

# *Actinidia chinensis* Planch root extract (acRoots) inhibits hepatocellular carcinoma progression by inhibiting EP3 expression

Tingting Fang · Jiayun Hou · Mingyan He ·  
Lingyan Wang · Minghuan Zheng ·  
Xiangdong Wang · Jinglin Xia

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**Abstract** A wide range of studies has demonstrated the potent anticancer activity of Chinese herbs. Here, we evaluated the anticancer activity and molecular mechanisms of *Actinidia chinensis* root extract (acRoots) on hepatocellular carcinoma (HCC). HepG2 HCC cells were treated with various concentrations of acRoots for 72 h and examined by mRNA expression profiling, revealing alterations in cellular immunity, inflammation, proliferation, cell cycle, and metabolic signaling responses. Further analysis of the altered genes in cellular immunity and inflammation gene clusters identified prostaglandin E receptor 3 (EP3) as a key regulator of gene expression in response to acRoots. Further analysis revealed inhibition of cell growth, migration, and invasion in HCC in response to acRoots, along with increased apoptosis due to downregulation of EP3

expression. Treatment with acRoots and EP3 antagonist L-798106 led to decreases in VEGF, EGFR, MMP2, and MMP9 expression in HCC cells, along with significant effects on growth, migration, invasion, and apoptosis; the effects were reversed/blocked by the EP3 agonist sulprostone. Taken together, these data clearly demonstrated that acRoots inhibit HCC cell invasion and metastasis via inhibition of EP3 expression, resulting in decreased activation of VEGF, EGFR, MMP2, and MMP9.

**Keywords** *Actinidia chinensis* · Hepatocellular carcinoma · Prostaglandin E receptor 3 · Immunity

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Tingting Fang, Jiayun Hou, Mingyan He, and Lingyan Wang contributed to the article equally as the first authors.

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T. Fang · M. He · J. Xia (✉)  
Liver Cancer Institute, Zhongshan Hospital, Shanghai, China  
e-mail: xiajinglin@fudan.edu.cn

J. Hou · L. Wang · M. Zheng · X. Wang (✉)  
Clinical Science Institute, Zhongshan Hospital, Shanghai, China  
e-mail: xiangdong.wang@clintransmed.org

J. Xia  
Minhang Hospital, Shanghai Medical School of Fudan University,  
Shanghai 201100, China

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors and the third leading cause of cancer mortality worldwide (Siegel et al. 2012), due in part to the high rate of metastasis seen with this cancer (Tang et al. 2004). Although curative hepatectomy has been shown to significantly improve survival times in patients with HCC, the long-term prognosis remains poor as a result of tumor invasiveness, frequent intrahepatic spread, and extrahepatic metastasis. Among patients with unresectable HCC, conservative treatments have been proven to be largely ineffective. Despite treatment advances, new systemic therapies for advanced HCC continue to exhibit both low efficacy and high toxicity. Development of new anticancer drugs

and adjuvants, including herbal medicines, is therefore essential for treatment of patients with HCC.

Recent studies examining the use of *Actinidia chinensis* Planch root extract (acRoots), a traditional Chinese medicinal herb used to treat various forms of cancer, conferred strong inhibition of tumor growth (Song et al. 2014; Cheng et al. 2015). Among the major compounds found in this extract, triterpenes such as corosolic acid have shown remarkable inhibition of HCC growth in vitro (Zhu et al. 2013; Cheng et al. 2015; Ku et al. 2015), while other compounds, including 2 $\beta$ ,3 $\beta$ ,23-trihydroxy-urs-12-ene-28-olic acid and polysaccharide have also been shown to exhibit significant anticancer effects (Song et al. 2014; Cheng et al. 2015). However, despite its apparent efficacy, the mechanisms underlying acRoots-mediated inhibition of HCC growth and metastasis remain largely unknown, limiting its development as a natural anticancer agent.

Immune and inflammatory responses have been shown to play a crucial role in the progression of HCC (Berasain et al. 2009; Diakos et al. 2014), although the molecular mechanisms underlying this activity remain unclear. Here, we examined immune and inflammatory gene expression profiles of HCC cells which were treated by acRoots to identify the potential mechanism underlying acRoots activity. From these analyses, we identified prostaglandin E receptor 3 (EP3) as a key regulator of gene expression altered in response to acRoots, with significant effects on tumor growth. Further analyses identified alterations in EP3, VEGF, EGFR, MMP2, and MMP9 signaling in HCC, which is modified in response to acRoots.

## Materials and methods

### Drug preparation

*A. chinensis* Planch roots were finely chopped and suspended in 10 volumes of double-distilled water. The mixture was then heated to 100 °C for 1 h and filtered. The entire extraction procedure was repeated two times, and the resulting extracts were pooled and equilibrated to 1 g/mL.

### Cell culture and reagents

Six human cell lines were used in this study. The normal liver epithelial cell line L02 and human hepatocellular

carcinoma cell lines Hep3B, HepG2, Huh7, and SMMC-7721 were obtained from the Cell Bank of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China. Human HCC cell lines MHCC97H and HCCLM3 were obtained from the Liver Cancer Institute at Fudan University, Shanghai, China. Cells were cultured in DMEM or MEM with high glucose (Gibco BRL, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco BRL, USA). All cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>. The EP3 receptor antagonist L-798106 and EP3 receptor agonist sulprostone were purchased from Sigma-Aldrich.

### Gene expression profiling

The human HCC cell line HepG2 was stimulated with acRoots (0, 1, 5, and 10 mg/mL) for 72 h and analyzed by microarray, as described previously (Xu et al. 2014). Briefly, total RNA was extracted from HCC cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was then amplified and labeled using the one color Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Labeled cRNA was purified using the RNeasy mini kit (Qiagen, GmbH, Germany). Slides were then hybridized with 1.65  $\mu$ g Cy3-labeled cRNA using the gene expression hybridization kit (Agilent) in a hybridization oven (Agilent) for 17 h, after which slides were washed in staining dishes (Thermo Scientific, Waltham, MA, USA) with gene expression wash buffer (Agilent) and scanned on an Agilent microarray scanner (Agilent) using the default settings. Data were extracted using Feature Extraction software 10.7 (Agilent). Raw data were normalized by a quantile algorithm using Gene Spring software 11.0 (Agilent).

### Data analysis

To identify all inflammation- and immune response-related genes regulated by acRoots, we developed the following filtering strategy. First, all inflammation- and immune response-related genes must be annotated as part of the inflammation and immune response Gene Ontology processes (GO 0006954 and GO 0006955) or their children (<http://amigo1.geneontology.org/>)

(Table S1). Second, genes regulated by acRoots in HepG2 cells must exhibit a change of at least twofold at any drug concentration relative to the untreated controls (Table S2). Finally, all inflammatory and immune response genes must exhibit a change of at least twofold in any of the drug concentration groups relative to the untreated controls (Table S3). The VLOOKUP function was used to overlay inflammatory and immune response genes from Table S1 with those differentially regulated by acRoots in Table S2, resulting in the final gene list presented in Table S3.

### Quantitative real-time PCR

RNA was extracted from cell lines using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and reverse transcribed using the PrimeScript RT Master Mix (Takara, Japan). SYBR green fluorescence-based qRT-PCR was performed according to the manufacturer's instructions (Takara, Japan); all primers used in our study are listed in Table 1. Relative mRNA expression levels were calculated using the  $-\Delta\text{Ct}$  method and expressed as  $2^{(-\Delta\text{Ct})}$  values based on threshold cycle (Ct) values after normalization to the internal control (GAPDH).

### Cell proliferation

Cells were plated at a density of 2000/well in 96-well culture plates and incubated with or without drug for the times indicated. Following incubation, 10  $\mu\text{L}$  CCK-8

solution (Dojindo, Japan) was added, after which the cells were incubated for an additional 2 h, and the OD value at 450 nm was measured using the Infinite 200 (Tecan, Switzerland). All experiments were performed at least three times.

### Migration and invasion

Cell migration assays were performed in 24-well Transwell plates using an 8.0  $\mu\text{M}$  pore polycarbonate membrane insert (Corning, USA). A total of  $1 \times 10^5$  cells were suspended in 100  $\mu\text{L}$  DMEM with high glucose (Gibco BRL, USA) with or without the corresponding drugs at the indicated concentrations and added to the upper chamber. The lower chamber was filled with 600  $\mu\text{L}$  DMEM with high glucose (Gibco BRL, USA) supplemented with 10 % FBS (Gibco BRL, USA). After incubation for 48 h, the cells on the upper surface of the membrane were removed, and the migrated cells on the lower surface were fixed in 4 % paraformaldehyde, stained with 0.1 % crystal violet for 15 min at room temperature, and counted (10 fields) under a  $\times 100$  objective. The mean  $\pm$  standard deviation (SD) was then calculated. Invasion assays were performed in a similar manner except that the cells were seeded onto Matrigel-coated filters (BD Biosciences, USA).

### Flow cytometry-based apoptosis

An annexin V-FITC/PI double staining assay was used to detect apoptotic cells according to the manufacturer's protocol. Briefly, HCC cells were plated at a density of  $3 \times 10^5$ /well in a six-well plate and incubated with or without the indicated concentrations of the drugs. After 48 h, cells were harvested and suspended in binding buffer, incubated with annexin-V-FITC (Miltenyi Biotec Inc., USA) and PI for 15 min at room temperature and immediately analyzed using the BD LSRFortessa flow cytometer (BD Bioscience).

### Statistical analyses

Statistical analyses were performed using SPSS 19.0 for Windows. Comparisons between groups were performed using Student's *t* tests. A two-sided  $p < 0.05$

**Table 1.** Primer sequences for qRT-PCR

Gene name	sequence
EP3	F: 5'CAACCTTTTCTTCGCCTCTG3' R: 5'ACTGGGCACTGGACTGAGAT3'
VEGF	F: 5'TTCCAGGAGTACCCTGATGA3' R: 5'TGAGGTTTGATCCGCATAAT3'
EGFR	F: 5'TCCTCCAGTGCCTGAATAC3' R: 5'TAATTTGGTGGCTGCCTTTC3'
MMP2	F: 5'AAGTCTGAAGAGCGTGAAGTTTGA3' R: 5'TGAGGGTTGGTGGGATTGGAG3'
MMP9	F: 5'AGTCCACCCTTGTGCTCTTCCC3' R: 5'TCTGCCACCCGAGTGTAACCAT3'
GAPDH	F: 5'TGACTTCAACAGCGACACCCA3' R: 5'CACCCTGTTGCTGTAGCCAAA3'

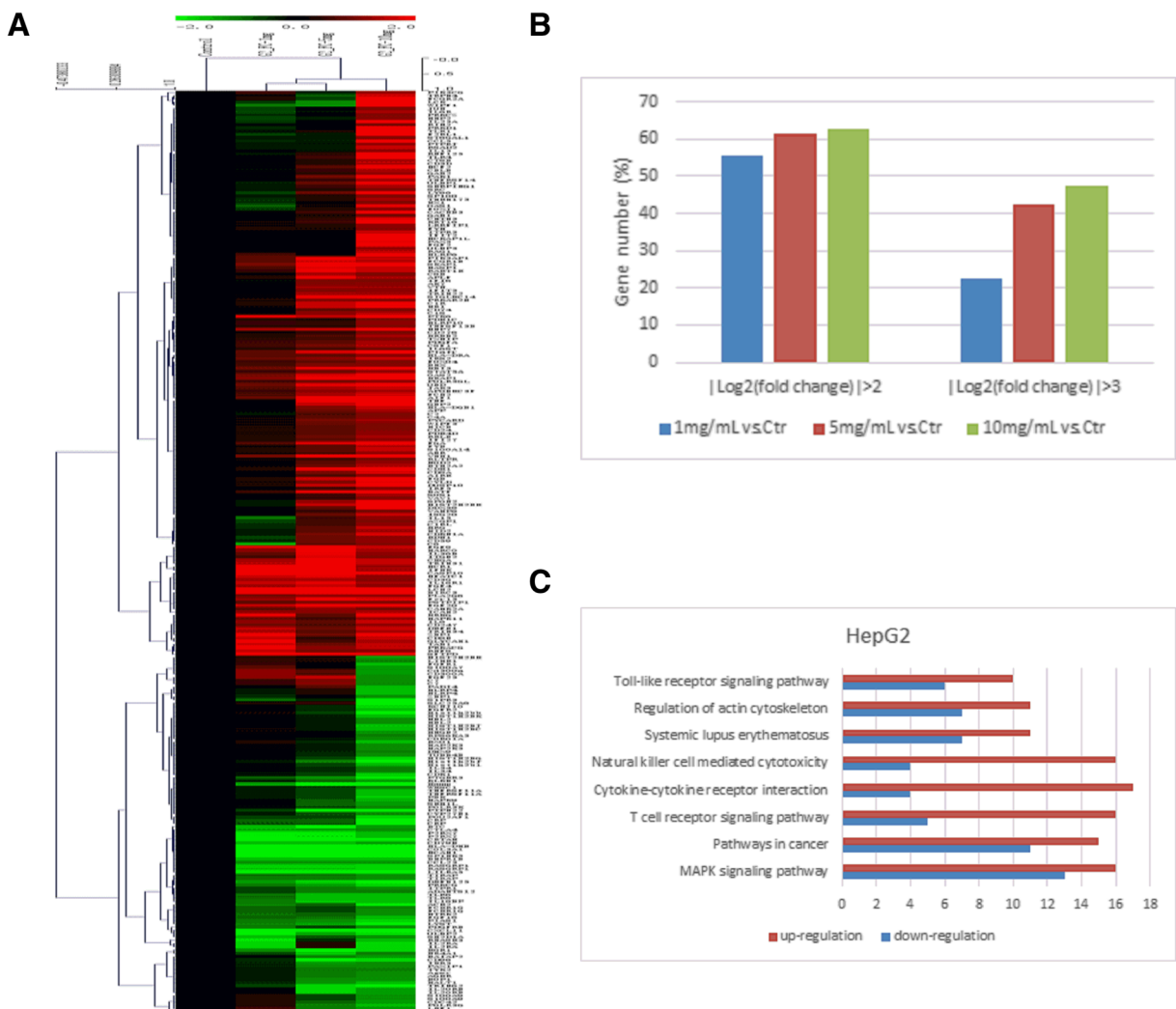
was considered statistically significant. All experiments were performed at least three times.

## Results

Effects of acRoots on inflammatory and immune response genes

Clear differences in gene expression profiles were seen between HepG2 cells treated with various concentrations of acRoots and untreated controls, including

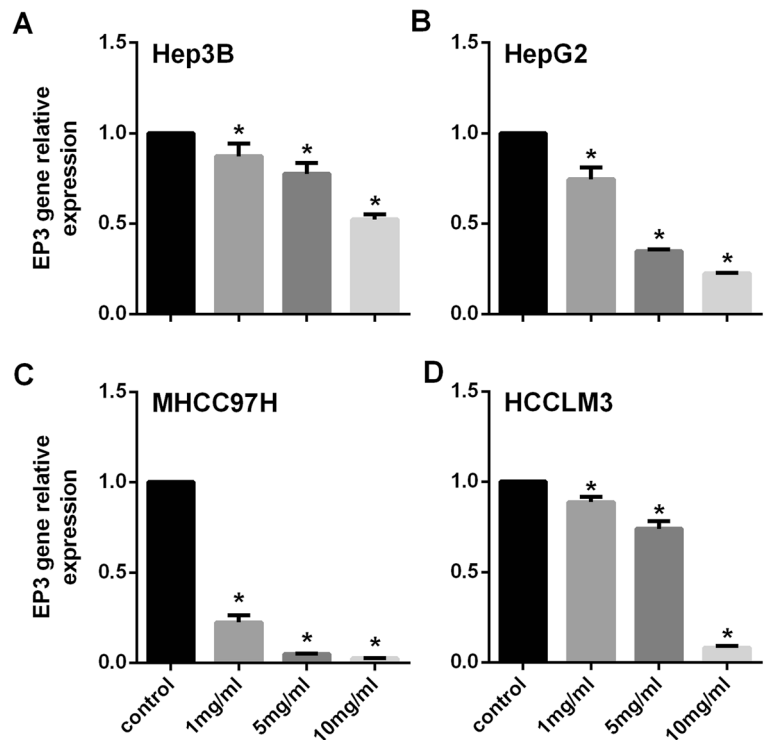
significant changes in cellular immunity, inflammation, proliferation, cell cycle, and metabolic pathways. Hierarchical clustering was used to identify inflammation- and immune response-related genes that were differentially expressed in response to acRoots (Fig. 1a), with higher concentrations resulting in more pronounced changes in gene expression (Fig. 1b). Inflammation- and immune-related genes differentially regulated by acRoots were analyzed using the DAVID database and compared with those found in the KEGG database. The eight most significantly affected pathways are shown in Fig. 1c.



**Fig. 1.** Relative expression of inflammation- and immune response-related genes in HepG2 cells following treatment with acRoots. **a** Hierarchical clustering was used to identify inflammation- and immune response-related genes that were differentially expressed between acRoots-treated HepG2 cells and

untreated controls. **b** Effects of drug concentration on mRNA fold changes. **c** Enrichment of KEGG pathways in response to acRoots was determined using DAVID software. Benjamini-Hochberg adjusted  $p$  values are shown for each indicated bar (\*\* $p < 0.01$ , \*\* $p < 0.05$ , \* $p < 0.1$ )

**Fig. 2.** EP3 expression decreased in response to increasing concentrations of acRoots in four HCC cell lines: **a** Hep3B, **b** HepG2, **c** MHCC97H, and **d** HCCLM3. Greater inhibition was seen in response to increasing drug concentrations ( $*p < 0.05$ )



acRoots inhibits EP3 expression in human HCC cell lines

A total of 366 inflammation- and immune response-related genes were differentially regulated at least twofold in HepG2 cells in response to acRoots. Analysis of these differentially regulated genes identified EP3 as a potential central regulator of these transcriptional responses.

To verify these results, four HCC cell lines, Hep3B, HepG2, MHCC97H, and HCCLM3, were stimulated with three concentrations (1, 5, and 10 mg/mL) of acRoots for 72 h. EP3 expression decreased in a linear manner in response to increasing concentrations of acRoots in all four HCC cell lines (Fig. 2). Based on these results, we selected the HepG2 and MHCC97H cell lines for follow-up experiments due to their greater susceptibility to acRoots. A final concentration of 10 mg/mL acRoots was chosen for all subsequent experiments.

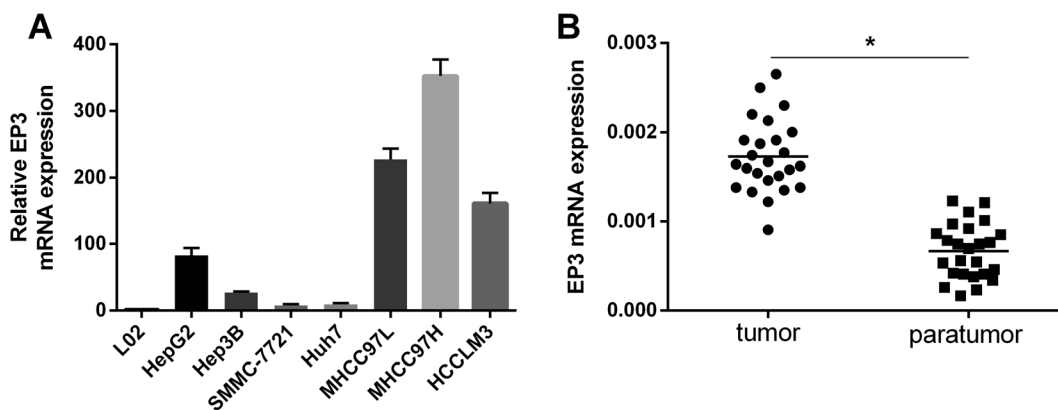
EP3 is expressed in human HCC cell lines and tissues

EP3 mRNA expression was assessed by qRT-PCR in human HCC cell lines with intermediate or high

metastatic potential (MHCC97L, MHCC97H, and HCCLM3), as well as those with low or no metastatic potential (Hep3B, HepG2, Huh7, and SMMC-7721); normal liver epithelial cell line L02 was used as a control. EP3 was shown to be constitutively expressed in human HCC cell lines (Fig. 3a), especially high expressed in metastatic potentials HCC cell lines: MHCC97L, MHCC97H, and HCCLM3 cell lines. Subsequent analysis of EP3 expression in primary HCC samples from 25 patients who had undergone curative hepatectomy also showed higher EP3 expression in tumor cells compared with peri-tumor tissues (Fig. 3b).

acRoots inhibits proliferation of HCC cells by decreasing EP3 expression

EP3 is a member of the PGE2 family, characterized by the presence of seven transmembrane domains. A growing body of evidence suggests that EP3 promotes tumor cell proliferation in many tumors (Amano et al. 2003; Yamaki et al. 2004; Ogawa et al. 2009). To verify the role of EP3 in tumor progression, HepG2 and MHCC97H cells were treated with various

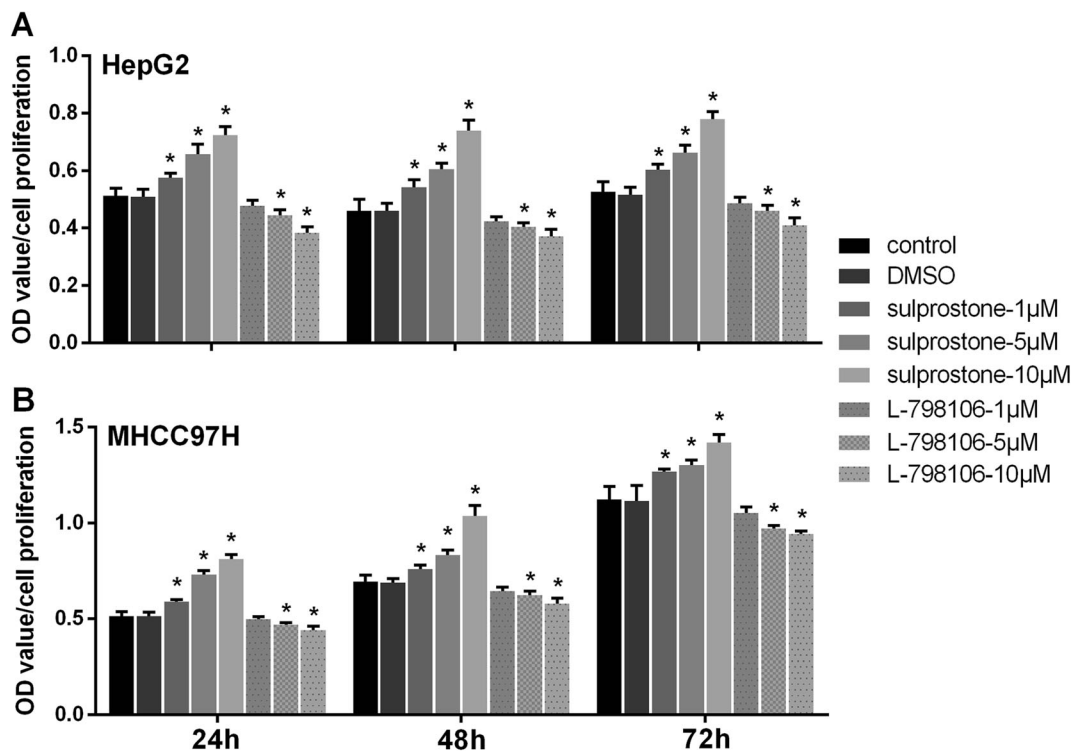


**Fig. 3.** **a** EP3 is constitutively expressed in human HCC cell lines. **b** EP3 expression was higher in tumor cells relative to peri-tumor tissue ( $*p < 0.05$ )

concentrations (1, 5, and 10  $\mu\text{M}$ ) of the EP3 receptor agonist sulprostone or selective antagonist L-798106 and examined for cell proliferation at three time points (24, 48, and 72 h) by CCK8 assay. Strong increases in cell proliferation were seen in response to sulprostone relative to untreated or DMSO vehicle controls, while receptor agonist L-798106 had the opposite effect (Fig. 4). Subsequent experiments

were performed using 10  $\mu\text{M}$  for each drug as a means of optimizing the effects of these compounds on cell proliferation.

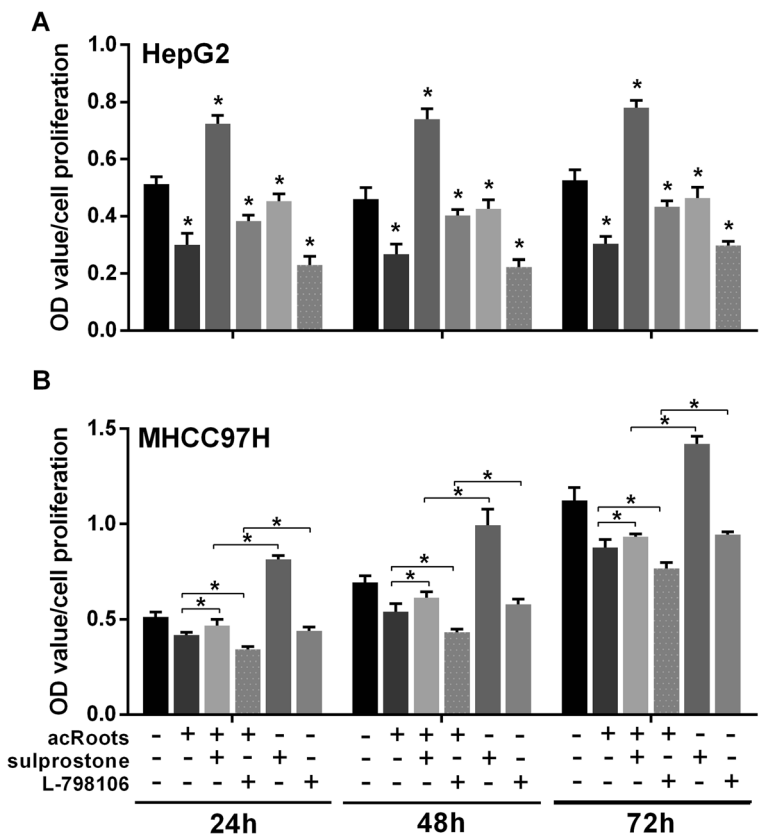
To assess the role of EP3 in the proliferation of HCC cells by acRoots, HCC cells were treated with 10 mg/mL acRoots with or without 10  $\mu\text{M}$  sulprostone or 10  $\mu\text{M}$  L-798106. Treatment with acRoots alone significantly inhibited HCC cell proliferation relative to that of the



**Fig. 4.** Proliferation of **a** HepG2 and **b** MHCC97H cells increased in response to sulprostone and decreased following treatment with L-798106 compared with untreated or vehicle (DMSO)-treated controls ( $*p < 0.05$ )



**Fig. 5.** Treatment with acRoots enhanced the inhibitory effects of L-798106 on **a** HepG2 and **b** MHCC97H cell proliferation and attenuated the proliferative effects of sulprostone (\**p* < 0.05)



controls. Addition of 10 μM sulprostone significantly attenuated the inhibitory effect of acRoots on HCC cell proliferation relative to cells treated with acRoots alone. Alternatively, addition of 10 μM L-798106 strongly enhanced acRoots-mediated suppression of HCC cell proliferation (Fig. 5). Taken together, these results suggest that acRoots inhibits the proliferation of HCC cells via downregulation of EP3 expression.

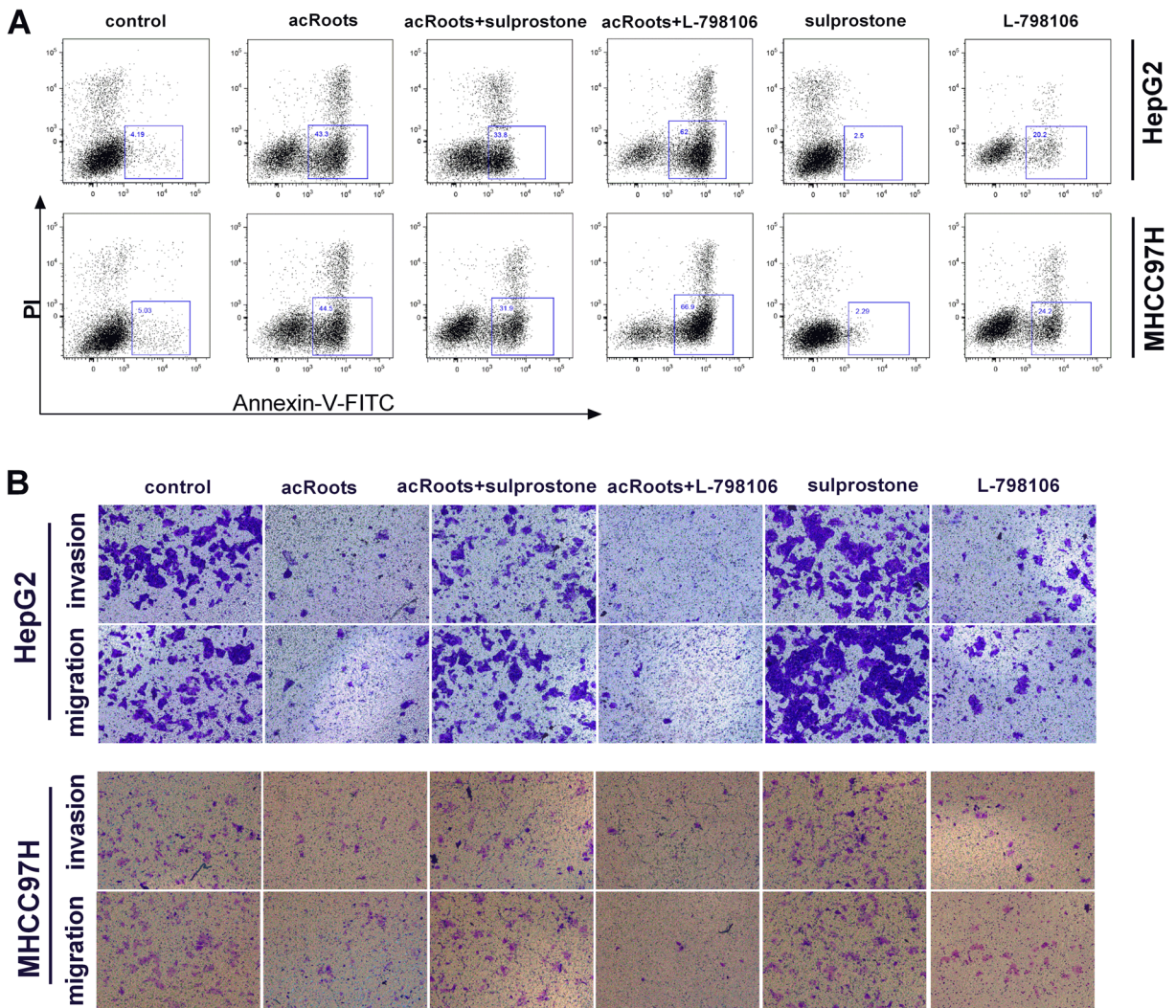
acRoots promotes apoptosis of HCC cells by decreasing EP3 expression

Next, we sought to assess the role of acRoots-mediated suppression of EP3 on apoptosis in HCC cells. HCC cells were treated with 10 mg/mL acRoots with or without 10 μM sulprostone or 10 μM L-798106. acRoots alone significantly increased HCC cell apoptosis compared with the negative controls. Addition of sulprostone strongly attenuated this effect, while the combination of

L-798106 significantly increased the rate of HCC cell apoptosis, relative to acRoots alone (Fig. 6a). These results show that acRoots promotes apoptosis of HCC cells via downregulation of EP3 expression.

acRoots inhibits invasion and migration of HCC cells by decreasing EP3 expression

The role of EP3 on HCC cell invasion and migration was assessed following treatment with 10 mg/mL acRoots with or without 10 μM sulprostone or 10 μM L-798106. acRoots alone significantly inhibited HCC cell migration and invasion relative to the controls. Addition of 10 μM sulprostone strongly attenuated this effect, while L-798106 further enhanced the inhibitory effects seen with acRoots alone (Fig. 6b). These results further confirm the link between acRoots and EP3, demonstrating a direct link between HCC cell invasion and migration and EP3 expression.



**Fig. 6.** **a** acRoots increased the rate of apoptosis and **b** inhibited the invasion and migration of HCC cells via the downregulation of EP3 expression ( $*p < 0.05$ )

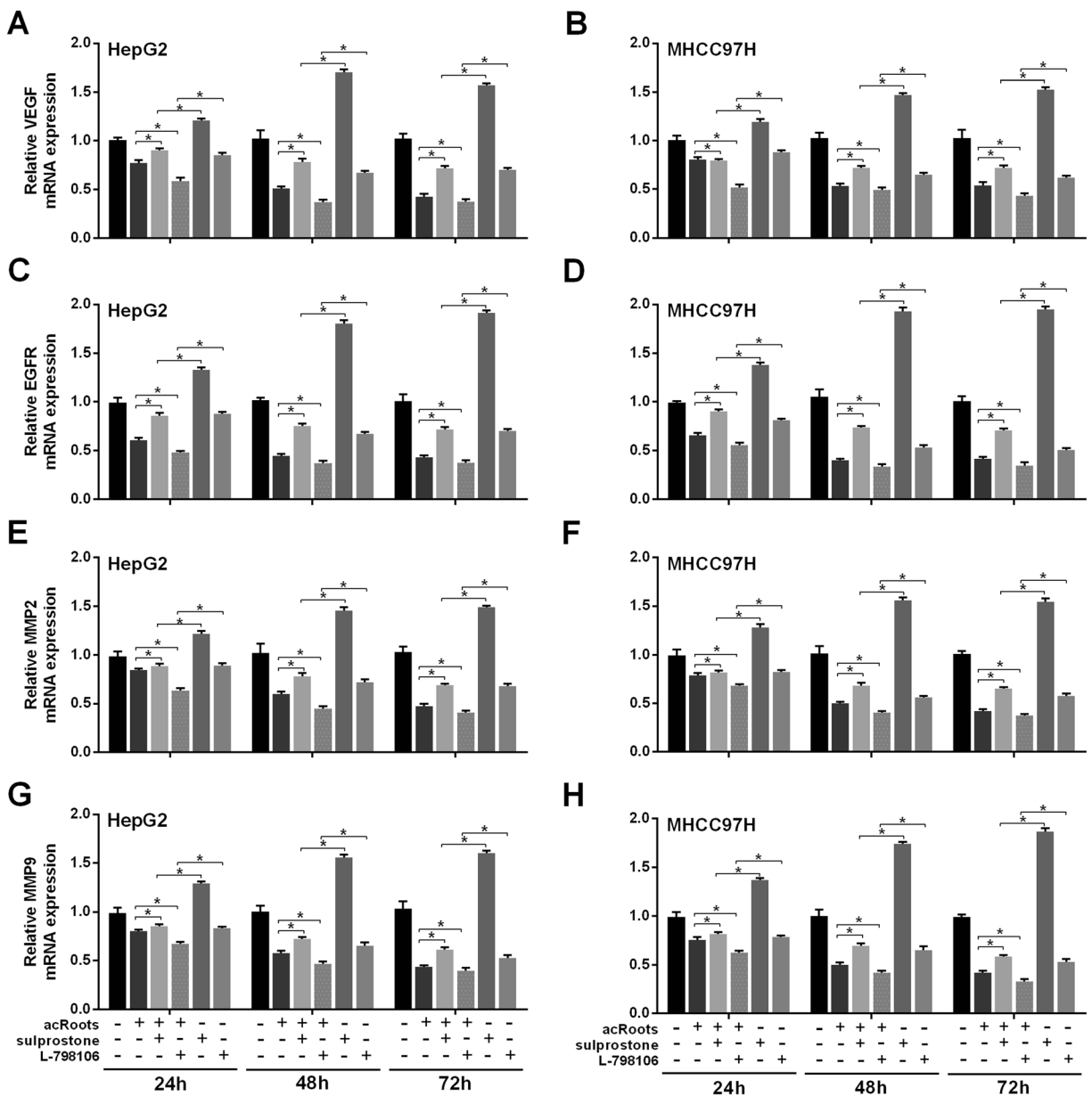
Molecules regulated by EP3 downregulation induced by acRoots in HCC cells

Activation of EP3 has been shown to induce expression of VEGF, EGFR, MMP2, and MMP9, resulting in increased tumor growth, angiogenesis invasion, and metastasis, indicating a central role for EP3 in tumor progression (Mendelsohn and Baselga 2006; Kerbel 2008; Kessenbrock et al. 2010). We therefore investigated whether increased expression of these molecules was also seen in response to EP3 activation in HCC cells. HCC cells were treated with increasing concentrations of sulprostone or L-798106 (1, 5, and 10  $\mu$ M)

for 24, 48, or 72 h and analyzed by qRT-PCR. Expressions of VEGF, EGFR, MMP2, and MMP9 were all increased in response to sulprostone in a dose-dependent manner; treatment with L-798106 had the opposite effect (Fig. 7). Based on these results, we choose 10  $\mu$ M and 48 h as the conditions for all follow-up experiments.

To further assess the role of EP3 activation on VEGF, EGFR, MMP2, and MMP9, HCC cells were treated with 10 mg/mL acRoots with or without 10  $\mu$ M sulprostone or 10  $\mu$ M L-798106 for 48 h. Treatment with acRoots alone decreased the expression of VEGF, EGFR, MMP2, and MMP9 relative to the controls. Addition of



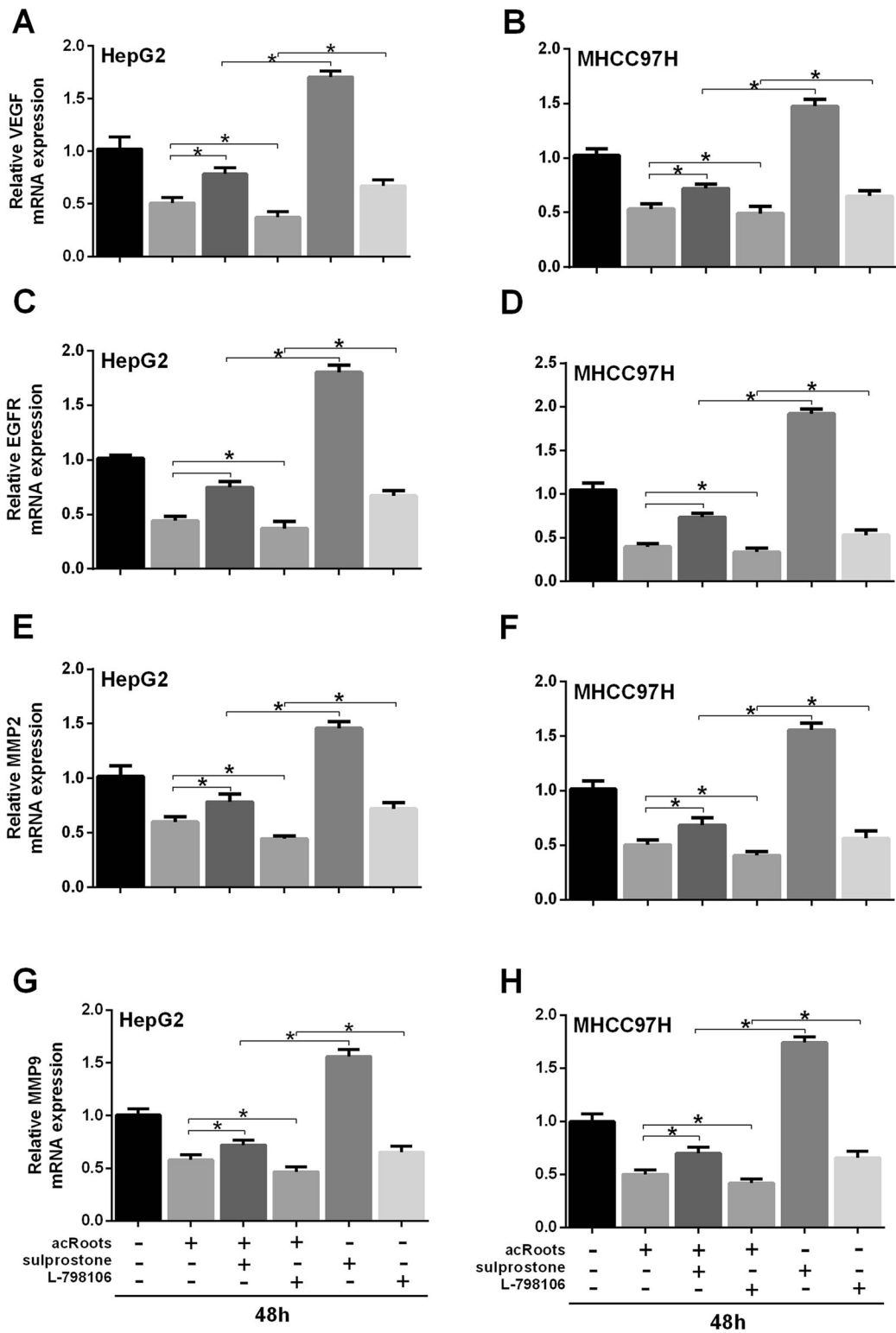


**Fig. 7.** Increased expression of **a** VEGF, **b** EGFR, **c** MMP2, and **d** MMP9 in HCC cells in response to sulprostone in a dose-dependent manner, relative to untreated or vehicle (DMSO)-treated controls; cells treated with L-798106 exhibited the opposite effect ( $*p < 0.05$ )

sulprostone attenuated this effect, while the combination of acRoots and L-798106 further suppressed the expression of VEGF, EGFR, MMP2, and MMP9 compared with acRoots alone (Figs. 8 and 9). These results indicated that acRoots inhibited the expression of VEGF, EGFR, MMP2, and MMP9 via downregulation of EP3 expression.

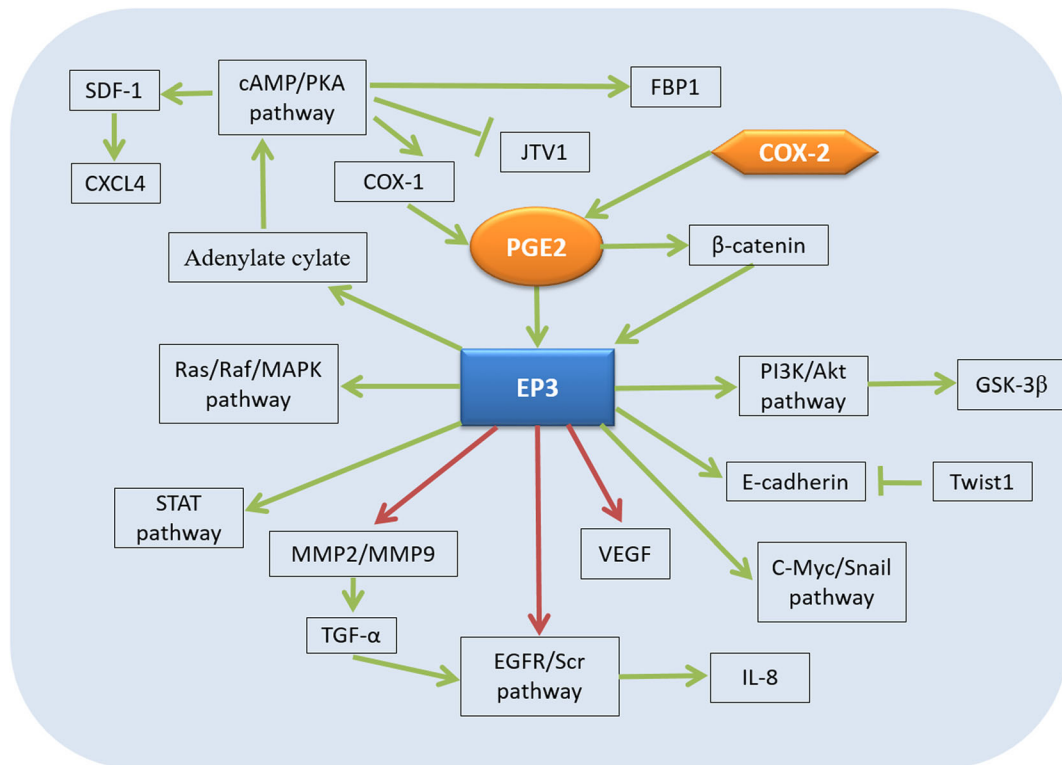
### Discussion

In the present study, we demonstrated that acRoots significantly affected tumor progress via downregulation of EP3 expression in HCC cells. To identify this potential mechanism of action, we first analyzed the inflammation- and immune response-



**Fig. 8.** Treatment with 10-mg/mL acRoots significantly inhibited expression of **a** VEGF, **b** EGFR, **c** MMP2, and **d** MMP9 compared with untreated controls. Addition of 10  $\mu$ M sulprostone

attenuated these effects, while addition of 10  $\mu$ M L-798106 strongly enhanced this inhibitory effect relative to acRoots alone ( $*p < 0.05$ )



**Fig. 9.** Roles of EP3 signaling pathway in cancer cells

related gene expression profiles associated with acRoots treatment in HepG2 cells across a range of concentrations, identifying EP3 as a key regulator of gene expression. Expression of EP3 itself was significantly attenuated in response to acRoots in a dose-dependent manner. To confirm this initial observation, we showed that EP3 was constitutively expressed in human HCC cell lines, especially highly expressed in metastatic potential HCC cell lines, MHCC97L, MHCC97H, and HCCLM3 cell lines, and with higher expression seen in tumors compared with the surrounding peri-tumor tissue. These results showed that EP3 might play an important role in the metastasis and progress of HCC. Finally, we assessed expression of EP3 in response to acRoots in the presence of EP3-specific agonists and antagonists, confirming that acRoots inhibits HCC cell proliferation, invasion, and migration and promotes apoptosis via down-regulation of EP3 expression.

EP3 is a member of the PGE2 family, characterized by the presence of seven transmembrane domains (Sugimoto and Narumiya 2007). A growing body of

evidence suggests that EP3 promotes tumor cell proliferation in many tumors (Kashiwagi et al. 2013; Ma et al. 2013; Hiyama et al. 2015; Jiang et al. 2015). EP3 is widely expressed throughout the body, with its transcripts detected in virtually all tissues in both rodents and humans (Sugimoto and Narumiya 2007). Overexpression of this gene is well-documented in cancer, including breast, lung, colorectal, and liver (Kang et al. 2011; Kim et al. 2011; Ma et al. 2013; Guillemllobat et al. 2016). From a pathogenesis standpoint, the growth- and metastasis-promoting activities of EP3 may stem from its ability to promote angiogenesis, in addition to cell proliferation, migration, and invasion (Miyata et al. 2013; Cheng et al. 2015; Du et al. 2015). These effects are also seen in response to activation of VEGF, EGFR, MMP2, and MMP9, particularly in tumor cells (Mendelsohn and Baselga 2006; Kessenbrock et al. 2010; Brown and Murray 2015; Claesson-Welsh 2016). VEGF is a key regulator of angiogenesis and cellular growth (Claesson-Welsh 2016), while overexpression of EGFR, a phenomenon seen in human HCCs, is closely linked to tumorigenesis (Komposch and Sibilja 2016). MMP-2 and MMP-9 are

particularly important because they play pivotal roles in the degradation of the extracellular matrix necessary for tumor invasion (Claesson-Welsh 2016). As activation of EP3 has been shown to affect expression of VEGF, EGFR, MMP2, and MMP9, EP3 expression may serve as an important regulator of tumor progression (Kang et al. 2011; Kim et al. 2011; Yokoyama et al. 2011).

Recent studies have demonstrated various antitumor properties in acRoots (Song et al. 2014; Cheng et al. 2015). Some components of acRoots, including corosolic acid, 2 $\beta$ , 3 $\beta$ , 23-trihydroxy-urs-12-ene-28-olic acid, and polysaccharide, have proven effective in treating a wide range of malignancies (Song et al. 2014; Cheng et al. 2015; Ku et al. 2015). In the present study, acRoots inhibited HCC cell proliferation, invasion, and migration, along with promoting apoptosis of HCC cells via the downregulation of EP3 expression. These effects appear to be mediated, in part, through the direct regulation of VEGF, EGFR, MMP2, and MMP9 expression by EP3, highlighting the significant role EP3 plays in immune and inflammatory responses.

Taken together, the data presented here show that acRoots significantly reduces EP3 expression, resulting in downstream inhibition of VEGF, EGFR, MMP2, and MMP9 expression. These changes in gene expression resulted in significant decreases in HCC cell proliferation, invasion, and migration, along with increases in HCC cell apoptosis. acRoots may therefore represent a promising new anticancer drug or adjuvant therapy for the treatment of HCC.

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