Crude Extracts of *Euchresta formosana* Radix Inhibit Invasion and Migration of Human Hepatocellular Carcinoma Cells

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Abstract. *Crude extracts of Euchresta formosana radix (EFR) have previously been observed to induce the suppression of liver cancer Hep3B cell growth and induce apoptosis in response to overexpression of reactive oxygen species, GADD153, Bax and caspase-3, and to decrease the levels of mitochondrial membrane potential in vitro. In this study, the effect of EFR on cell migration and invasion by the human liver hepatocellular carcinoma (HCC) cell line Hep3B was examined. Hep3B cells treated in vitro with EFR migrated and invaded less than cells treated with phosphate-buffered saline (PBS) as a control. EFR inhibited migration and invasion by down-regulating the production of RhoA and ROCK1, FAK, and matrix metalloproteinase-1, -2, -9 and -10 relative to PBS only. These results show that EFR inhibits invasion and migration by liver cancer cells by down-regulating proteins associated with these processes, resulting in reduced metastasis. Thus, EFR should be considered as a possible therapeutic agent for inhibiting primary tumor growth and preventing metastasis.*

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and the incidence of HCC and the mortality rate for patients with HCC has remained high over the past decade (1). The People's Health Bureau of Taiwan has demonstrated that about 8.99 people per 100,000 die per year of liver cancer in Taiwan. The causes of HCC include alcohol, smoking, mycotoxins and the human hepatitis virus (2). To date, the therapy for HCC has included surgery, radiotherapy and chemotherapy, has not been satisfactory.

Cancer development is a multi-step progression that eventually can enable tumor cells to move to other locations far from a given primary tumor mass, and this often leads to metastasis. More than 90% of cancer patients die not from their primary tumors but due to the development of metastases (3, 4). Cell movement through tissues or organs thus plays a crucial and primary role in cancer progression. The progression consists of tumor cell attachment to the extracellular matrix components, the degradation of the matrix by tumor cell-associated proteases and tumor cell advance into the region where the matrix component has been modified by proteolysis (5-7).

Migrating cells may show a decreased proliferation rate and less sensitivity to standard chemotherapy (7-10). Therefore, cancer therapy strategies must inhibit and/or reduce the spread of tumor cells by targeting the factors which regulate the invasive and migratory activity of these cells and so prevent metastasis.

The plant *Euchresta formosana* has long been used as a folk medicine in Chinese populations. Recent studies have shown that the crude extract of *Euchresta formosana* radix (EFR) has several biological activities including inhibiting the growth and proliferation of and promoting the cell cycle arrest and apoptosis of the human HCC cell line, Hep3B, which has been used extensively as a model to study the migration and invasion by tumor cells (28). However, no reports have addressed the effect of EFR on the invasion and migration of Hep3B cells. In this study, the effects of EFR on the proteins associated with invasion and migration in the human HCC cell line, Hep3B, were investigated.

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Materials and Methods

Preparation of plant extracts. The radixes of *Euchresta formosana* (EFR) were obtained from Taitung Dawu located in the east part of Taiwan in November 2003. They were identified by a taxonomist and a sample representing Herbarium No. CMU EF 0612 was deposited at the School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan. The radixes were air dried at room temperature, ground and kept in amber colored bottles until processed. The alcoholic extraction was conducted by percolating 400 g of the powdered plant material using 95% EtOH. The extraction was repeated five times at room temperature, and the percolate was then filtered through a Whatman filter paper No.1 and evaporated under reduced pressure to yield a brownish viscous residue (37.96 g). The prepared extracts were kept at –80˚C until used. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 50, 75, 100, 125, and 150 μg /ml.

Chemicals and reagents. Type I collagen, paraformaldehyde, crystal violet, propidium iodide (PI), potassium phosphates, triton X-100, Tris-HCl, trypan blue and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel™ Basement Membrane Matrix was a product of BD Biosciences (Bedford, MA, USA). Dimethyl sulfoxide (DMSO) and TE buffer were purchased from Merck Co. (Darmstadt, Germany). The 90% Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Cell culture. The human HCC cell line, Hep3B, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The Hep3B cells were maintained in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

In vitro invasion assay. The *in vitro* invasion assay was carried out according to the method of Huang *et al.* (11). Briefly, 24-well Transwell inserts with 8 μm porosity polycarbonate filters (Millipore, Billerica, MA, USA) were coated with 30 μg Englebreth-Holm-Swarm sarcoma tumor extract, (EHS Matrigel Basement Membrane Matrix), at room temperature for 1 hour to form a genuine reconstituted basement membrane. The Hep3B cells (10^4 cells/0.4 ml DMEM) were placed in the upper compartment and incubated with vehicle or EFR (50 or 75 μg /ml). The plates were then incubated at 37˚C for 24/48 hours in a humidified atmosphere with 95% air and 5% CO_2. The cells were then fixed with 4% paraformaldehyde in PBS and stained with 2% crystal violet. The cells on the upper surface of the filter were removed by wiping with a cotton swab, and the cells that penetrated through the matrigel to the lower surface of the filter were counted under a light microscope at x200 (12). Each treatment was assayed in duplicate, and three independent experiments were performed.

In vitro migration assay. Chemotactic directional migration was evaluated using a 24-well Transwell insert (13). Porous filters (8 mm pores) were coated with 30 μg type I collagen (Sigma) for 1 hour. The cells (10^4 cells/0.4 ml DMEM) were plated in the upper chamber in the presence or absence of EFR (50 or 75 μg/ml) and allowed to undergo migration for 24/48 hours. The non-migrated cells were removed from the upper chamber with a cotton swab, the filters were stained with 2% crystal violet, and the migrated cells adherent to the underside of the filter were counted under a light microscope at x200 (14). Each treatment was assayed in duplicate and three independent experiments were performed
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Wound assays. Hep3B cells were grown to confluent monolayers in 6-well plates. The cells were washed twice with PBS and a wound applied with a plastic pipette tip. The cells were washed twice with PBS, photographed under a phase-contrast microscope (time=0) then incubated in media with or without EFR (50 or 75 mg /ml) at 37˚C and 5% CO_2. After 24/48-hour incubation, the cells were gently washed with PBS and the wound area photographed (15). The assays were performed in triplicate.

Immunofluorescence. Untreated and EFR (125 μg/ml)-treated cells were fixed with 4% paraformaldehyde for 10 minutes and permeablized with 0.1% Triton X-100 for an additional 5 minutes at room temperature. After blocking with 1% BSA/PBS, the cells were incubated with primary antibodies overnight. The cells were treated with FITC-conjugated secondary antibodies and DAPI 1 μg/ml for 1 hour and 10 minutes, respectively (16). After washing, the images were observed and photographed by using a fluorescence microscope.

Western blotting for examining the effect of EFR on JNK-p, c-jun, c-jun-p, PKC, Raf-1, Ras, SOS-1, GRB2, VEGF, HIF-1α, ERK1/2, JNK1/2, p38-p, p38, MEKK3, MKK7, PI3K, AKT Thr348, AKT Ser473, COX1, COX2, iNOS, NF-κB p65, NF-κB p50, MMP-1, MMP-2, MMP-9, MMP-10, RhoA, ROCK-1 and FAK of Hep3B cells. The total proteins were collected from the Hep3B cells treated with or without 125 μg/ml of EFR for 6, 12, 24, 48 h before the JNK-p, c-jun, c-jun-p, PKC, Raf-1, Ras, SOS-1, GRB2, VEGF, HIF-1α, ERK1/2, JNK1/2, p38-p, p38, MEKK3, MKK7, PI3K, AKT Thr348, AKT Ser473, COX1, COX2, iNOS, NF-κB p65, NF-κB p50, MMP-1, -2, -9, -10, RhoA, ROCK-1 and FAK were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously (17-19).

Statistical analysis. Student's *t*-test was used to analyze the statistical analysis between the EFR-treated and control groups.

Results

Anti-invasion effect of EFR in Hep3B cells. The results shown in (Figure 1) indicated that Hep3B cells invaded from the upper to the lower chamber in the absence of EFR (control group). The Hep3B cells did not penetrate the EHS-coated filter in the presence of EFR. The quantification of cells in the lower chamber showed that EFR significantly inhibited Hep3B cell invasion (Figure 2A)

Anti-migration effect of EFR in Hep3B cells. The results shown in Figure 3 indicated that Hep3B cells migrated from the upper to the lower chambers in the absence of EFR (control group). Hep3B cells did not penetrate the collagen-

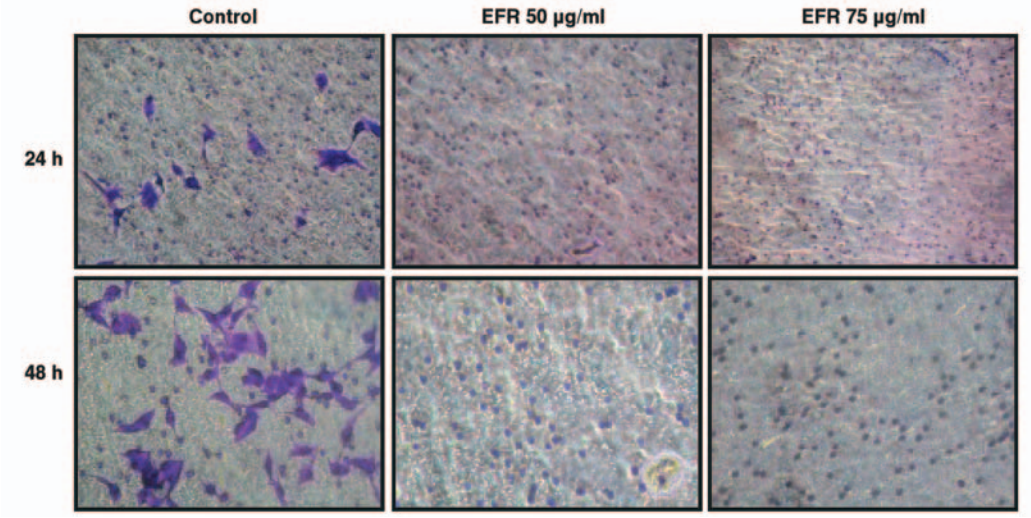

Figure 1. *Effects of EFR on the invasion of Hep3B cells in vitro. Cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and shown under a light microscope at x200.*

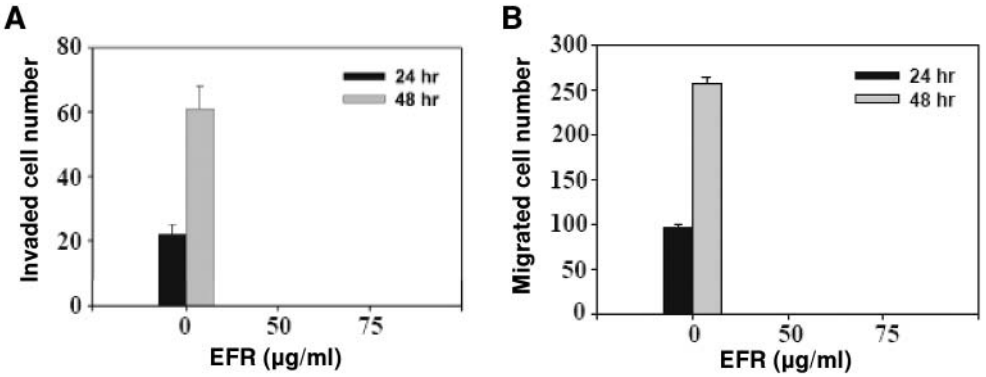

Figure 2. *Quantification of cells in the lower chambers, which was done by counting at x200. Columns, mean from three independent experiments. P<0.01, significant difference between EFR-treated groups and the control as analyzed by Student's t-test.*

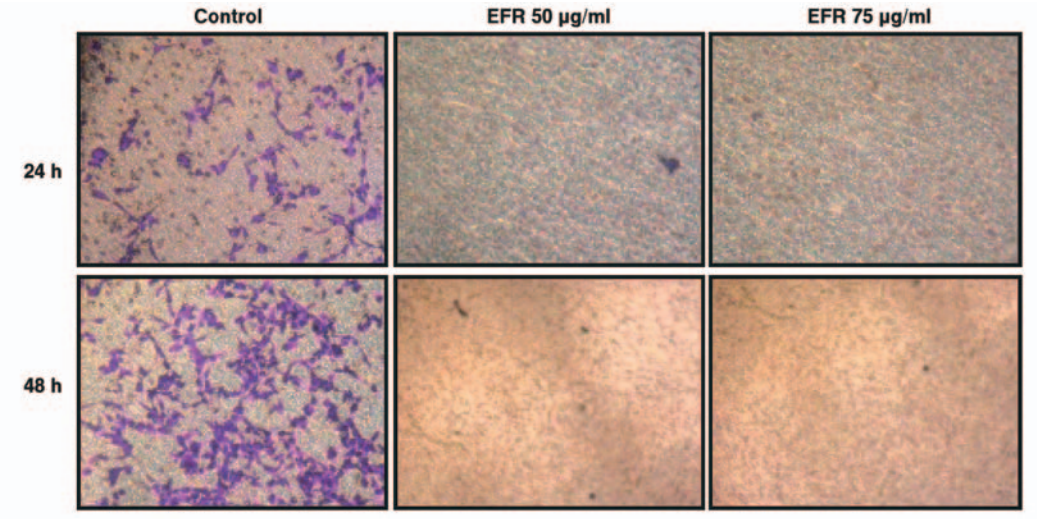

Figure 3. *Effects of EFR on the migration of Hep3B cells in vitro. The filters with a reconstituted collagen type I basement were stained with 2% crystal violet, and migrated cells adherent to the underside of the filter were observed under a light microscope at x200.*

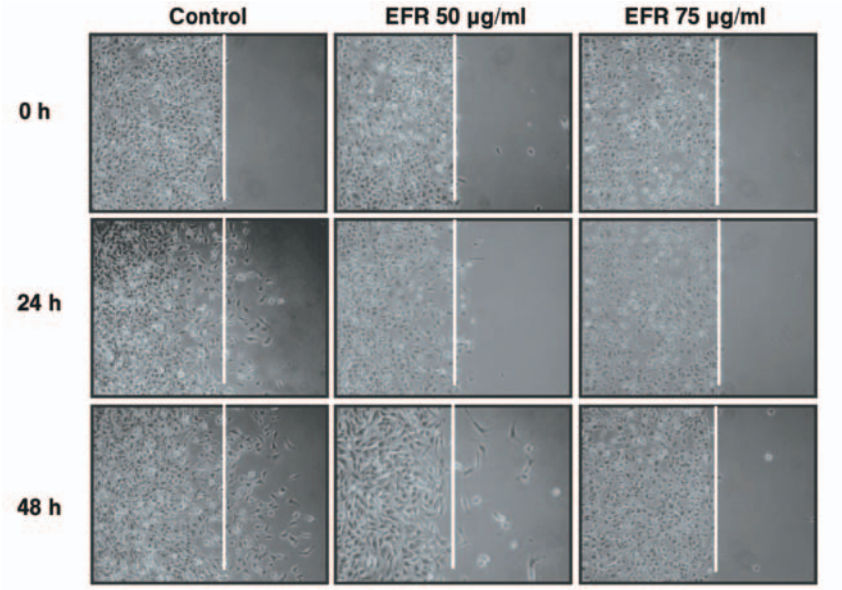

Figure 4. *In vitro wound closure. Confluent monolayers of cells were wounded with a scratch, rinsed to remove debris, and incubated in the absence or presence of EFR (50 and 75 µg/ml) as indicated for 24 and 48 hours. Photographs indicate relative wound closure as monitored by visual examination using a Nikon phase-contrast microscope. Fields shown are representative of the width of quadruplicate wounds made in triplicate cultures.*

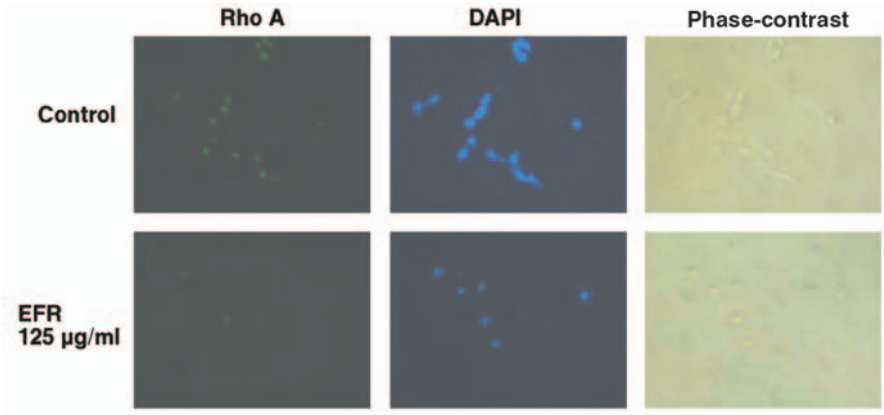

Figure 5. *Immunofluorescence showing RhoA localization. Cells were treated with or without EFR (125 µg/ml) and RhoA localization was represented as green fluorescence. DAPI staining indicated nuclear localization.*

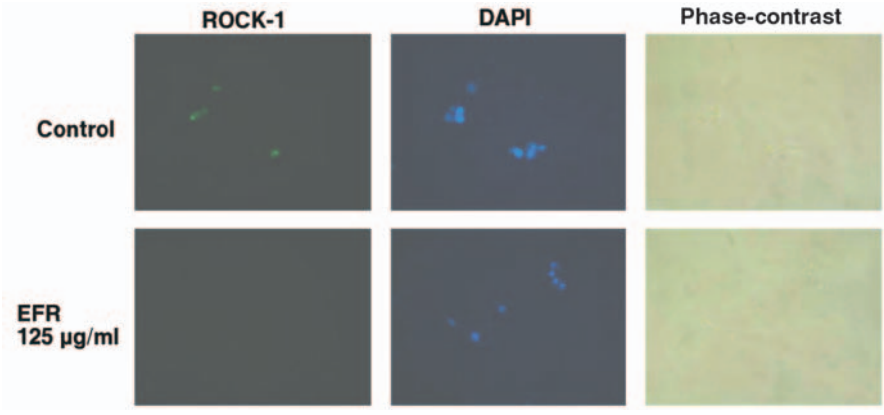

Figure 6. *Immunofluorescence showing ROCK-1 localization. Cells were treated with or without EFR (125 mg/ml) and ROCK-1 localization was represented as green fluorescence. DAPI staining indicated nuclear localization.*

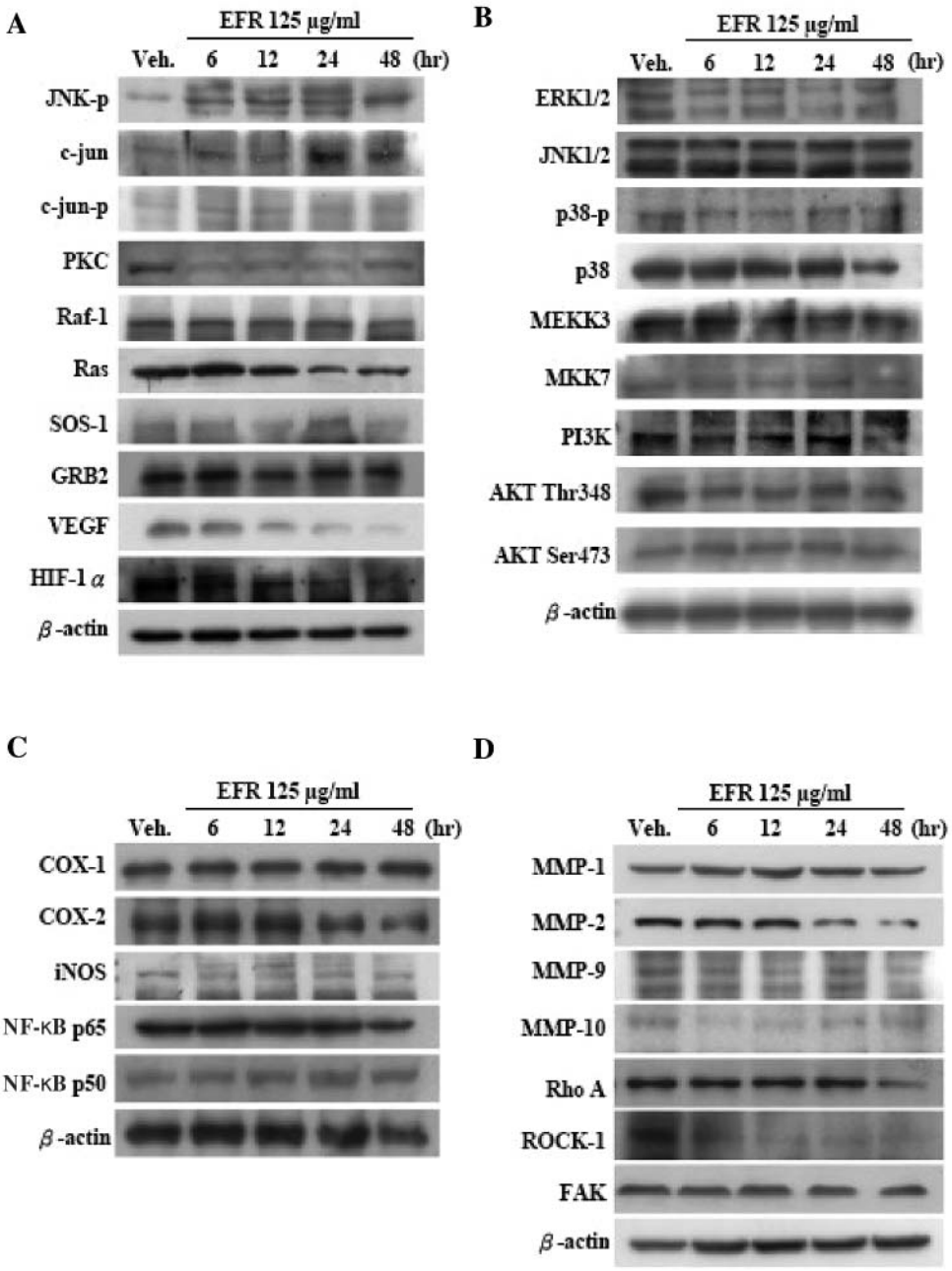

Figure 7. *Western blotting to examine the effect of EFR on the gene expression of Hep3B cells. The total proteins were collected from Hep3B cells treated with or without 125 μg/ml of EFR for 6, 12, 24, 48 h before the MMP protein expressions were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.*

coated filter in the presence of EFR. Quantification of the cells in the lower chamber showed that EFR significantly inhibited Hep3B cell migration (Figure 2B).

Effect of Hep3B cells on cell adhesion and migration in wound assays. The ability of cells to migrate into the wound area was assessed over 48 hours. There was evidence of active cell migration, as the width of the wound narrowed significantly over the 48 hour period in the control group. By contrast, the EFR-treated Hep3B cells did not significantly populate the wound area (Figure 4). The observations from the wound assays are consistent with the changes in morphology and adhesion of Hep3B cells, suggesting that the EFR could inhibit Hep3B cell migration into the wound.

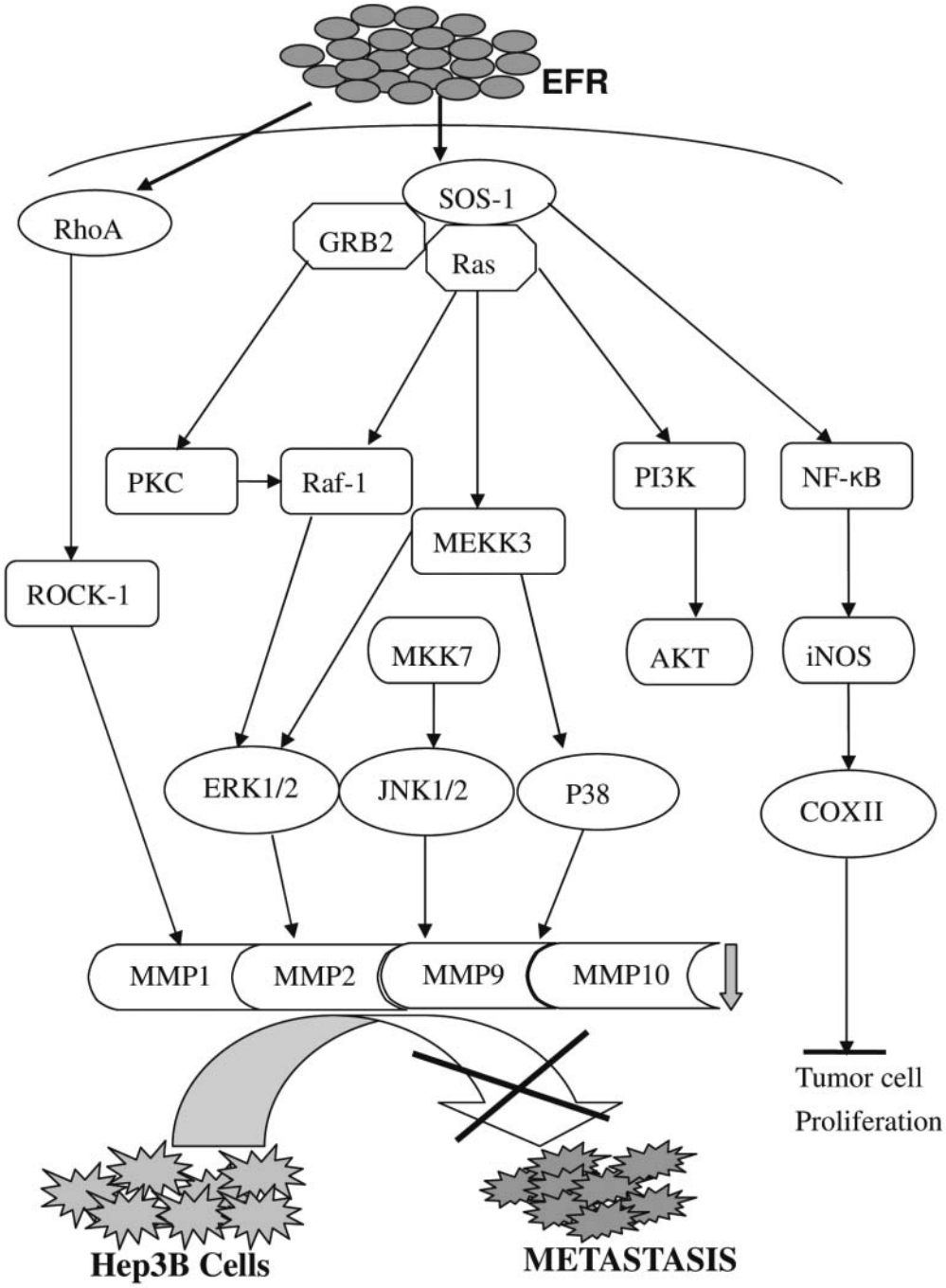

Figure 8. *The proposed scheme of mechanism of EFR action on Hep3B cells.*

Immunofluorescence of RhoA and ROCK-1. Prenylation of small GTPases leads to their attachment to the plasma membranes and exhibits their biological functions. As a result, we intended to determine whether the translocation of RhoA and ROCK-1 was affected by EFR. Immunostaining was applied to address this question. Immunostaining of RhoA and ROCK-1 is shown in Figures 5, 6. The localization of RhoA and ROCK-1 was not affected by the EFR treatment.

Western blotting for examining the effects of EFR on gene expression. The results of Western blot are shown in Figure 7. The decreases of the expressions of RhoA, ROCK-1,

FAK, MMP-1, 2, 9, 10, which may contribute to the inhibition of migration and invasion of the cells, were of particular interest (Figure 7).

Discussion

Metastasis is the most common factor for the poor prognosis of HCC and it remains a risk in some patients even if the tumor mass is surgically removed at an early stage. Many patients retain residual disease after surgery and this can eventually lead to metastasis. If metastasis can be successfully prevented, the prognosis is much improved. In our previous studies it was shown that EFR inhibited growth and induced apoptosis of HCC in a dose- and time-dependent manner. In the present study, we also confirmed that EFR inhibited the invasion and migration of Hep3B and the levels of proliferation-associated proteins were also examined. EFR inhibited the levels of NF-κB, iNOS and COX2. It has been reported that NF-κB, iNOS and COX2 are associated with proliferation of tumor cells (25, 26, 27) and inhibiting them can lead to inhibited proliferation of tumor cells. In the current studies, EFR suppressed metastasis-associated behaviors such as invasion and migration potently. It has been reported that cancer cell–matrix interaction is a critical step in the promotion of cell migration (20, 21) and proteolytic degradation of the extracellular matrix (ECM) is a critical event during tumor invasion and metastasis. It is well known that gelatinases such as MMP-2 and MMP-9 are directly involved in metastasis and that the suppression of gelatinase greatly contributes to the control of metastasis (22). Therefore, it is important to characterize the effect of EFR on the levels of MMPs in greater detail.

The Western blotting results indicated that EFR affected the Ras and p38 pathways leading to inhibition of the MMPs. This pathway has been reported to affect cell migration (29, 30) since MMPs mediate invasion and metastasis through degradation of the ECM and basement membrane, which enables tumor cells to invade surrounding tissues and enter the blood stream and thus travel to distant sites (23). MMP-2 and MMP-9 are considered to be particularly important targets for the development of anticancer drugs because they are associated with an aggressive, advanced, invasive or metastatic tumor phenotype (24). Our results (Figure 8) demonstrated that EFR inhibited expression of MMP-1, -2, -9 and -10, RhoA, ROCK-1 and FAK which co-incided with the inhibition of invasion and migration of the Hep3B cells after treatement with EFR. In addition, EFR inhibited expression of Raf-1, PKC and ERK are associated with invasion and migration (31, 32).

In conclusion, EFR inhibition of invasion and metastasis-associated proteases in a human hepatocellular carcinoma cell line (Hep3B) is reported. In addition, EFR inhibited degradation and cellular invasion of the ECM and basement membrane. This study provides additional information of the antimetastatic potential of EFR beyond its antitumor activity.

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