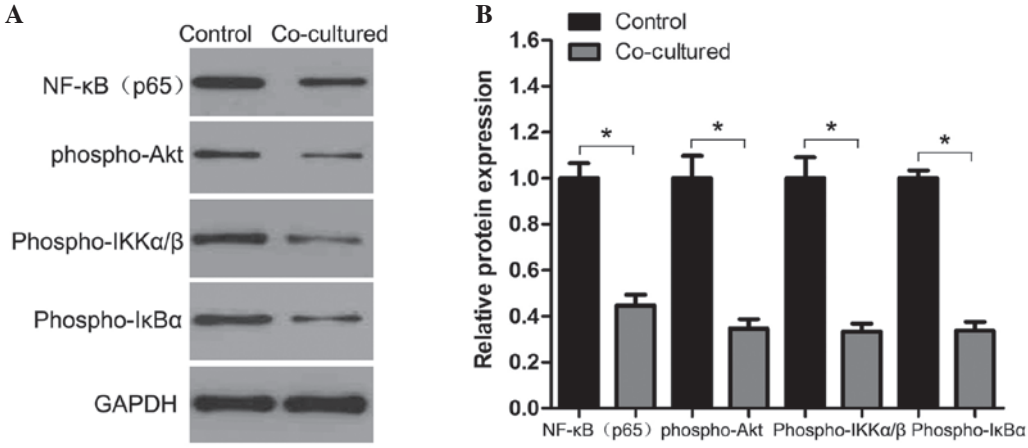

Figure 4. The effects of co-culturing CBRH-7919 cells and WB-F344 cells on the regulation of gene expression. (A) Western blots demonstrating the protein expression levels of NF-κB (p65), phospho-Akt, phospho-IKKα/β, and phospho-IκBα. (B) Quantified data of western blot analysis. *P<0.05.

downregulated in co-cultured CBRH-7919 cells, while the mesenchymal markers α-SMA and vimentin were upregulated when compared with control cells (P<0.05; Fig. 3B).

The tumor cell wound healing assay demonstrated that CBRH-7919 cells co-cultured with WB-F344 cell demonstrated a reduced invasion capacity compared with that of the control cells (P<0.05; Fig. 3C and D).

These results indicated that co-culture with WB-F344 cells suppressed the migration and invasion of CBRH-7919 cells via regulation of tumor cell EMT activity.

Akt/NF-κB signaling mediated the migration and invasion capacity of hepatoma CBRH-7919 cells. Since AKT and NF-κB are essential regulators of tumor cell properties, including metastasis (29,30), the present authors proposed that the Akt/NF-κB signaling pathway may be involved in the inhibition of hepatoma CBRH-7919 cell migration and invasion capacity through its pro-apoptotic role. As illustrated in Fig. 4, western blot analyses demonstrated that co-culture of hepatoma CBRH-7919 cells with WB-F344 cells resulted in the downregulation of NF-κB and phospho-Akt expression in CBRH-7919 cells.

Discussion

HCC is a leading cause of cancer-associated mortality in the world and has an extremely poor prognosis (31,32). The development and identification of novel therapeutic strategies to effectively control HCC metastasis may increase patient survival. Previous studies have indicated that stem cells may be useful to treat different types of human cancer (4-15). Regardless of their distinct origins, stem cells and tumor cells share a number of common properties, including the loss of contact inhibition and immortality, and therefore, similar signaling pathways may be involved in their control. It has been reported that the stem cell microenvironment is essential in preventing carcinogenesis by providing signals to inhibit cell proliferation and to promote differentiation (33). In the current study, it was demonstrated that co-culture with WB-F344 cells suppressed the growth, colony formation and tumor cell migration and invasion capacity of CBRH-7919 cells. Co-cultured CBRH-7919 cells also expressed downregulated levels of the epithelial markers E-cadherin and β-catenin and upregulated expression levels of the mesenchymal markers α-SMA and vimentin via downregulating NF-κB and phospho-Akt expression. In addition, the co-culture of CBRH-7919 cells with WB-F344 cells inhibited xenograft formation and growth in a nude mouse model. The current study therefore indicates that hepatocytic precursor (stem-like) WB-F344 cells regulate HCC cell phenotype and gene expression. However, this study is a proof-of-principle study and additional studies are needed to understand the underlying molecular mechanisms by which stem cells act on HCC cells.

The results of the present study are consistent with the effect of human mesenchymal stem cells on HCC cells, as previously reported (34). In the current study, suppression of the Akt/NF-κB signaling pathway after co-culturing CBRH-7919 cells with WB-F344 cells contributed to the suppression of HCC cell growth and invasion. Indeed, aberrant activation of the Akt/NF-κB signaling pathway has been widely investigated and reported in the literature. Akt and NF-κB proteins regulate a variety of cellular processes, including cell proliferation, apoptosis, invasion and angiogenesis (35-38). Furthermore, Akt and NF-κB are being extensively investigated as molecular targets for cancer therapeutics (38), as studies have demonstrated that repression of Akt and/or NF-κB activity inhibit cancer cell growth, migration and invasion, and sensitizes cancer cells to apoptosis (39-41). In agreement with these previous studies, the current data demonstrated that Akt-mediated phosphorylation of IKKα/β and IκBα was necessary for the activation of NF-κB, further mediating the metastasis potential of hepatoma CBRH-7919 cells in this co-culture system. In the resting state, the activity of NF-κB is sequestered in the cytoplasm as an inactive precursor complex with inhibitory κB (IκB). The biological activity of NF-κB is tightly regulated by IκBα. It appears that newly synthesized IκB rapidly re-associates with newly released NF-κB, thereby markedly reducing the amount of NF-κB translocated into the nucleus for the activation of cytokine genes. The activity of the NF-κB transcription factor is regulated by p-IκB through multiple intracellular signal transduction pathways (42).

In conclusion, hepatocytic precursor (stem-like) WB-F344 cells inhibited the growth, colony formation and

invasion capacity of hepatoma CBRH-7919 cells *in vitro* and *in vivo* by downregulating Akt/NF-κB signaling. However, further studies are required to confirm the results of the present study. Subsequent studies by the present authors will assess whether overexpressing genes in this signaling pathway overcome the activity of co-culturing CBRH-7919 cells with WB-F344 cells.

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