

# Casticin induces growth suppression and cell cycle arrest through activation of FOXO3a in hepatocellular carcinoma

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**Abstract.** Casticin, a polymethoxyflavone, has been reported to exert anticancer activities. The objectives of this study were to examine the molecular mechanisms by which casticin induces the growth inhibition and cell cycle arrest in human hepatocellular carcinoma (HCC) cells. The HCC cell lines Hep G2 and PLC/PRF/5 were cultured *in vitro*. The growth inhibitory effects of casticin were evaluated using clonogenic assays. The distribution of phases in the cell cycle was analyzed using flow cytometry (FCM) analysis with propidium iodide (PI) staining. Multiple molecular techniques, such as western blotting and gene transfection, were used to explore the molecular mechanisms of action. Our data demonstrated that casticin significantly inhibited cell viability and colony formation in HCC cells. Furthermore, it induced cell cycle arrest in the G2/M phase. Casticin inhibited phosphorylation of the FOXO3a protein and decreased the expression of FoxM1 and its downstream genes, such as cyclin-dependent kinase (CDK1), CDC25B and cyclin B and increased the expression of p27<sup>KIP1</sup>. Silencing of FOXO3a expression by small interfering RNA (siRNA) transfection clearly attenuated the inhibitory effects of casticin on FOXM1 expression and cell growth. Our findings provided clear evidence that casticin induces growth suppression and cell cycle arrest through inhibition of FOXO3a phosphorylation causing inactivation of FOXM1 in HCC cells.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related mortality, resulting in approximately 500,000 deaths per annum. Most HCC cases occur in either Eastern Asia (particularly in China) or sub-Saharan Africa (1). Currently, the prognosis for HCC is poor, as no effective therapy has yet

been developed (2). Therefore, developing effective therapeutic agents to treat HCC are of paramount importance.

Casticin is one of the main components of the fruits of *Vitex rotundifolia* L. Casticin has been widely used in Chinese traditional medicine as an anti-inflammatory drug for thousands of years (3). In recent years, increasing experimental evidence has been provided that casticin exhibits anti-carcinogenic activity in breast (4), cervical (5-7), lung (8), colon cancer (9) and HCC (10). It has been proposed that cell cycle arrest and apoptosis induced by casticin are the possible mechanisms for its anticancer effects. However, the precise underlying mechanisms have not been fully elucidated.

Forkhead box class O (FOXO) subfamily of forkhead transcription factors include FOXO1a/FKHR, FOXO3a/FKHRL1 and FOXO4/AFX (11,12). FOXO3a possesses a large number of functions, including cellular proliferation, transformation, differentiation and longevity. Recent studies suggest that the phosphorylation of FOXO3a at threonine-32 plays an important role in deciding the function of FOXO3a. The phosphorylation results in impairment of DNA binding ability and results in an increased binding affinity for the 14-3-3 protein (13). Newly formed 14-3-3-FOXO complexes are then exported from the nucleus (14), thereby inhibiting FOXO-dependent transcription. Dephosphorylation of active FOXO3a induces cell cycle arrest and apoptosis (15). It has been reported that FOXO3a may be a potentially important prognostic factor in HCC (16). Fei *et al* (17) demonstrated that arsenic trioxide induced the growth arrest of HCC cells involving FOXO3a expression and phosphorylation. Forkhead box M1 (FOXM1) belongs to the forkhead box transcription factor family and is a downstream target of FOXO3a (18). FoxM1 is a proliferation-associated transcription factor that is frequently upregulated in different types of cancers including HCC (19). Wang *et al* (20) demonstrated that FOXM1 was a novel target of a natural agent in pancreatic cancer. However, the intracellular mechanisms by which casticin inhibits growth in HCC cells through regulation of the FOXO3a/FOXM1 pathway have never been investigated.

In the present study, we demonstrated that casticin induced FOXO3a dephosphorylation and FOXM1 inactivation, leading to growth inhibition and cell cycle arrest in HCC cells. These results suggest that forkhead transcription factor FOXM1 is a downstream cellular target and a potential novel marker for casticin action and that casticin activates FOXO3a to suppress FOXM1 expression in HCC cells.

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## Materials and methods

**Chemicals.** Casticin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). It has a molecular weight of 374.3 ku, appears as yellow crystals and has a purity of 98.0%. Casticin was prepared in dimethyl sulfoxide (DMSO) as a 10 mmol/l stock solution and diluted in medium to the indicated concentration before use. The following items were purchased from Hunan Clonetimes Biotech Co., Ltd. (Changsha, China): RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), fetal bovine serum (Invitrogen Life Technologies), propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA), antibodies against FOXO3a, phospho-FOXO3a-Thr32 (Millipore), FoxM1, cyclin dependent kinase (CDK1), cyclin B, p27<sup>KIP1</sup>, cdc25B and  $\beta$ -actin (Santa Cruz Biotechnology, Inc.). Lipofectamine 2000 was purchased from Invitrogen Life Technologies. Protease inhibitor cocktail and all other chemicals were obtained from Sigma.

**Cells and cell culture.** Hep G2 (p53 wild-type) and PLC/PRF/5 (p53 mutant) cells were obtained from American Type Culture Collection (Rockville, MD, USA). They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Invitrogen Life Technologies) in an incubator containing 50 ml/l CO<sub>2</sub> at 37°C.

**Clonogenic assay.** Cells were plated in 24-well plates at a density of 300 cells/well for 24 h, prior to the addition of various concentrations of casticin (2.5, 5.0 and 10.0  $\mu$ mol/l). After 24 h of treatment, the drug-containing medium was removed and replaced with complete growth medium. Medium was changed every three days for 10 days until visible colonies formed. Colonies were simultaneously fixed and stained with Wright-Giemsa solution in methanol and manually counted. Individually stained colonies in each well were counted. The colony formation fraction was calculated as follows: Colony number/(number of cells seeded  $\times$  plating efficiency), where plating efficiency was equivalent to the colony number divided by the number of cells seeded in the drug-free medium.

**Cell cycle analysis.** Cell cycle analysis was performed using PI staining as described previously (21). Briefly, cells were washed in PBS and fixed in 90% ethanol. Fixed cells were then washed twice in PBS and stained in 50  $\mu$ M PI containing 5  $\mu$ g/ml DNase-free RNase for 1 h. They were analyzed by flow cytometry (FCM) using a FACScan (Coulter Epics XL-MSL; Beckman Coulter, Fullerton, CA, USA) and winMDI software.

**RNA interference.** A control non-specific small interfering RNA (siRNA) (UUCUCCGAACGUGUCACGdTdT) was purchased from Qiagen. FOXO3a siRNA (ACUCCGGGUCAGCUCCAC) was synthesized by Shanghai GenePharma Co. (Shanghai, China). Transfection of siRNA was carried out with Lipofectamine 2000 (Invitrogen Life Technologies) according to the procedure recommended by the manufacturer. Twenty-four hours after transfection, the cells were treated with DMSO (control) or casticin at the indicated concentrations for 24 h. The cells were then collected and processed for western blotting and clonogenic assay.

**Western blot analysis.** Desired cells (1 $\times$ 10<sup>6</sup>) were seeded in 100-mm culture dishes, allowed to attach by overnight incubation and treated with DMSO (control) or 2.5, 5.0 and 10.0  $\mu$ mol/l casticin for specified time periods. The cell lysates were prepared as described by us previously (22). The cell lysates were cleared by centrifugation at 14,000 rpm for 30 min. The lysate proteins were resolved by 10 or 12.5% SDS-PAGE and were transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with Tris-buffered saline containing 0.05% Tween-20 and 5% (w/v) non-fat dry milk. The membrane was then treated with the desired primary antibody for 1 h at room temperature or overnight at 4°C. Following treatment with the appropriate secondary antibody, the immunoreactive bands were visualized using the enhanced chemiluminescence method. The blots were stripped and re-probed with anti-actin antibody to normalize for differences in protein loading. Change in the level of desired protein was determined by densitometric scanning of the immunoreactive band and was corrected for  $\beta$ -actin loading control. Immunoblotting for each protein was performed at least twice using independently prepared lysates to ensure reproducibility of the results.

**Statistical analysis.** The database was set up with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA) to be analyzed. Data are represented as means  $\pm$  SD. The means of multiple groups were compared with one-way ANOVA, after the equal assessment of variance. The comparisons among the means were performed using the LSD method. Statistical comparison was also performed with the two-tailed t-test when appropriate. A P<0.05 was considered to indicate a statistically significant result.

## Results

**Casticin induces growth inhibition and cell cycle arrest in HCC cells.** Since the previous study demonstrated that casticin inhibited the viability of HCC cells using an MTT assay (10), we first examined the effect of casticin on the cell growth by a clonogenic assay. Fig. 1A and B shows that casticin treatment resulted in a significant inhibition of colony formation of Hep G2 and PLC/PRF/5 cells when compared to the control.

We next sought to evaluate the effects of casticin treatment on the phase distribution of the cell cycle using FCM after PI staining. As shown in Fig. 1C and D, casticin treatment caused a significant accumulation of cells in the G2/M phase and a marked decrease of cells in the G1/G0 phase when compared to control cells. These results revealed that casticin induced the growth inhibition and cell cycle arrest in the G2/M phase in HCC cells.

**Casticin downregulates the expression of FOXM1 in HCC cells.** It has been previously reported that the loss of FOXM1 expression induces the growth inhibition and cell cycle arrest in HCC cells (23). We investigated whether casticin regulates FOXM1 expression during casticin-induced growth inhibition in HCC cells. The results revealed that FOXM1 was overexpressed in the Hep G2 (Fig. 2A) and PLC/PRF/5 cell lines (Fig. 2B). Exposure of cells to 2.5, 5.0, 10.0  $\mu$ mol/l casticin for 24 h significantly reduced the expression of FoxM1 at the mRNA and protein levels (Fig. 2).

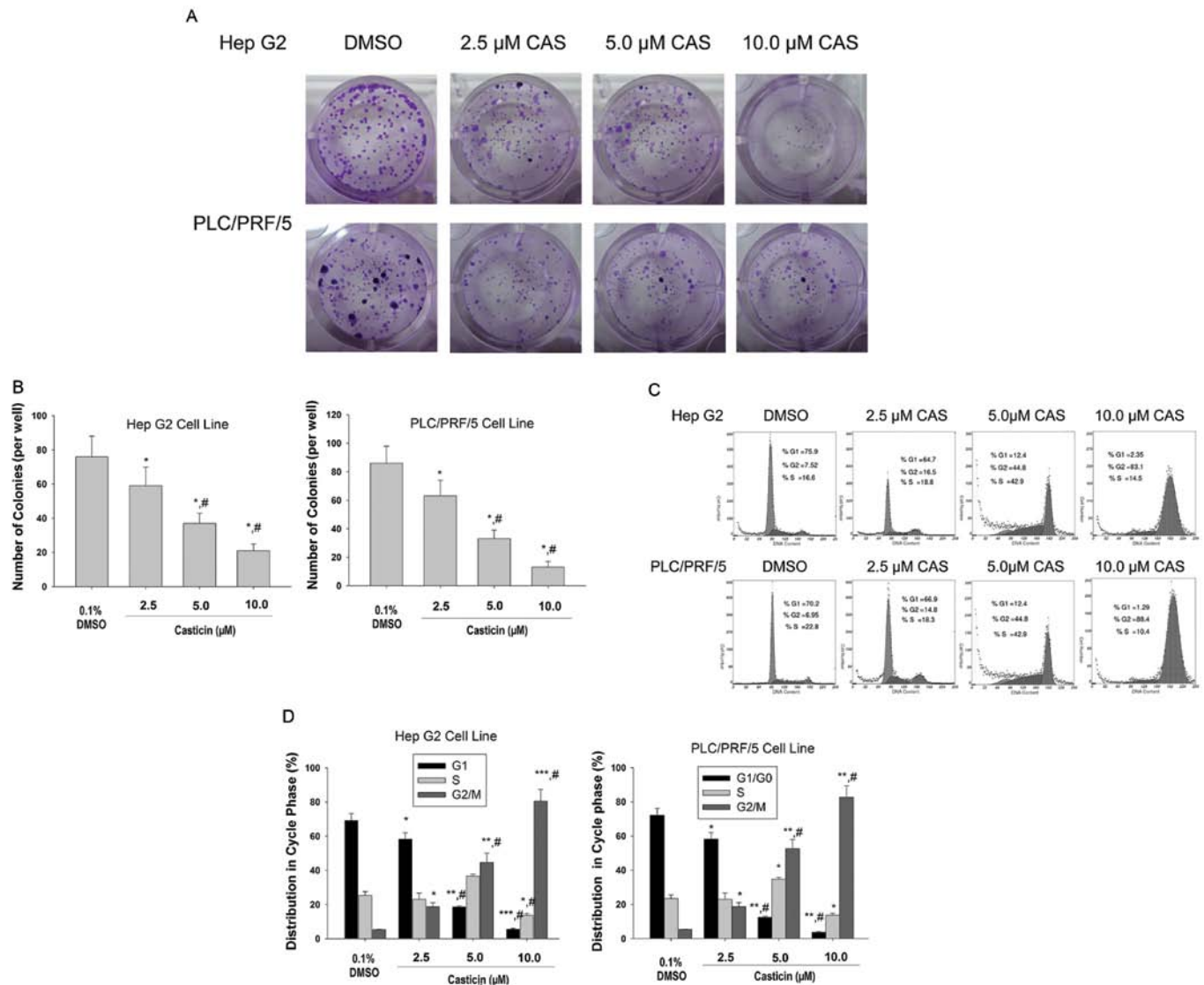


Figure 1. Casticin induces growth inhibition and cell cycle arrest in the G2/M phase in HCC cells. Cells were treated with the indicated concentrations of casticin for 24 h. (A and B) Casticin (CAS) decreased the colony number in the Hep G2 and PLC/PRF/5 cell lines. (C and D) Casticin increased the cell population in the G2/M phase in the Hep G2 and PLC/PRF/5 cell lines. Data and error bars are presented as means  $\pm$  SD. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 when comparing these treatments with DMSO. \* $P$ <0.05 when comparing these treatments with 2.5  $\mu$ M casticin.

*Casticin modulates the expression of downstream targets of FOXM1 in HCC cells.* To further confirm the effect of casticin on FOXM1 functional regulation, we assessed the expression of downstream target genes of FOXM1 in HCC cells after casticin treatment. It is well known that FOXM1 has several downstream target genes, such as CDK1, CDC25B, cyclin B1 and p27<sup>KIP1</sup>, for the regulation of growth and cell cycle arrest in cells. Western blot analysis results showed that casticin inhibited the expression of CDK1, CDC25B, cyclin B1 and increased p27<sup>KIP1</sup> in Hep G2 (Fig. 3A) and PLC/PRF/5 (Fig. 3B) cells at the protein level. These results provide molecular evidence suggesting that the casticin-induced growth inhibition and cell cycle arrest in HCC cells may be mediated via inactivation of the FoxM1 function.

*Casticin decreases the phosphorylation level of FOXO3a protein in HCC cells.* Since FOXO3a is the upstream regu-

lator of the FOXM1 transcription factor (24), we sought to examine the expression of phosphorylated FOXO3a protein in order to explain the mechanism for the effect of casticin on FOXM1 inhibition. Western blotting showed that treatment with casticin led to a decrease in the phosphorylation level of FOXO3a and a corresponding reduction in the expression of FOXM1 in HCC cells (Fig. 4A and B). These results indicate that the inhibition of FOXM1 expression by casticin may be associated with the inhibition of FOXO3a phosphorylation.

*Silencing of the FOXO3a gene attenuates casticin-mediated growth inhibition in HCC cells.* In order to confirm the relevance of the FOXO3a factor in the cellular growth inhibition response to casticin, we decided to perform gene silencing experiments. To this end, cells were generated in which FOXO3a protein expression was abrogated using siRNA

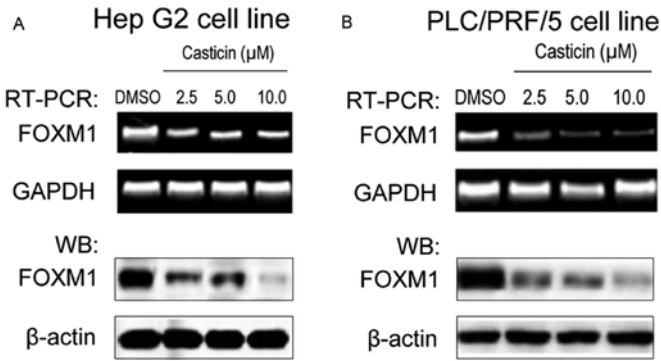


Figure 2. Downregulation of FOXM1 expression at the mRNA and protein levels by casticin in HCC cells. Cells were treated with the indicated concentrations of casticin for 24 h. (A) Casticin downregulated the expression of FOXM1 at the mRNA level (RT-PCR) and protein level (western blotting; WB) in the HepG2 cell line. (B) Casticin downregulated the expression of FOXM1 at the mRNA level (RT-PCR) and at the protein level (WB) in the PLC/PRF/5 cell line.

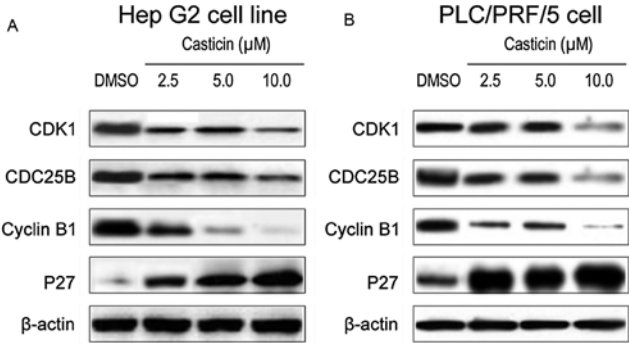


Figure 3. Decrease in the protein expression of FOXM1 downstream targets CDK1, CDC25B and cyclin B1 and increase in the protein expression of p27 by casticin in HCC cells. Cells were treated with the indicated concentrations of casticin for 24 h. (A) The expression of CDK1, CDC25B, cyclin B1 and p27 proteins was analyzed using western blotting in the Hep G2 cell line. β-actin was used as a loading control. (b) The expression of CDK1, CDC25B, cyclin B1 and p27 proteins was analyzed using western blotting in the PLC/PRF/5 cell line. β-actin was used as a loading control.

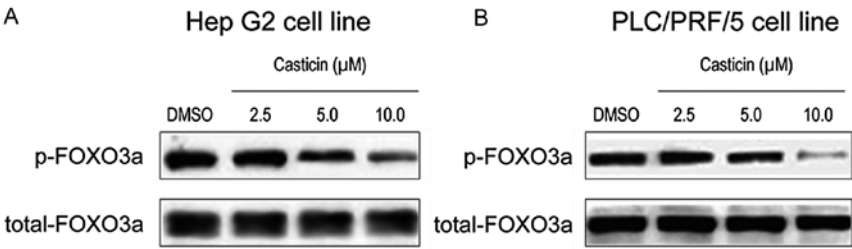


Figure 4. Reduction in the protein expression of phosphorylated FOXO3a by casticin in HCC cells. Cells were treated with the indicated concentrations of casticin for 24 h. (A) The protein expression of phosphorylated FOXO3a was analyzed using western blotting in the Hep G2 cell line. Total FOXO3a was used as a loading control. (B) The protein expression of phosphorylated FOXO3a was analyzed using western blotting in the PLC/PRF/5 cell line. Total FOXO3a was used as a loading control.

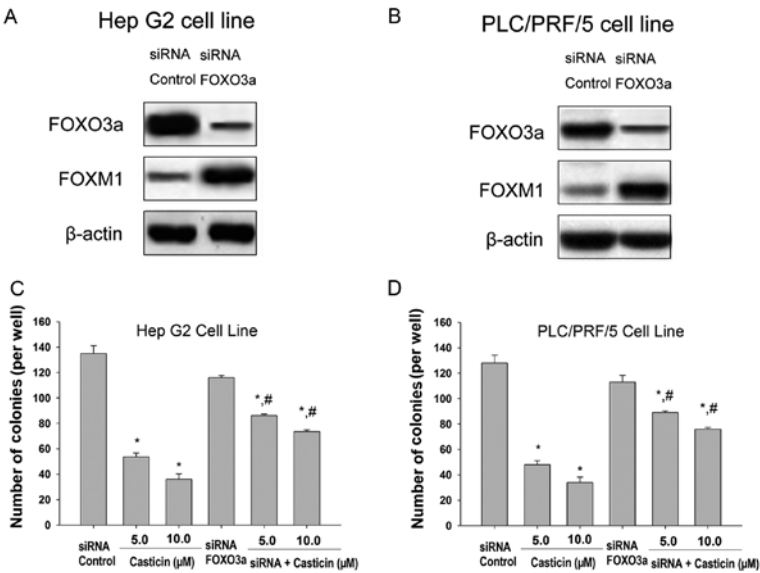


Figure 5. Depletion of FOXO3a by siRNA increased protein expression of FOXM1 and attenuated the inhibitory effects of casticin on colony formation in HCC cells. (A) Cells were transiently transfected with a control non-specific siRNA or a FOXO3a-targeted siRNA for 24 h. The expression of FOXO3a and FOXM1 proteins was analyzed using western blotting in the Hep G2 cell line. β-actin was used as a loading control. (B) Cells were transiently transfected with a control non-specific siRNA or a FOXO3a-targeted siRNA for 24 h. The expression of FOXO3a and FOXM1 proteins was analyzed using western blotting in the PLC/PRF/5 cell line. β-actin was used as a loading control. (C) Hep G2 cells were transiently transfected with the non-specific siRNA and the FOXO3a-targeted siRNA, respectively. Reduction in the colony numbers by casticin was attenuated by FOXO3a siRNA transfection. (D) PLC/PRF/5 cells were transiently transfected with the non-specific siRNA and the FOXO3a-targeted siRNA, respectively. Reduction in the colony numbers by casticin was attenuated by FOXO3a siRNA transfection. Data and error bars are presented as means ± SD. \*P<0.05 versus treatment with DMSO in transfected cells using the non-specific siRNA. #P<0.05 versus treatment with casticin at the same concentrations in transfected cells using the non-specific siRNA.

technology (Fig. 5A and B). A clear increase in the expression level of FOXM1 in the FOXO3a siRNA-transfected cells was noted (Fig. 5A and B). In addition, we found that the knockdown of FOXO3a significantly attenuated casticin-induced inhibition of growth of HCC cells (Fig. 5C and D). These findings indicate that casticin induces HCC cell growth inhibition by repressing FOXM1 through inducing FOXO3a activity.

## Discussion

Abnormal cell proliferation is an important characteristic of malignant tumors including HCC. This suggests that cell cycle arrest could be an effective method in the treatment of malignant tumors. We previously showed that casticin inhibits the viability of HCC cells (10). In this study, to evaluate the effect of casticin on HCC cells, we investigated cell growth inhibition, cell cycle arrest and the molecular mechanisms following treatment of human HCC cells with casticin *in vitro*. We detected HCC cell growth by the clonogenic assay. We further demonstrated that casticin significantly inhibited the colony formation in Hep G2 and PLC/PRF/5 cell lines. In addition, cell cycle analysis showed that the percentage of G2/M phase cells was significantly elevated in casticin-treated cells compared with DMSO-treated cells, indicating a G2/M phase cell cycle arrest. These findings are in line with those of other reports concerning casticin treatment in other types of cancer cells such as cervical cancer cells (5-7). For this reason, casticin-induced cancer cell growth inhibition was hypothesized to be mediated through an induction of cell cycle arrest in the G2/M phase in all cancers studied.

FOXM1, one member of the forkhead box transcription factor family, is a critical regulator of cell cycle progression and functions in cell proliferation, organogenesis, aging and carcinogenesis (25). *In vitro* loss of FOXM1 is associated with cell cycle arrest and leads to defective mitotic spindle integrity. Furthermore, *in vivo*, loss of FOXM1 has been reported to be embryonic lethal due to a failure to enter mitosis (26-28). Recent studies have shown that FOXM1 is overexpressed in several types of cancer, such as lung cancer, glioblastomas and gastric cancer. It is reported to have an important role in the development and progression of these cancers (29,30). FOXM1 expression is tightly associated with proliferation and is extinguished in differentiated or resting cells that have exited the cell cycle. In normal tissues, the expression of FOXM1 is restricted to embryonic tissues and a few adult endocrine glands (31). FOXM1 is strongly expressed in mouse fetal liver but not in the adult one. However, FoxM1 is highly expressed in HCC and HCC cell lines.

The above findings indicate that FOXM1 is a potential specific target for HCC therapy. Notably, a recent study showed that lower expression of FOXM1 in HCC was associated with prolonged disease-free survival after curative liver resection and validated FOXM1 as a prognostic marker for HCC (32). In the present study, we investigated the role and regulation of FOXM1 in response to casticin treatment in HCC cells. We found that casticin suppressed the expression of the transcription factor FOXM1 in Hep G2 and PLC/PRF/5 cells. The suppression of FOXM1 by casticin was also associated

with the downregulation of FOXM1 activity as revealed by the concomitant decrease in expression of the FOXM1 downstream targets, cyclin-dependent kinase (CDK1), cyclin B1, CDC25B and an increase in p27<sup>KIP1</sup>. These results suggest that FOXM1 affects the cell cycle of HCC cells by regulating the expression levels of CDK1, cyclins (cyclin B1) and CDKI (p27). In addition to the above mechanisms, a recent study suggested that cellular senescence caused by FOXM1 depletion may be involved in the inhibition of cell survival (28).

FOXO3a is a member of the forkhead box class O (FOXO) transcription factor family and an important regulator of FOXM1 activity and function (33). This study aimed to elucidate the involvement of FOXO3a during casticin-induced growth inhibition and cell cycle arrest in HCC cells. In this study, we showed that casticin inhibited FOXO3a phosphorylation in a concentration-dependent manner. Importantly, the silencing of the FOXO3a gene by siRNA transfection clearly attenuated the induction of cell growth and FOXM1 expression inhibition by casticin. These results showed that activation of FOXO3a contributed to HCC growth inhibition by casticin through downregulation of FOXM1 expression and inactivation of FOXM1 function. Emerging evidence has been provided that FOXO3a activation induces cell cycle arrest resulting in tumor suppression (34,35). Agents that activate FOXO3a may be novel therapeutic agents that can inhibit and prevent tumor growth and development in various cancer types. Moreover, activation of FOXO3a could enhance the effects of a series of chemotherapeutic drugs such as cisplatin and paclitaxel in various types of cancer (36,37). However, whether casticin enhances the sensitivity of cancer cells to chemotherapeutic drugs needs further investigation.

In conclusion, these results showed that downregulation of the expression levels of phosphorylated FOXO3a and FOXM1 in HCC cells by casticin decreased the colony formation ability and induced G2/M phase cell cycle arrest. Furthermore, a decrease in the FOXM1 expression level resulted in downregulation of CDK1, CDC25B and cyclins B1 along with upregulation of p27. The depletion of FOXO3a also reduced the effects of casticin. Our study provides clearly evidence that the FOXO3a/FOXM1 signaling pathway may serve as a new target for the natural flavonoid casticin in HCC therapy.

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