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The Tumor Suppressor Maspin Mediates E2F1-Induced Sensitivity of Cancer Cells to Chemotherapy

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Abstract

The E2F1 transcription factor is a critical downstream target of the tumor suppressor RB. When activated, E2F1 can induce cell proliferation and/or apoptosis. In addition, E2F1 overexpression sensitizes cancer cells to chemotherapeutic drugs. In a screen for genes that are regulated synergistically by E2F1 and chemotherapy in cancer cells, we identified the proapoptotic tumor suppressor gene *maspin* (mammary serine protease inhibitor) as a novel E2F1-regulated gene. In line with being an E2F-regulated gene, *maspin* expression is inhibited by short hairpin RNA directed against E2F1 and increases upon activation of endogenous E2F. Furthermore, *maspin* mRNA and protein levels are elevated upon activation of exogenous E2F1. Importantly, we show that E2F1-mediated upregulation of *maspin* is enhanced by chemotherapeutic drugs, and inhibition of *maspin* expression significantly impairs the ability of E2F1 to promote chemotherapy-induced apoptosis. Summarily, our data indicate that *maspin* is an important effector of E2F1-induced chemosensitization. *Mol Cancer Res; 8(3); 363–72. ©2010 AACR.*

Introduction

Members of the E2F family of transcription factors are downstream effectors of the tumor suppressor pRB. E2Fs play a pivotal role in controlling cell cycle progression mainly by determining the timely expression of genes required for entry into and progression through the S phase. Ectopic expression of E2F1 induces S-phase entry in quiescent cells (reviewed in refs. 1, 2) and, accordingly, early studies show that it can function as an oncogene, participate in transformation of primary rodent cells, and promote tumorigenesis in mice (reviewed in ref. 3). However, later studies reveal that, in addition, E2F1 exhibits properties of a tumor suppressor because E2F1-null mice develop tumors in several tissues (3). The tumor-suppressive activity of E2F1 is believed to be mediated, at least in part, by its ability to induce apoptosis. Ectopic expression of E2F1 results in apoptosis (4, 5). Moreover, E2F1-deficient mice have an excess of mature T cells due to a defect in thymocyte apoptosis (6), and loss of E2F1 suppresses apoptosis in RB-deficient mice embryos (7, 8). E2F1-induced apoptosis occurs through both p53-dependent and p53-independent pathways, and it has been shown that a number of E2F1-regulated genes, including *p14/p19ARF, p73, Apaf-1, caspases*, and BH3-only proteins, contribute to E2F-induced apoptosis (9-16).

E2F1 functions in the cellular response to DNA damage. Specifically, upon DNA damage, E2F1 is released from its inhibitor pRB (17) and phosphorylated by the damage response kinases ATM and Chk2. These phosphorylations stabilize E2F1 (18, 19). In addition, E2F1 is acetylated and it has been suggested that these modifications bias E2F1 to bind the promoter of its proapoptotic target *p73* (20). The physiologic role of E2F1 in mediating damage-induced cell death is shown by the observation that thymocytes and fibroblasts derived from E2F1 knockout mice exhibit reduced cell death in response to etoposide and flavopiridol, respectively (18, 21). Similarly, in cultured cells, short hairpin RNAs (shRNA) directed against E2F1 reduce chemotherapeutic drug–induced cell death (21-23), whereas E2F1 overexpression cooperates with chemotherapeutic drugs to augment apoptosis (24-29). E2F1-mediated sensitization to chemotherapy-induced apoptosis was found to be p53 independent (26), and presently the molecular mechanisms underlying this phenomenon are unclear.

We reasoned that the synergy between E2F1 and chemotherapy in inducing apoptosis might be due to their collaboration in upregulating expression of distinct apoptotic proteins. To investigate this hypothesis, we screened for E2F-upregulated genes that exhibit enhanced expression in the presence of genotoxic stress and identified the proapoptotic tumor suppressor *maspin*, not previously reported to be an E2F-regulated gene. In line with being an E2F-regulated gene, we find that *maspin* expression is inhibited

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by shRNA directed against E2F1 and increases upon activation of endogenous E2F. Furthermore, *maspin* mRNA and protein levels are elevated upon activation of exogenous E2F1. Importantly, in support of our hypothesis, this E2F1-mediated upregulation of *maspin* is enhanced by chemotherapeutic drugs, and acute downregulation of *maspin* inhibits cell death induced by the combination of E2F1 and chemotherapeutic drugs. Summarily, our data identify *maspin* as a novel E2F-upregulated gene and shows that the synergy between E2F1 and chemotherapy in inducing apoptosis is due to their collaboration in upregulating expression of distinct apoptotic proteins, such as Maspin.

Materials and Methods

Cell Culture

U2OS and SAOS2 osteosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Cells were maintained at 37°C in a humidified atmosphere containing 8% CO_2. To induce activation of ER-E2F-1, cells were treated with 4-hydroxytamoxifen (OHT) for the time and concentration indicated. In experiments where ER-E2F1 activation was combined with administration of chemotherapeutic drugs, OHT was added 16 h before addition of the chemotherapeutic drug. Two hours after drug administration, cell cultures were washed and replenished with fresh medium. Cycloheximide (Sigma) was added for the time and concentration indicated. For clonogenic assay, cells were preincubated with 70% ethanol for 15 min then stained with Giemsa (Merck) for 15 min.

Reverse Transcription-PCR and Quantitative PCR

Reverse transcription-PCR (RT-PCR) was done on total RNA prepared by the Tri Reagent method or the RNeasy kit (Qiagen) with the following primer pairs: *maspin*, 5′-TAATGTTGCTGGATCAGGAAGCCG and 5′-AAGCATAGTGCTGGGAAGAAGAGC; GAPDH, 5′ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA.

Real-time quantitative PCR (qPCR) was done using Absolute Blue SYBER Green Rox Mix (Thermo) and the following primer pairs: *maspin*, 5′-GGTCAGATCAACAACTCAATTAAGG and 5′-CCAACAAAGTAGGCAGCATTAAC; HPRT, 5′-TGACACTGGCAAAACAATGCA and 5′-GGTCCTTTTCACCAGCAACGT.

Western Blotting

Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40] in the presence of protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails I and II (Sigma). Equal amounts of protein, as determined by the Bradford assay, were resolved by electrophoresis in a SDS 12.5% polyacrylamide gel and then transferred to a membrane (Protran BA 85, S&S). The membrane was incubated overnight with one of the following primary antibodies: anti-Maspin (BD Pharmingen), anti-E2F1 (sc-251, Santa Cruz Biotechnology), anti–cleaved caspase-3 (Cell Signaling) or anti-tubulin (Sigma), anti–cleaved PARP1 (sc-8007, Santa Cruz Biotechnology), and actin (sc-1616r, Santa Cruz Biotechnology). Binding of the primary antibody was detected using an enhanced chemilluminescence kit (ECL Amersham).

Plasmids

The plasmids pBabe-neo-HA-ER-E2F1, pBABE-E7, and pBABE-E7Δ (21-35) have been described previously (30). shRNAs directed against human *maspin* (5′-GCACAGGGATTCTCACAATAG) or E2F1 (5′-GACGTGTCAGGACCTTCGT) were cloned into the retroviral vector pRETRO-SUPER (31).

Transfection and Infection Assays

Cells (2×10^6) of the packaging cell line 293T were cotransfected with ecotropic packaging plasmid pSV-E-MLV (10 μg), which provides packaging helper function, and the relevant shRNA expression plasmid (10 μg) using the calcium phosphate method in the presence of chloroquin (Sigma). After 8 h, the transfection medium was replaced with fresh Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Subsequently, cell supernatants containing retroviruses were collected. When performing infection, cells were incubated for 5 h at 37°C in 4.5 mL of retroviral supernatant supplemented with polybrene (8 μg/mL, Sigma H9268). Then, 5.5 mL of medium were added and after a further 24 h, the medium was replaced with fresh medium containing puromycin (2 μg/mL, Sigma P7130).

Fluorescence-Activated Cell Sorting Analysis

Cells were trypsinized and fixed by incubating in 70% ethanol at 4°C overnight. After fixation, cells were centrifuged for 4 min at 1,500 rpm before being incubated for 30 min at 4°C in 1 mL of PBS. Then, the cells were centrifuged again and resuspended in PBS containing 5 mg/mL propidium iodide and 50 μg/mL RNase A. After incubation for 20 min at room temperature, fluorescence intensity was analyzed by using a Becton Dickinson flow cytometer.

Results

E2F1 and Chemotherapeutic Drugs Synergize in Inducing Apoptosis of Human Cancer Cells

To investigate the mechanisms underlying the synergy between genotoxic agents and E2F1 in inducing apoptosis of cancer cells, we took advantage of a human osteosarcoma cell line, U2OS, that expresses a conditionally active E2F1, namely ER-E2F1 (32). Because one of the clinically relevant chemotherapeutic treatments for osteosarcoma is a mixture of doxorubicin and cisplatin,

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we chose to focus on these genotoxic agents (33). Initially, we surveyed the apoptotic effects of either activating E2F1 or adding genotoxic agents. ER-E2F1 was activated by the addition of OHT, and a mild activation of ER-E2F1 (5 nmol/L OHT) led to low levels of apoptosis; 10% and 13.4% of cells underwent apoptosis 24 and 48 h after E2F1 activation, respectively (Fig. 1A). Similarly, low levels of apoptosis were induced by sublethal doses of doxorubicin and cisplatin; only 5% and 8% of the cells underwent apoptosis after 24 and 48 h, respectively (Fig. 1A). As reported previously, this treatment results in a significantly increased percentage of cells in the G_2 phase of the cell cycle (Fig. 1A). Next, we examined the effect on U2OS cells of combining E2F1 activation with sublethal doses of genotoxic agents and observed substantially higher levels of apoptosis; 18% and 42% of the cells underwent apoptosis at 24 and 48 h, respectively (Fig. 1A and B). To corroborate that the cell death monitored by FACS is indeed apoptosis, we repeated the independent and combined treatments and assayed the levels of cleaved PARP1 and cleaved caspase-3. In line with the FACS data, increased cleavage of both proteins was detectable after the combined treatment of E2F1 activation plus chemotherapy (Fig. 1C). In addition, whereas E2F1 activation or chemotherapy administration caused reduced viability as determined by a clonogenic assay, the combined treatment of E2F1 activation plus chemotherapy resulted in an even more significant reduction in the number of colonies (Fig. 1D). Of note, parental U2OS cells lacking ER-E2F1 did not exhibit enhanced cell death in response to combined treatment of E2F1 activation and doxorubicin plus cisplatin administration (Supplementary Fig. S1A), supporting that E2F1 activation is required for the synergistic apoptotic effect.

Taken together, these data indicate that E2F1 sensitizes tumor-derived U2OS cells to chemotherapeutic drugs. Summarily, U2OS cells expressing conditionally active E2F1 and treated with doxorubicin and cisplatin represent a model system for investigating the synergy between E2F1 and DNA-damaging agents in inducing apoptosis of cancer cells.

Proapoptotic *Maspin* Exhibits Enhanced Upregulation when E2F1 Activation Is Combined with Chemotherapy

We reasoned that E2F1-dependent sensitization of cancer cells to chemotherapy could be due to enhanced upregulation of proapoptotic genes in response to the combination of E2F1 activation and chemotherapeutic drugs. To test this hypothesis, we used DNA microarray analysis to survey gene expression levels in U2OS cells after E2F1 activation or genotoxic treatment versus after E2F1 activation plus genotoxic treatment. In accord with our hypothesis, we found, in two independent experiments, that several genes were upregulated reproducibly upon E2F1 activation. These genes were upregulated further when E2F1 activation is combined with doxorubicin and cisplatin treatment. We chose to focus on one such gene, *maspin* (mammary serine protease inhibitor), not previously known to be an E2F1-regulated gene (Supplementary Table S1).

Maspin belongs to a family of serine protease inhibitors (serpins) and is characterized as a class II tumor suppressor based on its ability to inhibit cell invasion, promote apoptosis, and inhibit angiogenesis (34). To validate our microarray data, we analyzed *maspin* expression by real-time PCR. Likewise, we observed that E2F1 activation results in increased *maspin* mRNA levels (Fig. 2A) and, importantly, combining E2F1 activation with doxorubicin and cisplatin treatment leads to enhanced transcriptional upregulation of *maspin* (Fig. 2A). Next, Western blot analysis was used to discover if the expression changes seen at the mRNA level are also apparent at the protein level. Indeed, the protein levels of Maspin is significantly upregulated following E2F1 activation, and this upregulation is dramatically enhanced when E2F1 activation is followed by doxorubicin and cisplatin administration (Fig. 2B, compare lanes 1, 2, and 4). Notably, with parental U2OS cells (that do not express conditionally active E2F1), OHT treatment does not affect *maspin* mRNA levels and OHT treatment combined with doxorubicin and cisplatin does not result in enhanced upregulation of *maspin* RNA levels (Supplementary Fig. S1B). In conclusion, these data show that activation of exogenous E2F1 upregulates *maspin* expression and that when E2F1 activation is combined with genotoxic treatment, this upregulation of *maspin* expression is enhanced substantially. Therefore, in line with our hypothesis, chemotherapeutic drugs and exogenous E2F1 synergize in upregulating expression of a proapoptotic gene, namely the *maspin* gene.

Maspin Is a Novel E2F-Upregulated Gene

To our knowledge, this is the first time *maspin* has been evidenced as an E2F1-upregulated gene. To validate that *maspin* is regulated not only by exogenous E2F1 but also by endogenous E2F, we examined *maspin* expression in nontransformed WI38 cells before and after deregulation of endogenous E2F activity. Endogenous E2F activity was deregulated by infecting the cells with a retrovirus encoding human papilloma virus 16 (HPV16) E7 protein, which disrupts RB/E2F complexes and, thus, activates E2F. As a control, in parallel, cells were infected with a mutated E7 protein, E7D21-35, which does not bind RB family members and consequently does not deregulate E2F. As expected for a gene regulated by E2F, infection with E7, but not E7D21-35, results in increased *maspin* mRNA levels (Fig. 2C), implicating endogenous E2Fs in the regulation of *maspin* expression. To further test the involvement of endogenous E2F1 in controlling *maspin* expression, we studied the effect of E2F1 ablation on Maspin protein levels. Expression of a shRNA directed against E2F1 in U2OS cells results in significantly reduced endogenous Maspin levels, supporting that endogenous E2F1 regulates *maspin* expression (Fig. 2D).

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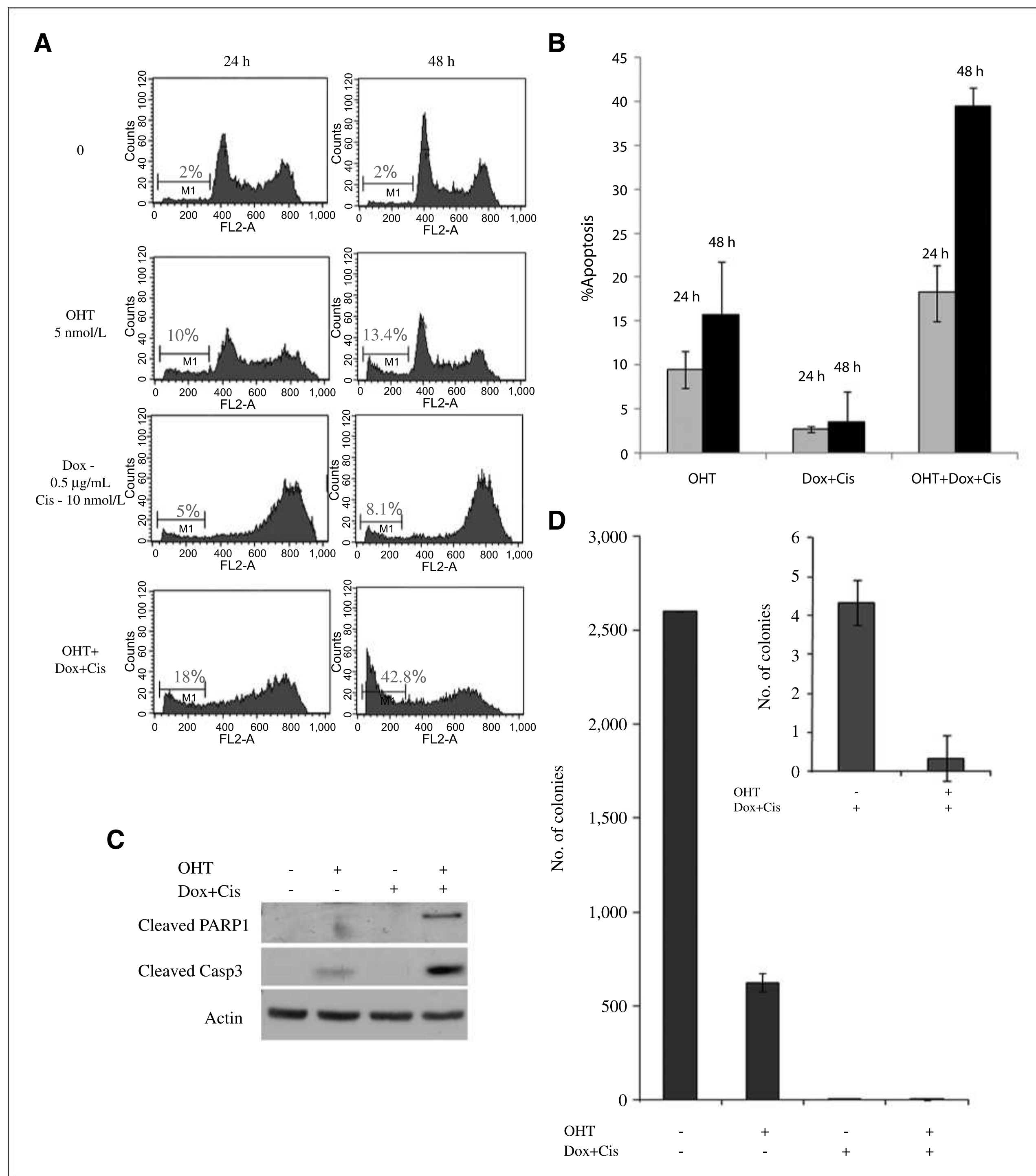

FIGURE 1. E2F1 and chemotherapeutic drugs synergize in inducing apoptosis of human cancer cells. A, U2OS cells stably expressing ER-E2F1 were left untreated (0) or preincubated with OHT (5 nmol/L) for 16 h and then treated or not with 0.5 μg/mL doxorubicin (Dox) and 10 μmol/L cisplatin (Cis). Two hours after chemotherapy administration, all cells were placed into a fresh medium and cultured for 24 or 48 h before being harvested for FACS analysis. The percentage of cells with sub-G_1 DNA content is indicated. B, the bar graph depicts the mean apoptotic fractions ± SD of the samples from two independent experiments, one of which is shown in A. C, protein extracts from cells described in A were subjected to Western blot analysis using antibodies against cleaved caspase-3, cleaved PARP1, or actin (used as a loading control). D, U2OS cells stably expressing ER-E2F1 were treated as described in A, collected 6 h after chemotherapy administration, and 10,000 of the cells from each treatment were cultured for an additional 2 wk. Then, all plates were Giemsa stained and the number of colonies was determined. The bar graph depicts the number of colonies ± SD from two independent experiments each made in triplicates. The inner graph is an enlargement of the right part of the bigger graph.

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Additionally, activation of exogenous E2F1 in the presence of the protein synthesis inhibitor cycloheximide resulted in an elevation of *maspin* mRNA levels albeit to a lesser extent (Fig. 2E), suggesting that upregulation of *maspin* by E2F1 is, most probably, a combination of direct and indirect effects.

E2F1 Enhances Chemotherapy-Induced Apoptosis Also in p53-Deficient Cells

E2F1 cooperates with p53 in inducing apoptosis (35) and p53 is activated by DNA damage (36). Therefore, it is possible that the synergy between E2F1 and chemotherapeutic drugs in inducing apoptosis is p53 dependent. To explore this possibility, we compared the apoptotic behavior of a human osteosarcoma cell line that lacks functional p53, SAOS2, with that of U2OS cells, which express wild-type *p53*. As can be seen in Fig. 3A, SAOS2 cells that express conditionally active E2F1 exhibit enhanced cell death when E2F1 activation is combined with doxorubicin and cisplatin administration in a similar manner to the U2OS cells. Similar data were obtained with another *p53*-null cell line, H1299 (data not shown). In addition, introduction of a *p53*-specific shRNA into U2OS cells, which significantly reduced p53 levels, did not affect the synergy between E2F1

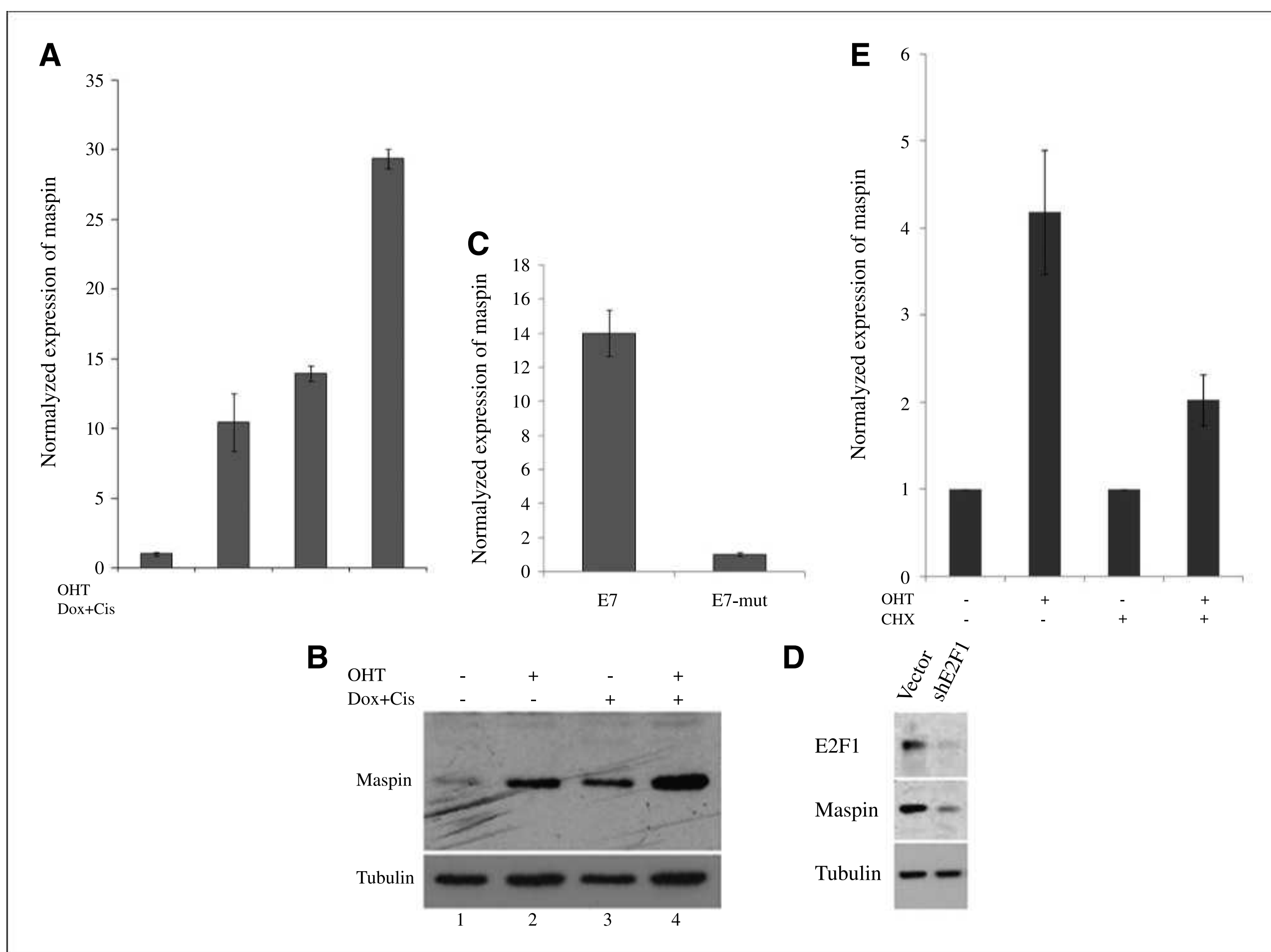

FIGURE 2. The tumor suppressor maspin exhibits enhanced upregulation when E2F1 activation is combined with doxorubicin and cisplatin administration. A, U2OS cells stably expressing ER-E2F1 were treated as in Fig. 1. RNA was extracted and maspin mRNA levels were determined by real-time RT-PCR and normalized to HPRT levels. B, protein extracts from cells described in A were subjected to Western blot analysis using antibodies against Maspin or tubulin (used as a loading control). C, WI38 cells were infected with a retrovirus expressing either wild-type E7 (E7) or a RB-binding deficient mutant of E7 (E7-mut). Total RNA was extracted from cells. Maspin mRNA levels were determined by real-time RT-PCR and normalized to HPRT levels. D, U2OS cells were infected with a control retrovirus (vector) or a retrovirus expressing a shRNA directed against E2F1 (shE2F1). Proteins were extracted from cells and subjected to Western blot analysis using antibodies against Maspin, E2F1, or tubulin. E, U2OS cells stably expressing ER-E2F1 were left untreated (0) or preincubated with OHT (5 nmol/L) for 10 h, then cells were treated or not with 10 μg/mL cycloheximide (CHX). RNA was extracted 16 and 20 h after cycloheximide administration. The bar graph depicts maspin mRNA levels at two time points ± SD as determined by real-time RT-PCR and normalized to HPRT levels.

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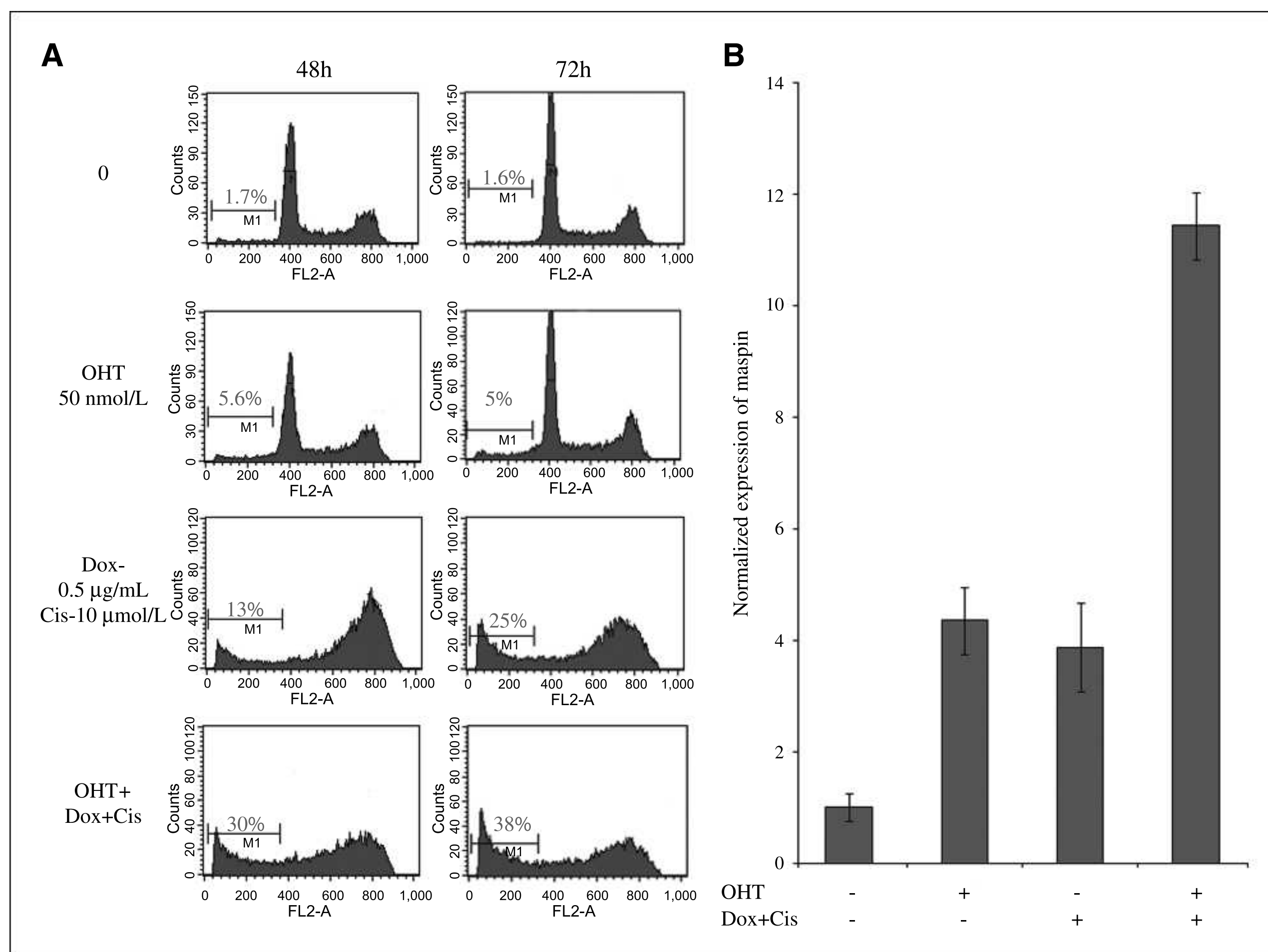

FIGURE 3. The effect of E2F1 on chemotherapy-induced apoptosis is p53 independent. A, SAOS2 cells stably expressing ER-E2F1 were left untreated (0) or preincubated with OHT (50 nmol/L) for 16 h and then treated or not with 0.5 µg/mL doxorubicin (Dox) and 10 µmol/L cisplatin (Cis). Two hours after chemotherapy, all cells were placed into a fresh medium and cultured for 48 or 72 h before being harvested for FACS analysis. The percentage of cells with sub-G_1 DNA content is indicated. B, RNA was extracted from SAOS2 cells treated as described in A, 10 h after chemotherapy administration, and maspin mRNA levels were determined by real-time RT-PCR and normalized to HPRT levels.

and chemotherapeutic drugs in inducing apoptosis (Supplementary Fig. S2). Taken together, these data indicate that the synergy between E2F1 and the chemotherapeutic drugs in inducing apoptosis is p53 independent.

Next, we wished to determine whether the synergy between E2F1 and chemotherapeutic drugs in inducing *maspin* expression is similarly p53 independent. We found that *maspin* RNA levels are elevated in SAOS2 cells after either administration of doxorubicin and cisplatin or activation of E2F1 and, importantly, combining these two treatments leads to enhanced transcriptional upregulation of *maspin* (Fig. 3B). Additionally, in U2OS cells containing a *p53*-specific shRNA, which significantly reduced p53 levels, we detected an increase in *maspin* mRNA and protein levels upon E2F1 activation and an enhanced increase in *maspin* mRNA and protein levels when E2F1 activation is combined with administration of doxorubicin and cisplatin (Supplementary Fig. S2B,C). Notably, upregulation of *maspin* expression is more modest in cells with no p53 or reduced p53 levels, suggesting that p53 contributes to E2F1-induced activation of *maspin*, which is probably a result of both direct and indirect effects.

These data indicate that enhanced *maspin* upregulation as well as enhanced apoptosis, both observed when E2F1 activation is combined with chemotherapy, are, to a large extent, p53-independent phenomena.

Maspin Activity Is Required for E2F1-Induced Chemosensitization

The aforementioned data establish that activation of ectopic E2F1 synergizes with sublethal DNA damage in inducing apoptosis in the human tumor-derived cell lines U2OS and SAOS2. Furthermore, consistent with its reported proapoptotic activity (34), the data reveal *maspin* to be upregulated following E2F1 activation and further

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upregulated when E2F1 activation is combined with administration of chemotherapeutic drugs. Thus, it seems that elevated *maspin* expression correlates with E2F1-induced chemosensitization. To test whether *maspin* is not just associated with but required for this phenomenon, we reduced *maspin* expression in tumor-derived cells and examined their E2F1-mediated sensitivity to chemotherapeutic drugs. *Maspin* expression was inhibited in U2OS cells expressing conditionally active E2F1 by introducing shRNA directed against *maspin*. Significantly reduced Maspin protein levels were confirmed by Western blot analysis (Fig. 4C). As expected, introduction of a control

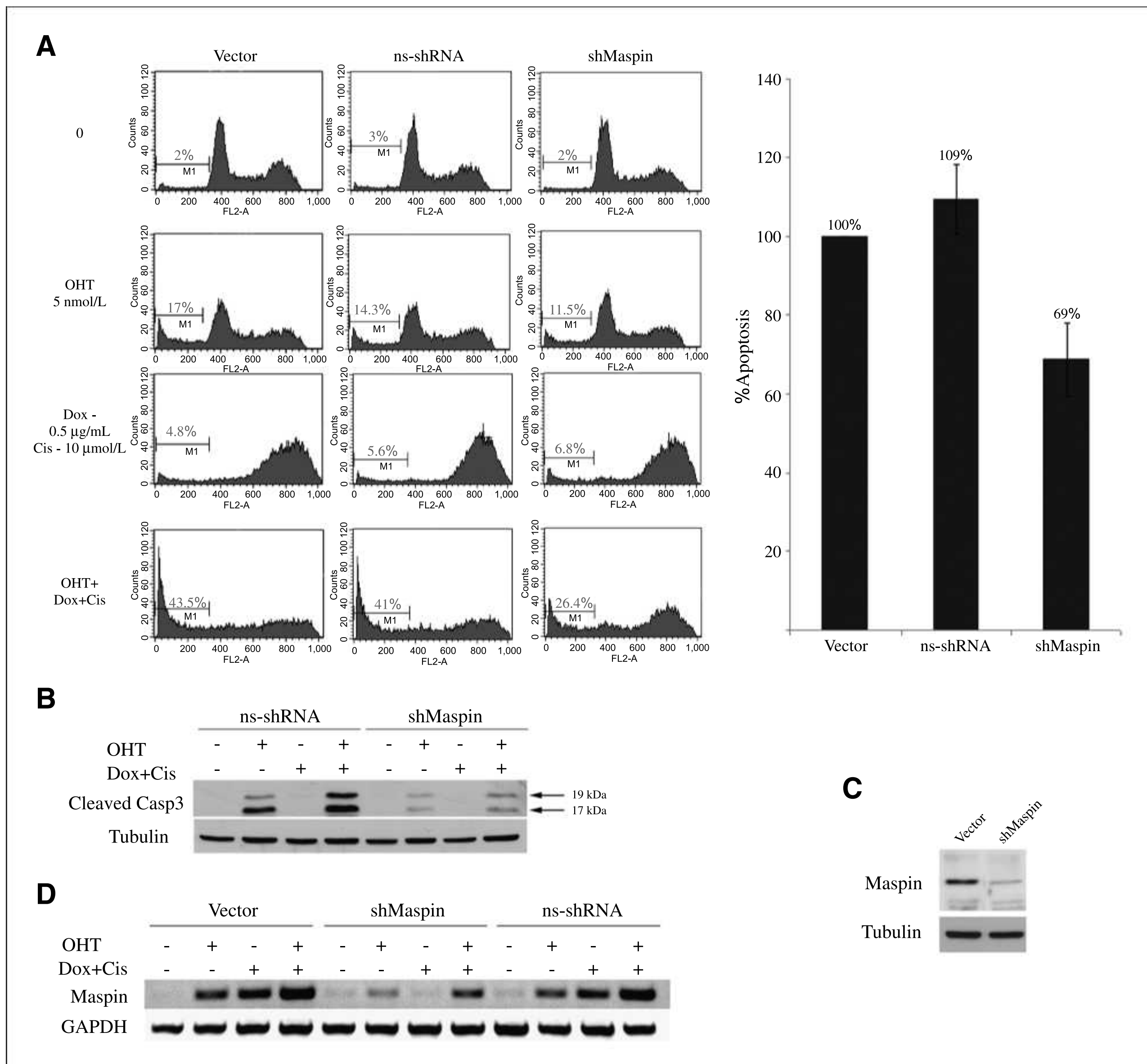

FIGURE 4. Maspin is required for E2F1-induced chemosensitization. A, U2OS cells stably expressing ER-E2F1 were infected with a control retrovirus (vector) or retroviruses expressing either a nonspecific shRNA (ns-shRNA) or a shRNA directed against maspin (shMaspin). Cells were incubated with either OHT or doxorubicin plus cisplatin or the combination of both as described in Fig. 1 and harvested for FACS analysis after 54 h. The percentage of cells with sub-G_1 DNA content is indicated. The bar graph shows the percentage of apoptosis in a given sample relative to the apoptosis in cells infected with the control retrovirus vector, which is depicted as 100%. The average of five independent experiments is presented. B, proteins were extracted from cells infected and treated as described in A and subjected to Western blot analysis using antibodies against cleaved caspase-3 or tubulin (used as a loading control). C, protein extracts from cells infected with a control retrovirus (vector) or retroviruses expressing a shRNA directed against maspin (shMaspin) were subjected to Western blot analysis using antibodies against Maspin or tubulin. D, RNA was extracted from cells treated as in A and subjected to RT-PCR analysis using primers specific for maspin or GAPDH.

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vector or a nonspecific shRNA has no effect on the enhanced levels of apoptosis induced when E2F1 activation is combined with doxorubicin and cisplatin treatment (Fig. 4A). In contrast, introducing a shRNA directed against *maspin* results in low levels of apoptosis in response to E2F1 activation combined with doxorubicin and cisplatin treatment (Fig. 4A). Similar data were obtained in five independent experiments, with apoptosis reduced on average by 30% (Fig. 4A). Moreover, the level of apoptosis after E2F1 activation alone is somewhat reduced by introduction of the shRNA directed against *maspin*, suggesting that Maspin may play a role in E2F1-induced apoptosis also in the absence of chemotherapy (Fig. 4A and B). These reductions in apoptosis levels were detectable both when assaying the percentage of cells with sub-G_1 DNA content (Fig. 4A) and when monitoring the amount of cleaved caspase-3 (Fig. 4B). Comparable results were obtained using a different shRNA directed against *maspin* (Supplementary Fig. S3), indicating that the effects observed after introduction of the two shRNAs directed against *maspin* are unlikely to be due to nonspecific shRNA targets. RT-PCR analysis of RNA extracted from these cells shows that shRNA directed against *maspin* severely impaired *maspin* RNA elevation following E2F1 activation alone, doxorubicin and cisplatin administration alone, and the combination of E2F1 activation with doxorubicin and cisplatin treatment (Fig. 4D). In light of these observations, we conclude that regulation of *maspin* expression plays a role in E2F1-induced sensitivity of transformed human cells to chemotherapeutic drugs.

Discussion

We establish here that ectopic expression of E2F1 sensitizes human osteosarcoma–derived cells to the commonly used chemotherapeutic drugs doxorubicin and cisplatin. We show that this E2F1-induced sensitization is p53 independent as it occurs to a similar extent in both p53-expressing and p53-deficient cells. Also, this E2F1-induced sensitization seems to be p73 independent as it occurs to a similar extent in both p53-expressing and p53-deficient cells in which p73 is inhibited (data not shown). Potentially, a clinically relevant corollary of these findings is that tumors expressing exogenous E2F1 should be sensitive to reduced concentrations of chemotherapeutic drugs. Activity of exogenous E2F1 was previously shown to cooperate with DNA-damaging agents in inducing apoptosis (25, 27, 29, 37, 38). However, the molecular mechanism (s) underlying this phenomenon were not fully elucidated. In this study, we show that E2F1 and the chemotherapeutic drugs cisplatin and doxorubicin converge on regulating expression of *maspin*. Indeed, downregulating *maspin* expression inhibits the cell death induced by E2F1 activation plus chemotherapy. Considering the proapoptotic function of Maspin, we suggest that Maspin mediates, at least in part, E2F1-induced chemosensitization. Of note, because inhibiting *maspin* expression does not fully abolish cell death induced by E2F1 activation plus chemotherapy, it seems likely that there are additional mediators of E2F1-induced chemosensitization.

While investigating the mechanism underlying E2F1-induced chemosensitization, we identified a novel E2F-upregulated gene, namely the proapoptotic tumor suppressor *maspin*. Our results that support this statement include the following: ectopic expression of E2F1 is associated with elevated *maspin* expression, E7-mediated deregulation of the endogenous RB/E2F pathway results in elevated *maspin* expression, and introducing a shRNA directed against E2F1 causes reduced *maspin* expression. The mitogen-activated protein kinase p38 was recently shown to influence positively *maspin* expression, most probably through activator protein 1 (39), and we have previously shown that E2F1 can activate p38 (40). However, whereas inhibition of the mitogen-activated protein kinase p38 reduces the basal levels of *maspin*, it does not affect E2F1-induced upregulation of *maspin* (Supplementary Fig. S4), indicating that p38 does not play a significant role in this upregulation. Also, *maspin* expression is regulated by the tumor suppressor p53 (41) and E2F1 can activate p53 through a number of pathways (2). Therefore, it is possible that E2F1-induced upregulation of *maspin* also occurs indirectly through p53. We found that upregulation of *maspin* expression by E2F1 happens also in p53-deficient cells, albeit to a lesser extent. Taking all these data into consideration, we suggest that *maspin* can be regulated both directly and indirectly by E2F1.

The tumor suppressor Maspin is a nonclassic serine protease inhibitor (serpine) that is downregulated or silenced in human tumors mainly through promoter hypermethylation (42-44). Maspin inhibits tumor growth, metastasis, and angiogenesis *in vivo* as well as tumor invasion *in vitro* (43, 45, 46). Moreover, Maspin is functionally linked to apoptosis in breast and prostate cancer (46, 47). Maspin protein is localized in the cytoplasm, cell membrane, and extracellular matrix; however, its effect on tumor cell apoptosis seems to stem from the cytoplasmic fraction (47) and is mediated, at least in part, by Bax (48). Elevated expression of *maspin* by itself does not induce apoptosis in tissue culture cells (47); however, *maspin* expression was shown to sensitize the apoptotic response of tumor cells to various drugs (39, 47-50). Notably, recent studies show that high expression of *maspin* is correlated with response to chemotherapy in a number of human primary tumors, including ovarian, colon, and head and neck carcinomas (51-53). In this study, for the first time, we characterize an E2F1/maspin link and show that *maspin* is a key mediator of E2F1-induced chemosensitization.

In conclusion, our study identifies the tumor suppressor *maspin* as a novel E2F1-regulated gene and suggests that it plays a role in E2F1-induced apoptosis and in particular in the cooperation between E2F1 and chemotherapy in inducing apoptosis. In addition, our results suggest that gene therapy involving ectopic expression of E2F1, and maybe also *maspin*, could dramatically improve the efficacy of anticancer chemotherapeutic drugs.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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