JMolMed

ORIGINAL ARTICLE

Inactivation of FoxM1 transcription factor contributes to curcumin-induced inhibition of survival, angiogenesis, and chemosensitivity in acute myeloid leukemia cells

Jing-ru Zhang · Fei Lu · Ting Lu · Wen-hao Dong · Peng Li · Na Liu · Dao-xin Ma · Chun-yan Ji

Abstract
Aberrant expression of forkhead box protein M1 (FoxM1) contributes to carcinogenesis in human cancers, including acute myeloid leukemia (AML), suggesting that the discovery of specific agents targeting FoxM1 would be extremely valuable for the treatment of AML. Curcumin, a naturally occurring phenolic compound, is suggested to possess anti-leukemic activity; however, the underlying mechanism has not been well elucidated. In this study, we found that curcumin inhibited cell survival accompanied by induction of G2/M cell cycle arrest and apoptosis in HL60, Kasumi, NB4, and KG1 cells. This was associated with concomitant attenuation of FoxM1 and its downstream genes, such as cyclin B1, cyclin-dependent kinase (CDK) 2, S-phase kinase-associated protein 2, Cdc25B, survivin, Bcl-2, matrix metalloproteinase (MMP)-2, MMP-9, and vascular endothelial growth factor (VEGF), as well as the reduction of the angiogenic effect of AML cells. We also found that specific downregulation of FoxM1 by siRNA prior to curcumin treatment resulted in enhanced cell survival inhibition and induction of apoptosis. Accordingly, FoxM1 siRNA increased the susceptibility of AML cells to doxorubicin-induced apoptosis. More importantly, curcumin suppressed FoxM1 expression, selectively inhibited cell survival as well as the combination of curcumin and doxorubicin exhibited a more inhibitory effect in primary CD34$^+$ AML cells, while showing limited lethality in normal CD34$^+$ hematopoietic progenitors. These results identify a novel role for FoxM1 in mediating the biological effects of curcumin in human AML cells. Our data provide the first evidence that curcumin together with chemotherapy or FoxM1 targeting agents may be effective strategies for the treatment of AML.

Key message
- Curcumin inhibited AML cell survival and angiogenesis and induced chemosensitivity.
- Aberrant expression of FoxM1 induces AML cell survival and chemoresistance.
- Inactivation of FoxM1 contributes to curcumin-induced anti-leukemic effects.
- Curcumin together with FoxM1 targeting agents may be effective for AML therapy.

Keywords Acute myeloid leukemia · Angiogenesis · Chemosensitivity · Curcumin · FoxM1

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous and aggressive disorder characterized by a highly proliferative accumulation of dysfunctional and immature myeloblasts in the bone marrow (BM). Despite improvement in treatment protocols, a large number of AML cases are resistant to chemotherapy and have poor prognosis [1]. It is now widely accepted that the defects in the core machinery of the apoptotic pathway contribute to chemoresistance [2]. Moreover, we have previously shown that angiogenesis is important for leukemogenesis and chemosensitivity in AML [3, 4]. Thus, exploration of novel, reliable, and less toxic therapeutic agents is urgently needed to prevent AML chemoresistance/progression and

improve patient survival rates. Identifying molecular mechanisms involved in leukemogenesis and chemoresistance may therefore be the key to providing such novel treatment approaches.

Forkhead box protein M1 (FoxM1), an oncogenic transcription factor, has been demonstrated to promote the proliferation of AML cells through the modulation of cell cycle progression [5]. FoxM1 is known to be a key cell cycle regulator of G1-S and G2-M transition by upregulation of S-phase kinase-associated protein 2 (SKP2) and cyclin-dependent kinase (CDK) units through downregulation of p21 and p27 [6, 7]. FoxM1 is also reported to regulate transcription of genes essential for cell cycle progression and mitotic entry, including cyclin B1 and Cdc25B [8]. Emerging evidence suggests that FoxM1 signaling is frequently upregulated in human malignancies [5, 9]. Furthermore, downregulation of FoxM1 could lead to the inhibition of cell growth, invasion, and angiogenesis as well as the enhancement of chemosensitivity [10–12]. These data suggest that FoxM1 signaling plays critical roles in the development and progression of human cancers including AML. Therefore, inactivation of FoxM1 may represent a promising strategy for the treatment of AML.

An ideal chemopreventive/therapeutic agent should target the aberrant signaling pathways involved in tumor development and progression to restore normal growth control while minimizing toxicity in normal tissues. Curcumin, a naturally occurring phenolic compound present in *Curcuma longa*, is considered to possess anti-tumor effects and had no toxicity in normal cells [13]. Previous studies by our group and others have demonstrated that curcumin can suppress growth of various cancer cells in vitro and in vivo by causing cell cycle arrest and apoptosis induction through simultaneously modulating multiple targets, including survivin and Bcl-2 [13–15]. Curcumin has also been proven to be a powerful chemosensitizer for tumors [13, 16, 17]. Furthermore, curcumin is reported to inhibit angiogenesis by downregulation of vascular endothelial growth factor (VEGF) and affect remodeling of the extracellular matrix through inactivation of matrix metalloproteinases (MMPs) [14, 18, 19]. Recently, accumulating evidence suggests that FoxM1 could regulate VEGF and MMPs critically involved in the processes of tumor angiogenesis and progression [20, 21]. However, no reports have been published regarding the role of curcumin in the regulation of FoxM1 in leukemia. Our preliminary study demonstrated that curcumin treatment concomitantly attenuated FoxM1 messenger RNA (mRNA) and protein expression in leukemia cells, which led us to conduct the current study to determine whether inactivation of FoxM1 could contribute to curcumin-induced inhibitory effects in human AML.

In this study, our data identify a contributory role for FoxM1 in the effects of curcumin on AML cells. These results provide supportive evidence that inactivation of FoxM1 by curcumin, either alone or in combination with chemotherapy, might be a novel targeted approach for the treatment of AML.

Materials and methods

Cell lines, patient samples, and experimental reagents

Human AML cell lines HL60, Kasumi, NB4, KG1, and human umbilical vascular endothelial cells (HUVECs) were cultured in a 5 % CO_2 atmosphere at 37 °C. Bone marrow mononuclear cells (BMMCs) were isolated by Ficoll-Hypaque density gradient centrifugation and enriched using a human CD34 MicroBead Kit (Miltenyi Biotec, Auburn, CA). The detailed information on cell culture, sample preparation, and experimental reagents are available in Supplementary methods online.

Cell survival and colony formation assay

Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan) and soft agar colony formation assay were conducted to assess cell survival as described previously [3, 12, 13].

Cell cycle and apoptosis assay

Cell cycle distribution by flow cytometric analysis and apoptosis detection by Annexin V/PI staining were performed as described [2, 22].

Real-time reverse transcription-PCR and Western blot analysis

The primers of human FoxM1, cyclin B1, CDK2, SKP2, Cdc25B, p21, p27, survivin, Bcl-2, VEGF, MMP-2, MMP-9, and GAPDH used for real-time reverse transcription (RT)-PCR are shown in Supplementary Table 2 [14]. Protein levels were examined by Western blot using the antibodies against human FoxM1, cyclin B1, CDK2, Bcl-2, VEGF, MMP-2, MMP-9, and β-actin [3, 22]. Detailed methods for total RNA isolation, complementary DNA (cDNA) synthesis, real-time RT-PCR, and Western blot are provided in Supplementary methods online.

Gelatin zymography analysis and enzyme-linked immunosorbent assay

The effects of curcumin on the gelatinolytic activities of MMP-2 and MMP-9 and VEGF protein levels were examined by gelatin zymography and ELISA, respectively (Supplementary methods online).

Matrigel in vitro HUVEC tube formation assay

Curcumin-treated cells were cultured in serum-free medium, and then the conditioned media were collected for HUVEC tube formation assay (Supplementary methods online).

Plasmids, siRNA, and transfections

HL60 and KG1 cells were stably transfected with a FoxM1 cDNA plasmid or control vector alone and maintained under G418 selection as described previously [10, 11]. FoxM1 siRNA or the scrambled control siRNA was transfected using Lipofectamine 2000 [5]. The sequences of FoxM1 siRNA were as follows: FS 1# 5′-CUCUUCUCC CUCAGAUA UAdTdT-3′ and FS 2# 5′-GGACCACUUUCCUAC UUdTdT-3′. After 48 h of transfection, the cells were collected for cell survival and apoptosis assay, tube formation assay, Western blot, gelatin zymography, and ELISA analysis as described above.

Statistical analysis

Data were expressed as mean±SD from three independent experiments. Statistical analyses were carried out using the Student's t test with GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). P values <0.05 were considered statistically significant. Assessment of the combined effect of drugs was performed using CalcuSyn software (Biosoft, Cambridge, UK).

Results

Curcumin inhibited AML cell survival

Our previous data demonstrated that curcumin caused marked inhibition of osteosarcoma cell growth [14]. Here, we determined whether curcumin could suppress AML cell survival in a variety of cultured cell lines (HL60, Kasumi, NB4, and KG1 cells). As detected by CCK-8 assay, the treatment of AML cells for 12, 24, and 48 h with 5, 10, 20, 30, and 40 μM of curcumin resulted in cell survival inhibition (Fig. 1). The half maximal inhibitory concentration (IC50) of curcumin at 48 h was found to be 20.51±3.71, 22.79±3.77, 20.86±3.48, and 18.54±2.34 μM in HL60, Kasumi, NB4, and KG1 cells, respectively.

Curcumin induced G2/M cell cycle arrest and apoptosis in AML cells

To further examine the effect of curcumin on cell survival in more detail, we conducted a cell cycle analysis using AML cells treated with 10 μM of curcumin for 24 h. As illustrated in Fig. 2a, curcumin induced an accumulation of cells in the G2/M-phase fractions. The G2/M-phase fraction increased from 23.92, 19.35, 16.73, and 18.84 % in control cells to 34.59, 33.21, 25.07, and 36.47 % in curcumin-treated HL60, Kasumi, NB4, and KG1 cells, respectively. These results indicate that the decreased survival in curcumin-treated cells is at least partially a result of the cell cycle arrest, which is consistent with our previously published reports [14].

Next, we investigated whether survival inhibition induced by curcumin was also accompanied by induction of apoptosis. Treatment with 20 μM of curcumin for 48 h resulted in apoptotic rates of 22.92±2.47, 23.26±5.38, 24.03±3.33, and 26.52±4.76 % in HL60, Kasumi, NB4, and KG1 cells, respectively (Fig. 2b), suggesting that curcumin could induce AML cell apoptosis.

Downregulation of the FoxM1 expression by curcumin

FoxM1 signaling is suggested to play important roles in cellular proliferation and apoptosis [5, 23]. To further clarify the molecular mechanism involved in curcumin-induced AML cell cycle arrest and apoptosis, alterations in the FoxM1 signaling were investigated. FoxM1 expression in HL60, Kasumi, NB4, and KG1 cells treated with increasing concentrations of curcumin for 24 and 48 h were assessed using real-time RT-PCR analysis. Compared with control, FoxM1 mRNA levels were downregulated by curcumin in all four cell lines (Fig. 3a and Supplementary Fig. 1). To confirm our results, we also did Western blot analysis. Indeed, we observed a lower level of FoxM1 protein in the curcumin-treated AML cells (Fig. 3b). Together, we conclude that curcumin was effective in inhibiting the transcription and translation of the FoxM1 gene.

Curcumin reduced the expression of FoxM1 downstream target genes

To further verify the inactivation of FoxM1 signaling by curcumin, we also detected the expression of FoxM1 downstream target genes in AML cells after curcumin treatment. As shown in Fig. 3a, b and Supplementary Fig. 1, depletion of FoxM1 expression in the curcumin-treated AML cells was accompanied by a significant reduction in the mRNA or protein levels of cyclin B1, CDK2, SKP2, Cdc25B, survivin, and Bcl-2. In contrast, suppression of FoxM1 expression by curcumin resulted in increased levels of p21 and p27. These results provide evidence for a potential role of FoxM1 during curcumin-induced AML cell cycle arrest, apoptosis, and survival inhibition.
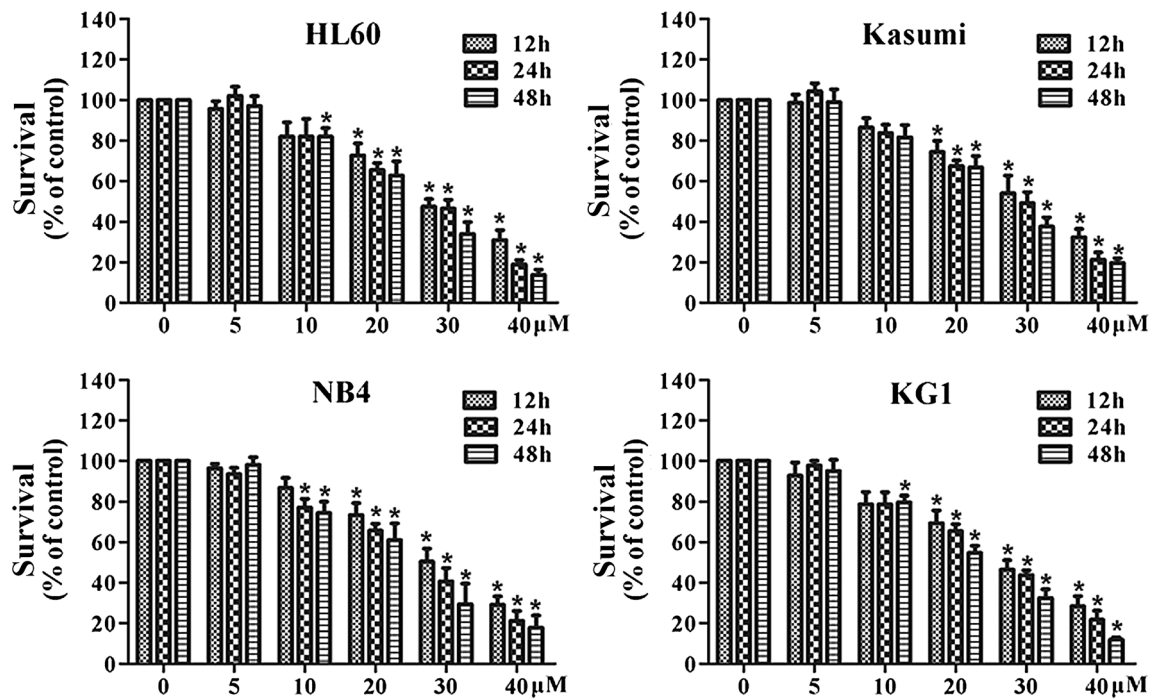
Fig. 1 Curcumin suppressed AML cell survival in a dose- and time-dependent manner as assessed by CCK-8 assay. HL60, Kasumi, NB4, and KG1 cells were treated with 5, 10, 20, 30, and 40 μM of curcumin for 12, 24, and 48 h (control is cells treated with DMSO). Cell survival in curcumin-treated cells is expressed as the percentage of DMSO-treated samples. Results represent the mean±SD of three individual experiments from six determinations per experiment for each experimental condition. *$P<0.05$

Curcumin decreased the expression and activities of VEGF, MMP-2, and MMP-9

It is well known that VEGF and MMPs are transcriptionally regulated by FoxM1 [20, 21]. Thereafter, we tested whether the downstream effect of curcumin induced by inactivation of FoxM1 was associated mechanistically with VEGF and MMP reduction. Compared with control cells, the mRNA and protein expression of VEGF, MMP-2, and MMP-9 were dramatically reduced in curcumin-treated AML cells (Fig. 3a, b and Supplementary Fig. 1). Next, we explored whether curcumin lead to a decrease in MMP activities. Gelatin zymography analysis demonstrated that both MMP-2 and MMP-9 activities were reduced in curcumin-treated cells (Fig. 3c). Most importantly, curcumin also resulted in a decrease in the levels of VEGF secreted in the culture medium as detected by ELISA assay (Fig. 3d).

### Curcumin inhibited the angiogenic effect of AML cells

Our previous study has indicated that VEGF, MMP-2, and MMP-9 are critically involved in the processes of AML cell angiogenesis [3], and since FoxM1 plays crucial roles in tumor angiogenesis [10, 11, 20, 21], we further conducted an in vitro tube formation assay to evaluate the effects of curcumin on angiogenesis. Conditioned media from HL60 and KG1 cells (Fig. 4a) as well as VEGF (Fig. 4b) were able to significantly induce the tube formation of HUVECs in 8 h

incubation (compared with Fig. 4b, medium only as negative control). Importantly, conditioned media from HL60 and KG1 cells treated with curcumin inhibited tube formation compared with the medium from cells treated with DMSO (Fig. 4a, control). To exclude the possibility that tube formation inhibition by conditioned media from curcumin-treated AML cells was mediated through the direct effect of curcumin on tube formation, HUVECs grown in growth factor-reduced Matrigel were treated with 10 μM of curcumin. Curcumin had no effect on tube formation within 8 h of incubation (compared with Fig. 4b, DMSO control). Taken together, these data suggest that curcumin could exert pronounced angiogenesis-inhibitory effect on AML cells, in agreement with the decreased secretion of VEGF, MMP-2, and MMP-9.

Overexpression of FoxM1 by cDNA transfection partially rescued curcumin-induced inhibition of cell survival and angiogenesis

Subsequently, we investigated the effect of FoxM1 overexpression on curcumin-induced survival and angiogenesis inhibition. FoxM1 cDNA plasmid-transfected HL60 cells were treated with 10 μM curcumin for 24 h. Upregulation of FoxM1 by cDNA transfection showed overexpression of FoxM1 protein (Fig. 4c), and this overexpression in FoxM1 alleviated curcumin-induced cell survival and angiogenesis inhibition to a certain degree (Fig. 4d, e). Additionally, FoxM1 overexpression rescued a curcumin-induced decrease in the

Fig. 2 Curcumin induced G2/M phase arrest (**a**, 10 μM for 24 h) and apoptosis (**b**, 20 μM for 48 h) in HL60, Kasumi, NB4, and KG1 cells as measured by flow cytometry analysis. Results in (**a**) are plotted as the percentage of cells in each cell cycle phase. Results in (**b**) are plotted as the percentages of early (AV+PI−) and late (AV+PI+) apoptotic cells in each quadrant. Percent of total (early+late) apoptotic cells are presented as mean±SD of three separate experiments. *P<0.05
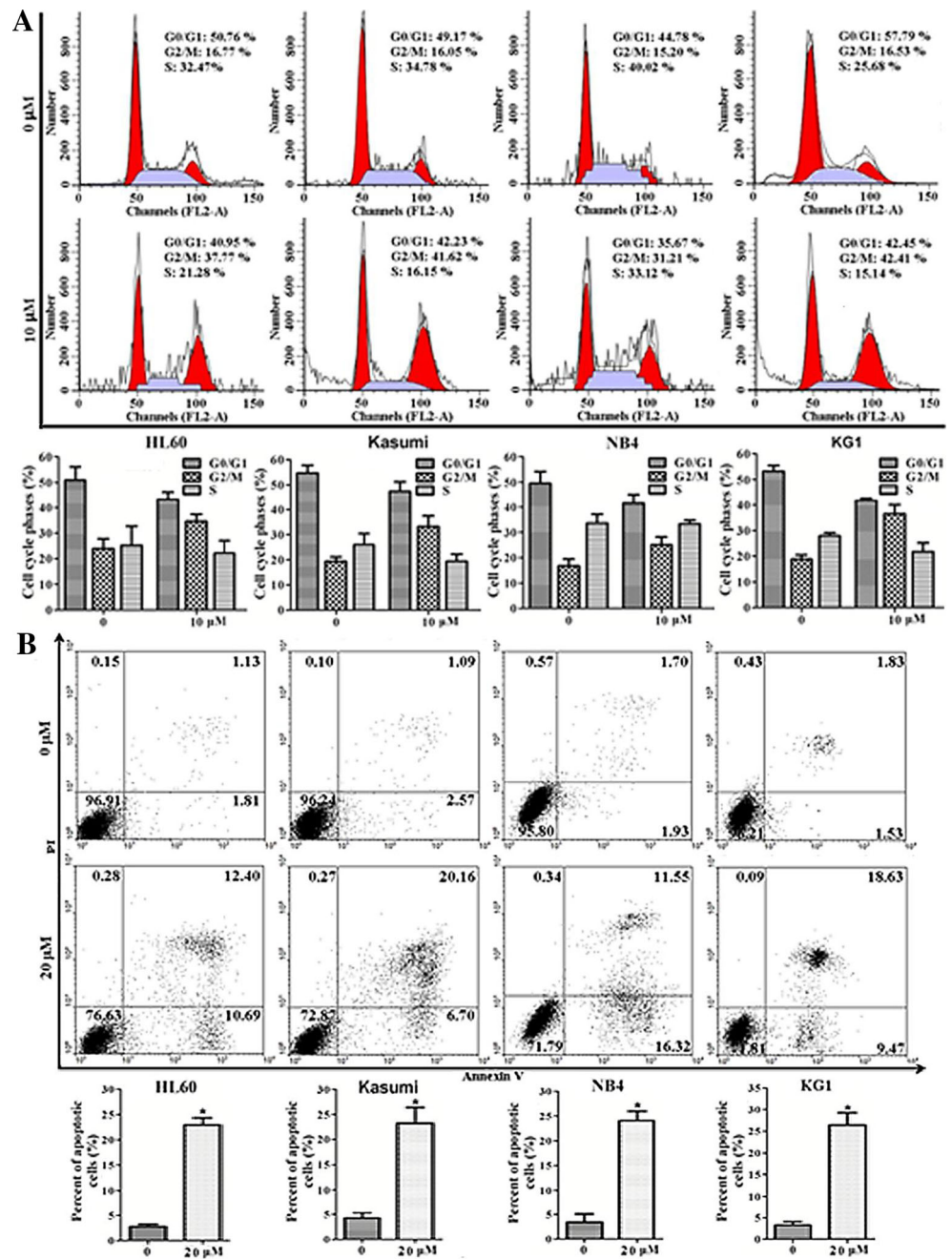

activities of VEGF, MMP-2, and MMP-9 (Fig. 4f) as well as the expression of cyclinB1 and CDK2 (Fig. 4g). These results provide mechanistic evidence suggesting that AML cell survival and angiogenesis inhibition by curcumin is in part due to inactivation of FoxM1 signaling.

Downregulation of FoxM1 by siRNA potentiated curcumin-induced cell survival inhibition and apoptosis

To further confirm the role of FoxM1 in survival and apoptosis of AML cells, we conducted a gene knockdown experiment in KG1 cells. As shown in Fig. 5a, downregulation of

FoxM1 by siRNA showed reduced expression of FoxM1 protein, and FoxM1 siRNA plus curcumin induced inactivation of FoxM1 activity to a greater degree, compared with curcumin alone. Moreover, the knockdown of FoxM1 promoted a curcumin-induced inhibition in the activities of VEGF, MMP-2, and MMP-9 (Fig. 5b). We also found that FoxM1 siRNA significantly inhibited KG1 cell survival, and this downregulation potentiated curcumin-induced survival inhibition (Fig. 5c). Furthermore, FoxM1 siRNA-transfected KG1 cells were significantly more sensitive to curcumin-induced apoptosis (Fig. 5d). Thus, these data indicate that downregulation of FoxM1 by siRNA inhibited cell survival
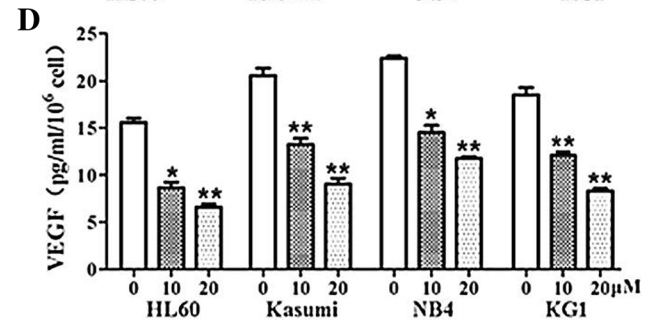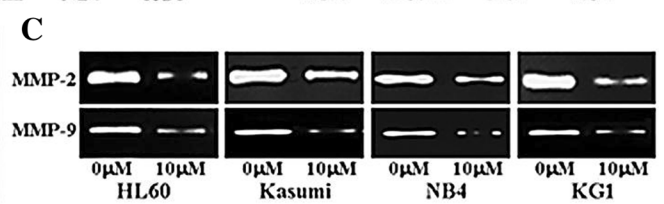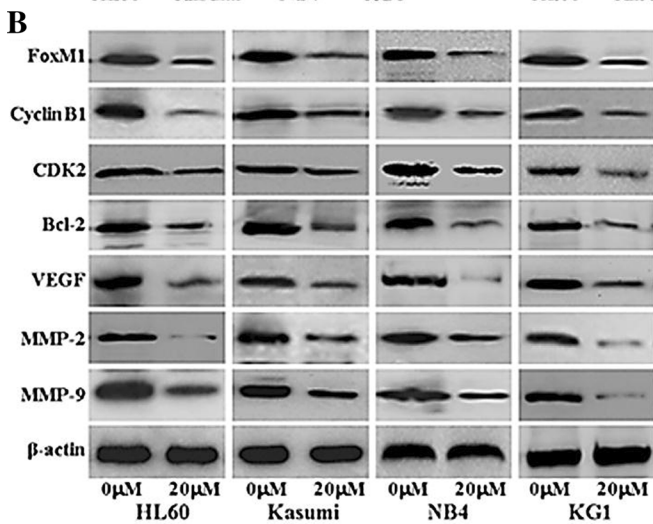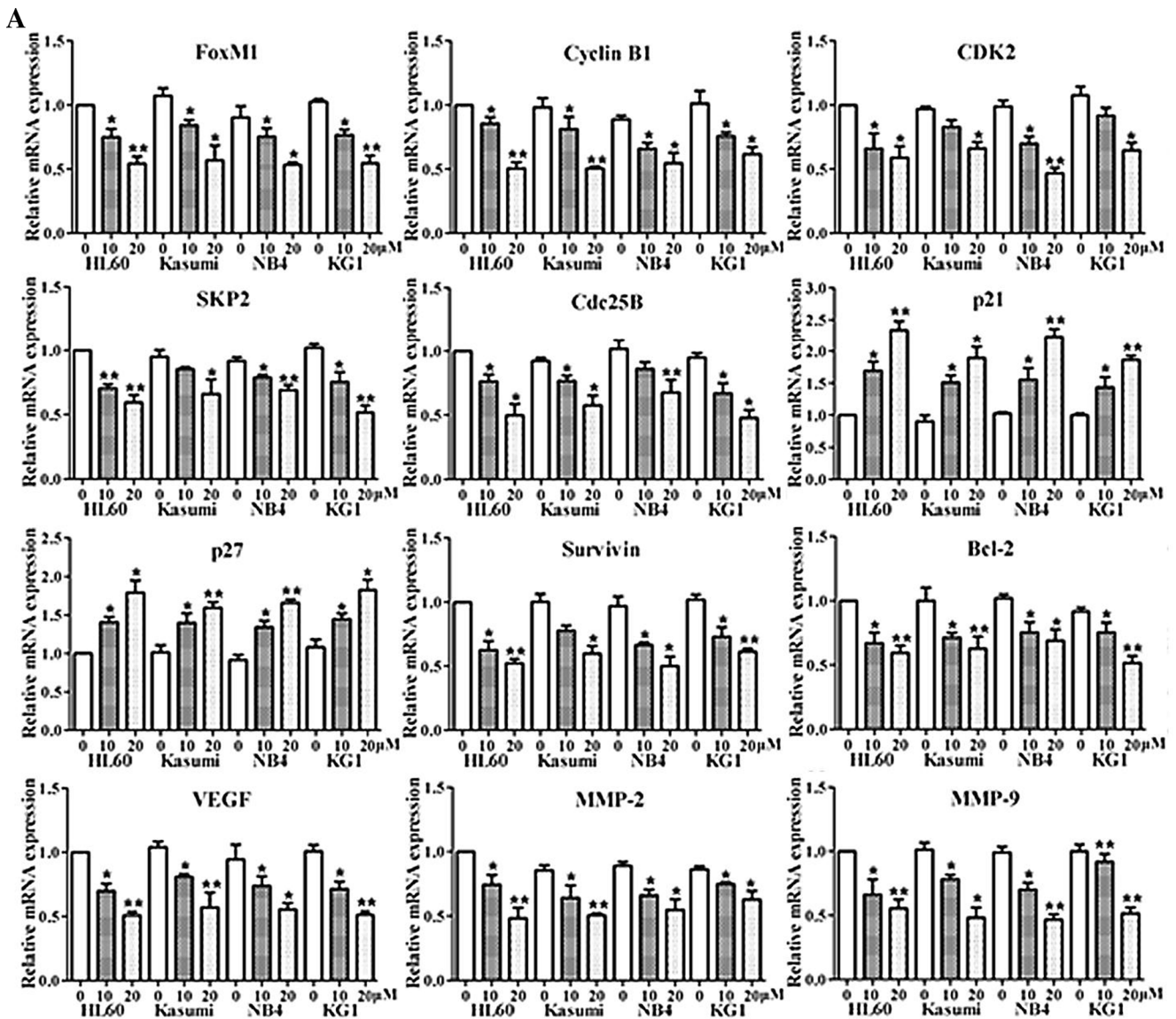
◀ **Fig. 3** Curcumin downregulated the expression of FoxM1, cyclin B1, CDK2, SKP2, Cdc25B, survivin, Bcl-2, VEGF, MMP-2, and MMP-9 mRNA, whereas upregulated p21 and p27 mRNA (**a**, 10 and 20 μM for 48 h); reduced FoxM1, cyclin B1, CDK2, Bcl-2, VEGF, MMP-2, and MMP-9 protein (**b**, 20 μM for 48 h) as well as decreased the activities of MMP-2, MMP-9 (**c**, 10 μM for 48 h) and VEGF (**d**, 10 and 20 μM for 48 h) in HL60, Kasumi, NB4, and KG1 cells. Real-time RT-PCR, Western blot, gelatin zymography analysis, and ELISA assay were performed as described in the "Materials and methods". GAPDH was used to normalize the mRNA level and results are presented as the percentage relative to control (cells treated with DMSO). β-actin was used as a sample loading control. Data are expressed as mean±SD from three independent experiments. *P<0.05; **P<0.01

and induced apoptosis of AML cells, and the combination of FoxM1 siRNA and curcumin showed a more potent effect.

Suppression of FoxM1 with siRNA induced apoptosis and increased the susceptibility of AML cells to doxorubicin-induced apoptosis

We further tested the effect of FoxM1 siRNA on apoptosis and doxorubicin sensitivity. As shown in Fig. 5e, FoxM1 siRNA-induced apoptosis in 48 h (22.63±4.63 % in HL60 and 27.07±6.91 % in KG1) was similar to that in curcumin-treated HL60 and KG1 cells (22.92±2.47 and 26.52±4.76 %, Fig. 2b), respectively. More importantly, downregulation of FoxM1 by siRNA increased the susceptibility of these cell lines to doxorubicin-induced apoptosis (44.98±2.07 % in HL60 and 40.25±3.69 % in KG1), compared to doxorubicin only (15.09±1.77 % in HL60 and 9.63±2.91 % in KG1, Fig. 5f). Additionally, both FoxM1 siRNA and curcumin also enhanced sensitivity to cytarabine-induced apoptosis in HL60 cells (Supplementary Fig. 2).

Curcumin suppressed FoxM1 expression and inhibited cell survival in primary CD34+ AML cells ex vivo

To further evaluate the survival-inhibitory effects of either curcumin and/or doxorubicin on primary CD34+ AML cells, we performed CCK-8 assay in the sorted CD34+ BMMCs from five AML patients and three healthy donors. As shown in Fig. 6a, curcumin significantly decreased the amount of viable CD34+ AML cells, but only exhibited modest lethality in normal CD34+ hematopoietic progenitors. Nevertheless, primary CD34+ AML cells were insensitive to doxorubicin (Fig. 6a). Curcumin induced remarkable apoptosis in CD34+ AML cells, but minimal apoptosis in normal CD34+ progenitors (Fig. 6b). Additionally, colony formation in CD34+ AML cells treated with 20 μM of curcumin was also suppressed (Fig. 6c). Notably, Western blot analysis showed weak expression of FoxM1 in normal CD34+ progenitors, while a higher FoxM1 level was detected in CD34+ AML cells and curcumin was able to decrease the FoxM1 protein levels

(Fig. 6d). The combined effect of curcumin and doxorubicin was also examined in eight AML patients and six healthy donors. We found that 20 or 30 μM of curcumin together with doxorubicin (20 μg/l) exhibited a more survival-inhibitory effect in CD34+ AML cells, whereas normal CD34+ progenitors were less susceptible to the combined effects (Fig. 6e). Together, these data demonstrated that curcumin suppressed FoxM1 expression, selectively inhibited cell survival as well as the combination of curcumin and doxorubicin showed a more inhibitory effect in primary CD34+ AML cells, while showing limited lethality in normal CD34+ hematopoietic progenitors.

Discussion

Emerging evidence has shown that curcumin exhibits anti-tumor effects against a wide variety of cancers [15]. In the current study, we demonstrated that curcumin elicited an anti-leukemic effect via induction of apoptosis and G2/M cell cycle arrest as well as reduction of the angiogenic effect of AML cells. To elucidate the molecular mechanisms of survival and angiogenesis inhibition by curcumin, we investigated the activity of FoxM1 signaling, which plays a critical role in the process of cell survival and angiogenesis [10, 11].

FoxM1 expression is regulated by oncogenes and tumor suppressors as well as by proliferation and anti-proliferation signals [23–25]. Since these factors are often mutated, overexpressed, or lost in human cancer, the normal control of FoxM1 expression by them provides the basis for deregulated FoxM1 expression in tumors. Accordingly, FoxM1 is upregulated in a variety of human malignancies, including AML [5, 12, 23–25]. Consistently, we observed that FoxM1 protein was overexpressed in CD34+ cells derived from AML patients, and treatment with curcumin reduced FoxM1 protein expression in those cells. Therefore, FoxM1 may represent an attractive target, and thus, the development of novel agents that will target FoxM1 is likely to have a significant therapeutic impact on human cancer.

Chemopreventive agents may be useful for targeted inactivation of FoxM1 and are likely to have beneficial effects toward cancer therapy because chemopreventive agents are typically known to be non-toxic, unlike many synthetic compounds [26, 27]. In this study, we demonstrated for the first time that the natural non-toxic agent curcumin inhibited the expression of FoxM1 and its target genes in AML cells. Overexpression of FoxM1 by cDNA transfection attenuated curcumin-induced AML cell survival inhibition. These data indicate that AML cell survival inhibition by curcumin could be partly mediated via inactivation of FoxM1 activity. Furthermore, the combination of FoxM1 siRNA and curcumin further inhibited AML cell survival to a greater degree. Thus,
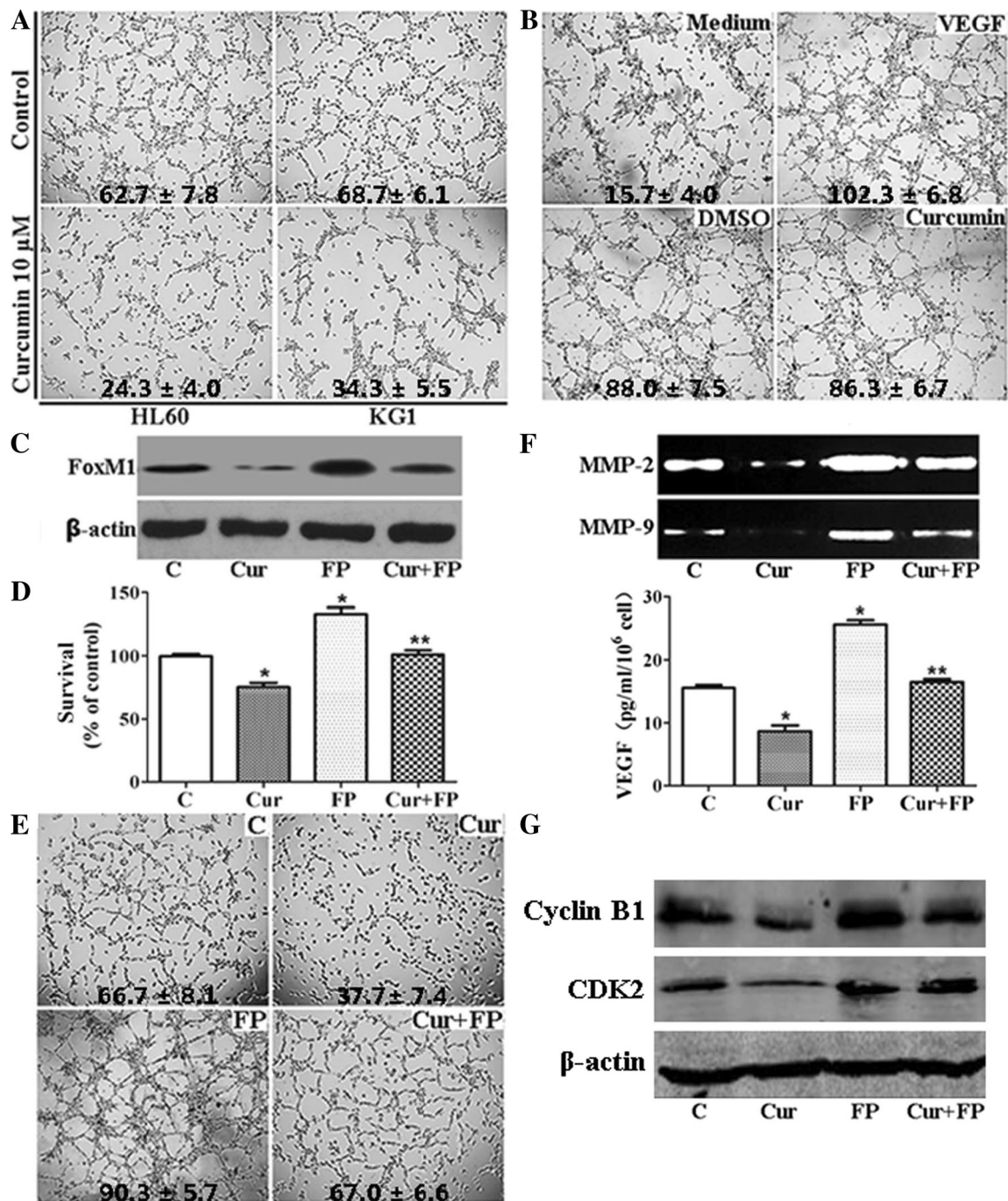
Fig. 4 Curcumin inhibited the angiogenic effect of HL60 and KG1 cells (**a** and **b**, 10 μM for 48 h). FoxM1 cDNA plasmid increased the expression of FoxM1 protein (**c**), and this overexpression significantly promoted cell survival (**d**) in HL60 cells. Upregulation of FoxM1 by cDNA transfection abrogated cells from curcumin-induced cell survival and angiogenesis inhibition to a certain degree (**d** and **e**), as well as rescued a curcumin-induced decrease in the activities of MMP-2, MMP-9, and VEGF (**f**) as well as the expression of cyclin B1 and CDK2 (**g**). *C* control, *Cur* 10 μM curcumin, *FP* FoxM1 cDNA plasmid, *Cur+FP* 10 μM curcumin+FoxM1 cDNA plasmid. FoxM1 cDNA plasmid-transfected HL60 cells were treated with 10 μM curcumin for 24 h. Conditioned media from curcumin-treated cells were collected for the HUVEC tube formation, gelatin zymography analysis, and ELISA assay, and the transfected cells were collected for Western blot and cell survival analysis as described in the "Materials and methods." Quantification of angiogenic activity was calculated by measuring the tubule/capillary length using Image-Pro Plus software. β-actin was used as a sample loading control. Data are expressed as mean±SD from three independent experiments. *$P<0.05$ relative to control; **$P<0.05$ relative to FoxM1 cDNA or curcumin

we strongly believe that downregulation of FoxM1 by siRNA together with curcumin treatment could be a possible therapeutic approach for better management of AML survival.

However, curcumin has been shown to modulate different molecular targets in cancer cells, and thus, the various mechanisms such as inactivation of the AP-1 transcription factor
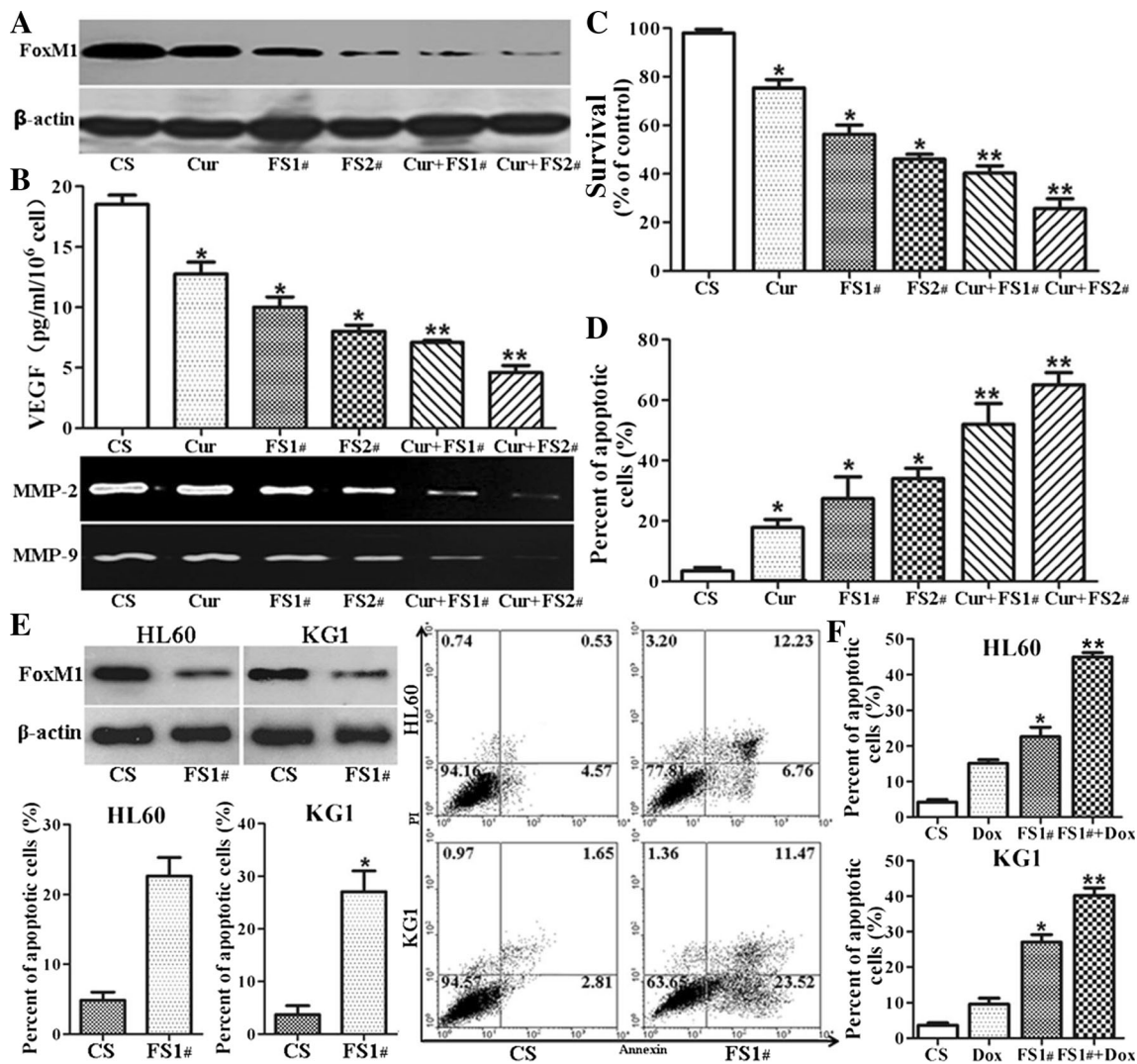
Fig. 5 FoxM1 siRNA decreased the expression of FoxM1 protein (**a**), and this downregulation significantly promoted a curcumin-induced inhibition in the activities of VEGF, MMP-2, and MMP-9 (**b**) in KG1 cells. Downregulation of FoxM1 by siRNA potentiated curcumin-induced survival inhibition to a greater degree compared to curcumin alone (**c**). FoxM1 siRNA-transfected KG1 cells were significantly more sensitive to curcumin-induced apoptosis (**d**). Suppression of FoxM1 by siRNA induced apoptosis (**e**) and increased susceptibility to doxorubicin (**f**) in HL60 and KG1 cells. *CS* control siRNA, *Cur* 10 μM curcumin, *FS* FoxM1 siRNA, *Cur+FS* 10 μM curcumin+FoxM1 siRNA, *Dox* 20 μg/l doxorubicin. FoxM1 siRNA-transfected KG1 cells were treated with 10 μM curcumin for 24 h. The transfected cells were collected for Western blot analysis and cell survival and apoptosis assay, and conditioned media from curcumin-treated cells were collected for ELISA assay and gelatin zymography analysis as described in the "Materials and methods." β-actin was used as a sample loading control. Data are expressed as mean±SD from three independent experiments. *$P<0.05$ relative to control siRNA; **$P<0.05$ relative to FoxM1 siRNA or curcumin or doxorubicin

could be involved in the combined effect of FoxM1 silencing and curcumin [28].

It is now well accepted that overexpression of FoxM1 in tumors could overcome the G2/M checkpoint imposing progression of cells through cell cycle conferring proliferative advantage [5, 6, 29]. Since curcumin inhibits FoxM1 and decreased cell survival, we assumed cell survival inhibition could be attributed to the induction of cell cycle arrest. Indeed, curcumin was effective in inducing G2/M arrest, demonstrating that FoxM1 downregulation by curcumin strongly inhibited AML cell survival. Moreover, our observations

showed that the expression of cyclin B1, CDK2, SKP2, and Cdc25B was downregulated, whereas p21 and p27 were up-regulated in curcumin-treated AML cells. Together, these results provide evidence that curcumin affects AML cell cycle partially by regulating the expression of cell cycle regulatory proteins.

In addition, anti-apoptotic survivin and Bcl-2 are highly expressed in a variety of cancer cells, especially quiescent leukemia CD34+ cells [13, 30]. Notably, FoxM1 is reported to regulate survivin expression [26, 27], and survivin inhibition is critical for Bcl-2 inhibitor-induced apoptosis [31].
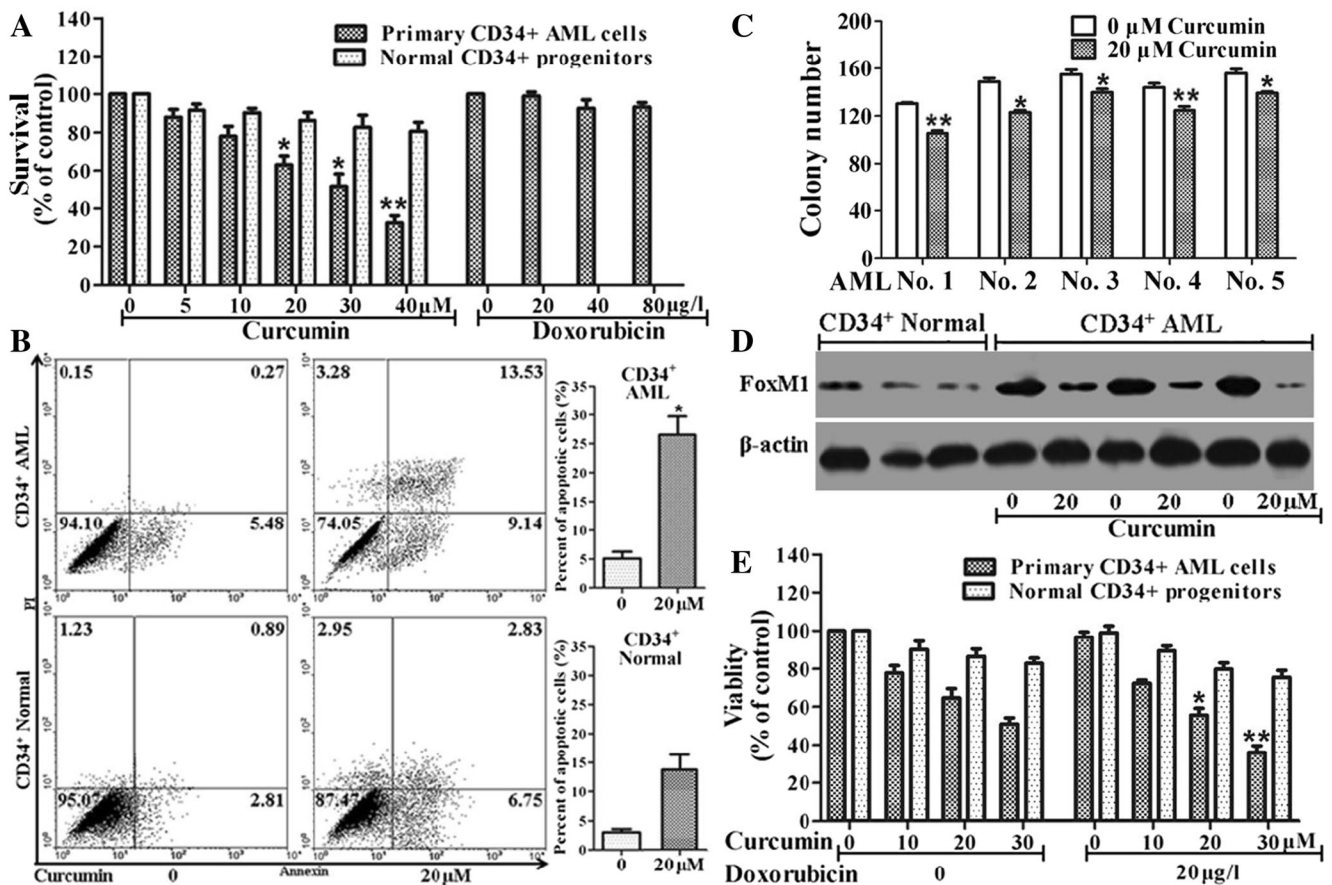
Fig. 6 Curcumin selectively inhibited cell survival (**a**), induced apoptosis (**b**), deceased colony formation (**c**), and suppressed FoxM1 protein expression (**d**), as well as together with doxorubicin exhibited a more survival-inhibitory effect (**e**) in primary CD34$^+$ AML cells, while showing limited lethality in normal CD34$^+$ hematopoietic progenitors. **a**–**d** Five CD34$^+$ AML (patient nos. 1–5) and three CD34$^+$ normal samples (donor nos. 1–3) were treated with different concentrations of curcumin (0, 5, 10, 20, 30, and 40 μM) for 24 h. The same CD34$^+$ AML samples were also exposed to doxorubicin (0, 20, 40, and 80 μg/l) for 24 h. The CD34$^+$ cells with different treatment were collected for cell survival, apoptosis and clonogenic assay, and Western blot analysis as described in the "Materials and methods." β-actin was used as a sample loading control. **d** eight CD34$^+$ AML and six CD34$^+$ normal samples were exposed to different concentrations of curcumin (0, 10, 20, and 30 μM), doxorubicin (20 μg/l), and their combination for 24 h. Data are expressed as mean±SD from three independent experiments. *$P<0.05$; **$P<0.01$

Accordingly, our results demonstrated that curcumin efficaciously induced apoptosis of AML cells, accompanied by downregulation of FoxM1, survivin, and Bcl-2. Together with curcumin treatment, repression of FoxM1 by siRNA could further induce apoptosis. In view of these findings, we could reasonably infer that FoxM1 inactivation by curcumin results in the downregulation of its target genes, which are mechanistically associated with induction of apoptosis by curcumin.

FoxM1 signaling is known to promote angiogenesis in certain tumor models [21]. Moreover, FoxM1 inactivation could be mechanistically associated with the downregulation of VEGF and MMPs, resulting in tumor cell angiogenesis and invasion inhibition [10, 11]. Our results also demonstrated that downregulation of FoxM1 by curcumin inhibited the expression and activities of VEGF, MMP-2, and MMP-9, leading to the inhibition of AML cell angiogenesis. Furthermore, FoxM1 siRNA promoted curcumin-induced angiogenesis inhibition, associated with further downregulation of these genes. Therefore, our data provide a possibility that inactivation of FoxM1

by curcumin could potentiate anti-angiogenic activities partly through suppression of VEGF, MMP-2, and MMP-9 in AML cells.

Accumulating evidence has shown that curcumin potentiates the effects of chemotherapeutic drugs such as bortezomib, daunorubicin, 5-fluorouracil, and cisplatin [13, 16, 17, 32, 33]. Indeed, our study demonstrated that curcumin significantly reduced cell viability and together with doxorubicin exhibited a more survival-inhibitory effect in association with decreased FoxM1 expression in primary CD34$^+$ AML cells, but not in normal CD34$^+$ progenitors. Accordingly, suppression of FoxM1 by siRNA also increased the susceptibility of AML cells to doxorubicin-induced apoptosis, indicating that FoxM1 inactivation plays an important role in this curcumin-induced chemosensitizing effect.

In conclusion, our present findings indicate that curcumin may function as a potent inhibitor of FoxM1, inhibited cell survival and angiogenesis as well as induced apoptosis, which may lead to chemosensitization of AML cells. Importantly,

curcumin selectively inhibited cell survival as well as the combination of curcumin and doxorubicin exhibited a more inhibitory effect in primary CD34^{+} AML cells, accompanied by inactivation of FoxM1. Taken together, the results from our in vitro and in vivo experiments clearly suggest that FoxM1 inhibition, especially by natural agents as shown here, might be possible strategies to sensitize AML cells to conventional chemotherapy and to inhibit tumor growth. Several clinical trials have demonstrated the potential therapeutic efficacy of curcumin [34]. Nevertheless, its poor bioavailability has limited its clinical applications, and numerous techniques such as the use of synthetic analogs, derivatives, and different formulations have been explored to improve its bioavailability [34, 35]. Further in-depth investigations are warranted to elucidate the potential significance of combination treatment with curcumin together with chemotherapy or FoxM1 targeting agents in preclinical animal models for the successful treatment of human AML.

Conflict of interest All the authors declared no competing interests.

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