Eps8 decreases chemosensitivity and affects survival of cervical cancer patients

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Abstract

The oncoprotein Eps8 facilitates proliferation in fibroblasts and colon cancer cells. However, its role in human cervical cancer is unclear. By immunohistochemical staining and Western blotting, aberrant Eps8 expression was observed in cervical carcinoma compared with normal cervical epithelial cells. Clinicopathologic analysis of 45 patients indicated that Eps8 expression was associated with parametrium invasion and lymph node metastasis, two major poor prognostic factors for early-stage cervical cancer. Kaplan-Meier analysis of cervical cancer specimens also indicated an inverse relationship between the level of Eps8 and the patients' survival rate. Using small interfering RNA of *eps8*, we observed reduced proliferation and tumorigenesis in Eps8-attenuated HeLa and SiHa cells cultured in dishes or inoculated in mice. Furthermore, diminished Eps8 impeded G_1-phase progression in HeLa and SiHa cells that might be attributable to reduced expression of cyclins D1, D3, and E, elevated accumulation of p53 and its downstream target $p21^{Waf1/Cip1}$, and suppressed hyperphosphorylation of retinoblastoma. Alteration of these cell cycle–related proteins could be reversed by ectopic Eps8, implicating that the effect of Eps8 on the mentioned cell cycle modulators was specific. Notably, the augmented expression of p53 by diminished Eps8 was at least due to its decreased turnover rate. Concurrent with p53 up-regulation and the decrement of Src and AKT activity, Eps8-attenuated HeLa and SiHa cells exhibited increased chemosensitivity to cisplatin and paclitaxel. Together, our findings implicate the involvement of Eps8 in chemoresistance and show its importance in prognosis of cervical cancer patients. [Mol Cancer Ther 2008;7(6):1376–85]

Received 12/11/07; revised 4/14/08; accepted 4/15/08.

Grant support: China Medical University grant CMU95-306 (M-C. Maa) and National Health Research Institute grants NHRI-EX-96-9517BI and NHRI-EX-97-9517BI (T-H. Leu).

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doi:10.1158/1535-7163.MCT-07-2388

Introduction

Cervical cancer is the second most common malignancy among women (1). It accounts for ~12% of all cancers in women. Each year, there are ~500,000 diagnoses of cervical cancer and ~200,000 deaths because of that (2, 3). Cervical carcinoma evolves slowly from cervical intraepithelial neoplasias to invasive carcinomas. More than 90% of cervical cancers are associated with human papillomavirus (HPV) infection (4). Therefore, HPV is deemed as the primary risk factor in cervical cancer development. A series of epidemiologic and laboratory-based studies reveal that only the high-risk HPV, including types 16, 18, 31, 33, 45, and 51, are associated with cervical intraepithelial neoplasia (5, 6), which may lead to cervical carcinoma (7). Remarkably, HPV16 and HPV18 are detected in >65% of all HPV DNA-positive cervical cancer (8, 9). This strong association between HPV and cervical cancer applies to both squamous cell carcinoma and adenocarcinoma. To date, E6 and E7 proteins encoded by high-risk HPV are key players in HPV-mediated carcinogenesis (7). However, they are necessary but not sufficient for cervical cancer formation (10, 11). Therefore, further understanding the oncogenesis related to HPV infection may provide the opportunity for better prevention and treatment of cervical carcinoma.

Retinoblastoma (Rb) and p53 are tumor suppressors guarding cell cycle progression, especially in G_1- to S-phase transition. Rb is differentially phosphorylated by cyclin-dependent kinases (cdk) in G_1 phase (12, 13). In the presence of growth factor stimulation, quiescent cells reenter the cell cycle and Rb is hypophosphorylated by cyclin D-cdk4/6 complexes in early G_1 phase (12). It is the hypophosphorylated Rb that associates with E2F transcription factors (E2F) and represses the transcription of their target genes required for DNA replication and the progression of cells from G_1 to S phase (12, 14). When cyclin E-cdk2 further phosphorylates Rb into the hyperphosphorylayted form in late G_1 phase (12), E2F are released and turn on their downstream target genes. As a pivotal transcription factor for cell cycle arrest and apoptosis, the expression level of p53 can be modulated by Mdm2-mediated ubiquitination and proteosome degradation in normal cells (15, 16). In response to environmental hazards like UV irradiation and genotoxicants, DNA damage–responsive kinases stabilize p53, leading to gene expression of $p21^{Waf1/Cip1}$ (17), an inhibitor of the cdk in G_1

(cdk2, cdk4, and cdk6) and G_2 (cdc2) phases, which ultimately results in G_1 arrest (18, 19). Extensive DNA damage may further activate p53-dependent pathways, causing cell apoptosis through intrinsic and extrinsic mechanisms (20, 21). In cervical cancer cells, p53 (22, 23) and Rb (24) are documented to interact with E6 and E7, respectively, which results in their degradation and abnormal cell proliferation.

Eps8 is a substrate of both receptor (25) and nonreceptor (26) tyrosine kinases. Its expression is elevated by v-Src, which is critical in v-Src-mediated transformation (27). Recently, we confirm that Eps8 can mediate the activation of Src and participates in colon cancer formation and progression (28). Because stabilization and activation of Src family kinases has been speculated to contribute to E6-mediated cell proliferation (29), the implication of Eps8 in the carcinogenesis of cervical cancer is attempted. In this study, we observed that, compared with neighboring normal cervical epithelia, Eps8 was increased in cervical carcinoma. Studies of control and *eps8* small interfering RNA (siRNA)–expressing cells derived from HeLa (HPV18 DNA-positive; ref. 30) and SiHa (HPV16 DNA-positive; ref. 30) cervical caner cells indicated that Eps8 played a critical role in cell proliferation in culture and tumor formation in mice. By virtue of (*a*) down-regulation of cyclins D1, D3, and E, (*b*) elevation of p53 and $p21^{Waf1/Cip1}$, and (*c*) decrease of hyperphosphorylated Rb, diminished Eps8 could effectively arrest cell cycle at G_1 phase. Consistently, ectopically expressed murine Eps8 could reverse these changes in Eps8-attenuated cells. Besides, via up-regulation of p53 and reduced activation of Src and AKT, diminished Eps8 also sensitized both HeLa and SiHa cells to chemotherapeutic agents, such as cisplatin and paclitaxel. Further statistical analyses indicated that early-stage cervical cancer patients with higher Eps8 expression tended to have parametrium invasion, lymph node metastasis, and decreased survival rate. Thus, a pathologic role of Eps8 in cervical cancer progression was confirmed.

Materials and Methods

Cells and Lysate Preparation

Human cervical carcinoma cells (HeLa and SiHa) were maintained in DMEM with 10% fetal bovine serum and penicillin-streptomycin. Cultured cells were lysed in modified radioimmunoprecipitation assay buffer as described previously (31). The cell debris was removed after centrifugation at 10,000 rpm for 10 min and protein concentration in the supernatant was determined by protein assay kit (Bio-Rad).

Antibodies and Immunoblotting

The following antibodies were used: cyclin E, HPV 16 E6, HPV 18 E6, HPV 16 E7, HPV 18 E7, Eps8, and actin (Santa Cruz Biotechnology); Eps8 (Transduction Laboratories), Src (GD11), Pi-Tyr^{416} Src (Upstate); p53 (Calbiochem); and $p21^{Waf1/Cip1}$, Pi-Ser^{473} AKT, AKT, cyclin D1, cyclin D3, Rb Pi-Ser^{807}/Ser^{811} (Cell Signaling Technology). Western immunoblots were done with respective antibodies and visualized by enhanced chemiluminescence (Amersham) as described previously (31).

Surgical Specimens of Cervical Cancer and Immunohistochemistry

From January 1999 to September 2001, 110 consecutive patients with carcinoma of uterine cervix were scheduled for radical hysterectomy and pelvic lymphadenectomy at National Cheng Kung University Hospital (Taiwan). The clinical staging and histologic classification was based on the criteria of the International Federation of Gynecology and Obstetrics classification and the WHO classification, respectively. Patients who had undergone conization of the cervix before radical hysterectomy and who had unusual histopathology such as clear cell, adenosquamous carcinoma, and small cell carcinoma were excluded from this study. Immunostaining was done with anti-Eps8 antibody at 4°C overnight. The LASB 2 kit (DakoCytomation) was used to detect the resulting immune complex and the activity was visualized using aminoethyl carbazole as a substrate. Finally, sections were counterstained with hematoxylin and mounted with Glycergel mounting medium (DakoCytomation). At least five sections from each patient's specimen were analyzed for histology and Eps8 expression. Each section was examined blindly by two investigators trained in gynecologic pathology. Eps8 staining was evaluated manually or by image analysis with a CoolSnap-Pro color digital camera (Roper Scientific) and Image-Pro Plus 4.1 software (Media Cybernetics) over 15 to 20 high-power fields. The distribution of immunohistochemical stains of Eps8 in each specimen was graded as low or high according to its presence was in <50% or >50% of tumor area. To analyze the clinical outcome by Eps8 differential expression, the survival data of low- and high-grade patients (excluding the other causes of death) were assessed by Kaplan–Meier methods and differences were compared by the log-rank statistic. Differences between values were considered significantly when $P < 0.05$.

Cell Line Generation

The construction of *eps8* siRNA expression construct, pS-hEps8 (5′-GATCCCGCTGTGATGCATTCATGCATTCAA-GAGATGCATGAATGCATCACAGCTTTTTTGGAAA-3′), was described previously (28). To generate HeLa and SiHa cells stably expressing *eps8* siRNA or the nonspecific negative control, DNA of pS-hEps8 or the negative control pSilencer (it contains sequences not present in human genome and is provided by Ambion) was individually transfected into these cells using LipofectAMINE Plus method followed by hygromycin (400 μg/mL) selection for ~2 weeks (28). To generate cells expressing Myc-Eps8, HeLa cells expressing *eps8* siRNA were transfected with pCMV-Myc-Eps8 (32) followed by G 418 selection.

Cell Proliferation Assay

Cells stably expressing *eps8* siRNA or the nonspecific control and their respective HeLa and SiHa parental cells were plated at a density of 2×10^5 per 60-mm dish in the beginning. After 1, 2, and 3 days, total number of cells was counted using a hemocytometer and plotted.

Chemotherapy Compounds and Drug Sensitivity Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay]

Cisplatin and paclitaxel were purchased from Calbiochem and dissolved in DMSO to 100 and 5 mmol/L, respectively. Cells (2×10^3) were seeded onto 96-well culture plates overnight and treated with various concentration of either cisplatin or paclitaxel for 24 and 48 h. The cell viability was assayed by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL; Sigma) containing serum-free medium and incubating the cells for 3 h at 37°C in a CO_2 incubator followed by cell lysis with 100 μL DMSO/well. Percentage of cell viability was determined by measuring the absorbance at 595 nm and normalizing the value with a corresponding untreated control. All experiments were done in quadruplicate for at least three times.

Flow Cytometry

For cell cycle analysis, unsynchronized cells were cultured in 10% FBS for 1 or 2 days and then harvested, fixed in 95% ice-cold ethanol, and stored at -20°C overnight. After being washed with PBS, cells were stained for DNA in a solution containing 0.5 mL RNase (Sigma-Aldrich; 1 mg/mL) and 0.5 mL propidium iodide (500 μg/mL) for 30 min in the dark followed by flow cytometric analysis (Becton Dickinson FACScan system). The fraction of cells present in each cell cycle phase (G_1, S, and G_2-M) was determined by WinMDI software (Scripps Research Institute) of DNA histograms. Apoptotic cells appear in the sub-G_1 fraction.

Tumorigenicity in Severe Combined Immunodeficient Mice

Cells ($\sim 1 \times 10^7$) in 0.1 mL sterile PBS were injected s.c. into the hip of 4- to 5-week old female severe combined immunodeficient mice (NOD/LtSz-Prkdc<scid>/J). One week later, mice were checked every 2 or 3 days and the tumor(s) formed was measured with a micrometer as described previously (32). After ~5 or 9 weeks, tumors were excised and weighed. The mouse experiments were done according to the ethical guidelines for laboratory animal use and approved by the Institutional Animal Study Committee.

Statistical Analysis

Unless indicated, all values were expressed as mean ± SD of triplicate experiments. The significance of difference was evaluated by Kruskal-Wallis one-way ANOVA on ranks followed by Student's-Newman-Keuls' pairwise multiple comparison tests. Differences were considered significant if $P < 0.05$.

Results

Eps8 Expression in Tumor Tissues Is Associated with Clinical Outcome

To investigate the pathologic characteristics of Eps8 in cervical carcinoma, we examined the expression level of Eps8 in surgical specimens of early-stage cervical cancer (stage Ib-IIa) by immunohistochemical staining. As shown in Fig. 1A, Eps8 protein was scanty in normal or noncancerous cervical epithelial tissues of all surgical specimens examined ($n = 45$). In contrast, cervical cancer tissues clearly expressed Eps8 protein with different amounts. We grouped the patients by Eps8 grading and analyzed the survival data accordingly. Group 1 had its Eps8 staining in <50% of tumor area (low grade of Eps8 expression), whereas group 2 had its Eps8 staining in >50% of tumor area (high grade of Eps8 expression; Fig. 1B). When compared with group 1, group 2 exhibited a significantly higher percentage of parametrium invasion and pelvic lymph node metastasis, which are two major poor prognostic factors for early-stage cervical cancer (Table 1). Consistently, increased Eps8 expression was associated with the poor disease-free survival (DFS) and overall survival (OS; Fig. 1C). The 44-month DFS and OS of the patients ($n = 45$) whose surgical specimens were studied were 79% and 81%, respectively. The 44-month DFS and OS of group 1 were 94% and 95%, respectively. In striking contrast, the 44-month DFS and OS of group 2 were significantly decreased to 66% and 70%, respectively.

Eps8 Attenuation Results in Decreased Cell Proliferation of HeLa and SiHa Cells Both *In vitro* and *In vivo*

HeLa and SiHa are two widely studied human cervical carcinoma cell lines. Compared with the primary culture cells of normal cervical epithelia derived from three patients, significantly augmented expression of Eps8 was observed in both HeLa and SiHa cells (Fig. 2A). To study the role of Eps8 in cervical cancer, nonspecific and *eps8*-specific siRNA-bearing plasmids were introduced into HeLa and SiHa cells to obtain the corresponding control and siRNA cells, respectively. As shown in Fig. 2B, Eps8 attenuation resulted in a significantly reduced proliferation of both HeLa and SiHa cells. The prominent inhibitory effect of reduced Eps8 expression on cervical cancer cell proliferation *in vitro* suggested that Eps8 might promote tumor growth *in vivo*. To substantiate this hypothesis, female severe combined immunodeficient mice were s.c. inoculated with Eps8-attenuated HeLa or SiHa cells and their respective parental and control cells. As shown in Fig. 2C, all these mice grew s.c. tumors. However, mice inoculated with Eps8 knockdown cells developed smaller tumors than those inoculated with parental or control HeLa and SiHa cells.

Involvement of Eps8 in the Cell Cycle Regulation of HeLa and SiHa Cells

By flow cytometric analysis, Eps8 knockdown slowed down G_1-phase progression and reduced the cell population at S and G_2-M phases (Fig. 3). For 24-h cultured HeLa and SiHa cells, Eps8 attenuation increased the proportion of G_1 cells from 53% to 67% and from 62% to 68%, respectively. Similar phenomenon was also observed in 48-h cultured HeLa, SiHa, and their derived cells. It is well documented that accelerated proliferation of cervical cancer cells can be attributed to E6- and E7-mediated inactivation of p53 and Rb, respectively. To elucidate the mechanisms responsible for G_1 arrest triggered by diminished Eps8, a variety of well-known cell cycle modulators

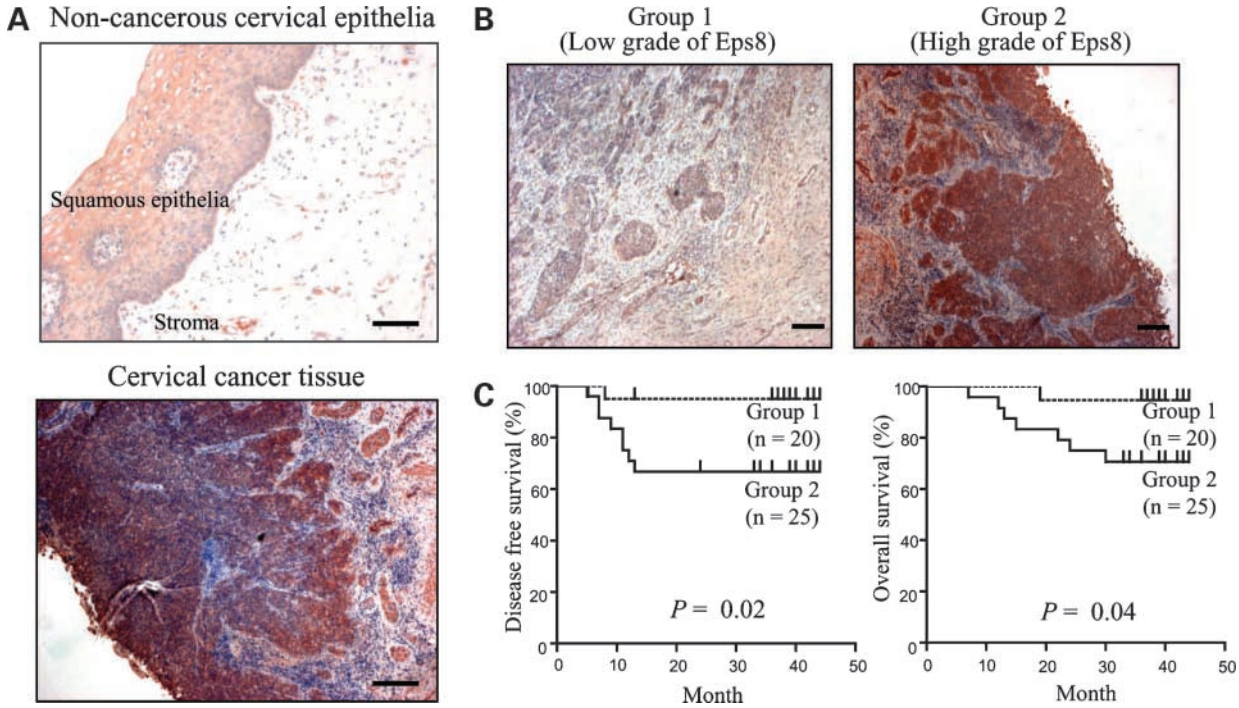

Figure 1. Increased Eps8 expression in cervical carcinoma is associated with poor prognosis of cervical cancer patients. **A,** patterns of Eps8 expression in surgical specimens of cervical cancer. Eps8 expression was obvious in the cervical cancer tissues (*bottom*). In contrast, Eps8 protein was nearly undetectable in the adjacent normal or noncancerous cervical tissues (*top*). Bar, 30 μm. **B,** representative pictures of cervical cancer samples with different Eps8 abundance. As described in Materials and Methods, group 1 (*left*) indicates that the distribution of Eps8 staining is <50% of tumor area, whereas group 2 (*right*) indicates that the distribution of Eps8 staining is >50% of tumor area. Bar, 30 μm. **C,** Eps8 expression is inversely correlated with DFS and OS of early-stage cervical cancer patients (Ib-IIa). Kaplan-Meier analyses of DFS (*left*) and OS (*right*) of patients in group 1 ($n = 20$) and group 2 ($n = 25$) within 44 mo were indicated.

in HeLa, SiHa, and their derived cells were examined. Whereas the levels of E6 and E7 were unaltered, augmented expression of p53 and $p21^{Waf1/Cip1}$ was observed in HeLa- and SiHa-based siRNA cells compared with their parental and control cells (Fig. 4). The increased p53 expression in Eps8-attenuated HeLa cells was at least due to its decreased turnover rate (half-life was from ~12 to ~40 min), which could be reverted by ectopic Eps8 (Fig. 5C). Meanwhile, Eps8 knockdown led to reduction of cyclins D1, D3, and E, which might further cause decreased Rb Pi-Ser^{807}/Ser^{811} (the specific target of cyclin D1–dependent cdk4 complex; ref. 12) and Rb hyperphosphorylation (Fig. 4). Remarkably, when the level of Eps8 was partially restored in HeLa-based siRNA-1 and siRNA-2 cells, a significant recovery of cyclins D1/D3/E, Rb Pi-Ser^{807}/Ser^{811}, and hyperphosphorylated Rb as well as decreased expression of p53 and $p21^{Waf1/Cip1}$ were observed (Fig. 5A). Given that Eps8 could augment the activity of Src and AKT (proteins that are important in cell proliferation and survival) in colon cancer cells (28);

Table 1. Clinical characteristics of cervical cancer patients grouped by the differential Eps8 staining

Characteristics	Group 1 ($n = 20$)	Group 2 ($n = 25$)	P
Age (y), mean (range)	46 (33-61)	45 (32-63)	0.95
International Federation of Gynecology and Obstetrics staging, *n* (%)			
Ib	19 (95)	22 (88)	0.62
IIa	1 (5)	3 (12)	
Histology, *n* (%)			
Squamous cell carcinoma	16 (80)	18 (72)	0.73
Adenocarcinoma	4 (20)	7 (28)	
Parametrium invasion, *n* (%)	1 (5)	8 (32)	<0.05
Lymph node metastasis, *n* (%)	2 (10)	11 (44)	<0.05

NOTE: Group 1 indicates that the distribution of Eps8 staining is <50% of tumor area, whereas group 2 indicates that the distribution of Eps8 staining is >50% of tumor area.

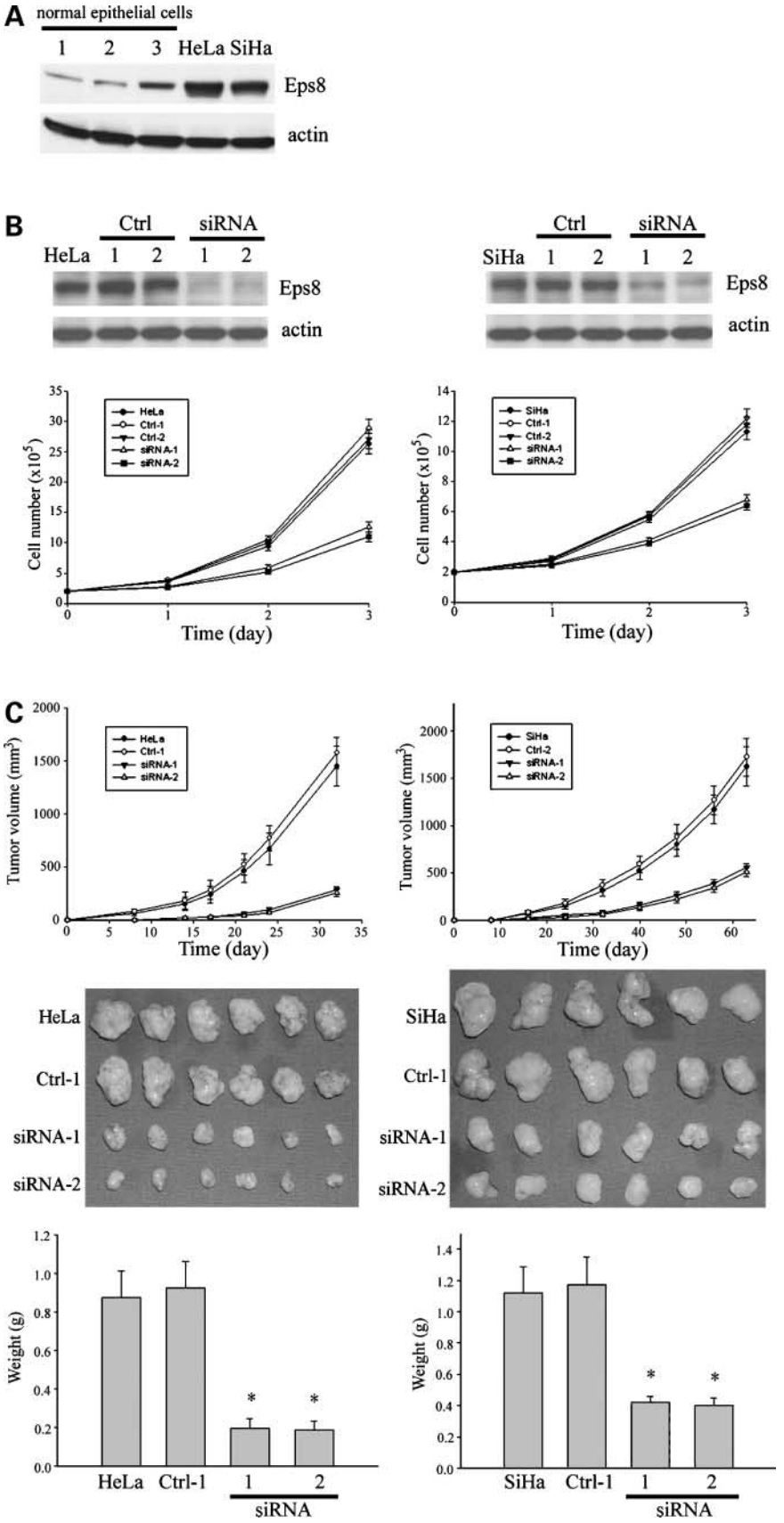

Figure 2. Eps8 attenuation resulted in decreased cell proliferation of HeLa and SiHa cells both *in vitro* and *in vivo*. **A,** overexpression of Eps8 in HeLa and SiHa cervical carcinoma cells. Equal amount (100 μg) of lysates prepared from HeLa, SiHa, and primary cultured cells derived from three normal-appearing epithelia were resolved by SDS-PAGE and immunoblotted with antibodies as indicated. **B,** Eps8 expression affects cell proliferation of HeLa and SiHa cells. Equal amount (100 μg) of lysates prepared from HeLa, SiHa, and their derived control cells (Ctrl-1 and Ctrl-2) or *eps8* siRNA-expressing cells (siRNA-1 and siRNA-2) were resolved in SDS-PAGE and immunoblotted with antibodies as indicated (*top*). For cell proliferation, indicated cells were plated at a density of 2×10^5 per 60-mm dish at the beginning and then were counted by hemocytometer after 1-, 2-, or 3-day culture and plotted (*bottom*). Similar results were obtained at least twice. **C,** equal number (1×10^7) of cells of HeLa (*left*), SiHa (*right*), and their derived cells were injected s.c. into the hip of severe combined immunodeficient mice ($n = 6$) as described in Materials and Methods. The tumor volume was measured every week since the seventh day of postinjection and was plotted (*top*). After 32 or 63 days, HeLa- and SiHa-derived tumors were excised, photographed (*middle*), weighted, and plotted (*bottom*). The weights of the tumors excised are mean ± SD in two independent experiments. *, $P < 0.05$.

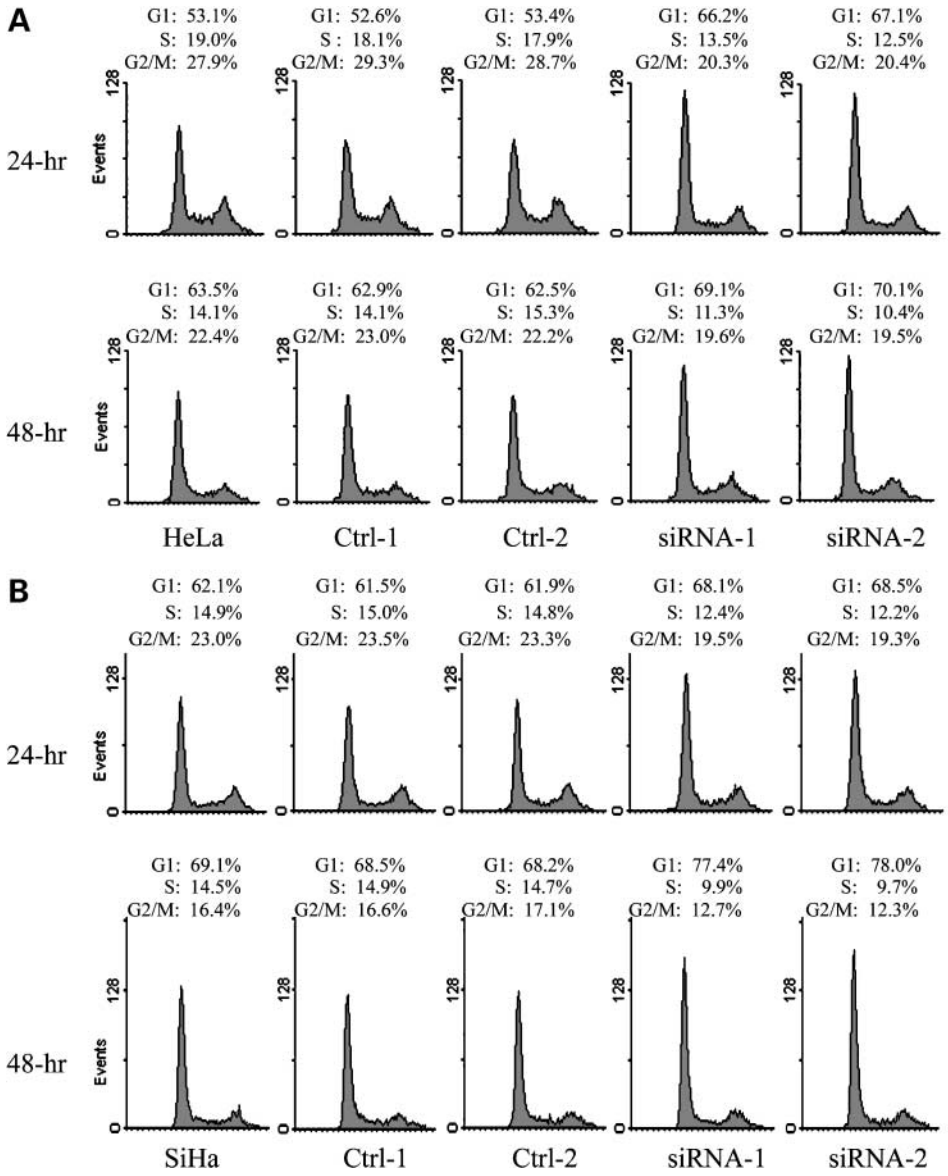

Figure 3. Eps8 attenuation impeded G_1-phase cell cycle progression of cervical cancer cells. HeLa (**A**), SiHa (**B**), and their derived cells were cultured for 24 h (*top*) or 48 h (*bottom*) and trypsinized for flow cytometric analysis as described in Materials and Methods. Notably, no obvious sub-G_1 population was detected in Eps8-attenuated HeLa or SiHa cells. Representative of three independent experiments with similar results.

therefore, its effect on these two kinases in HeLa cells was examined. As shown in Fig. 5A, the decrement of Src and AKT activity as reflected by Src Pi-Tyr^{416} and AKT Pi-Ser^{473}, respectively, were observed in response to Eps8 knockdown. Ectopic Eps8 could recover Src and AKT activity (Fig. 5A). Concurrent with these results, ectopic Eps8 also alleviated the suppressed proliferation in siRNA cells (Fig. 5B). These findings suggested that Eps8 could accelerate cellular growth through activation of Src and AKT as well as inactivation of both p53- and Rb-mediated pathways in cervical cancer cells.

Eps8 Attenuation Sensitizes HeLa and SiHa Cells to the Cytotoxicity of Cisplatin and Paclitaxel

Finally, we focused on the implication of Eps8 in chemosensitivity of HeLa and SiHa cells. Cisplatin and paclitaxel, which individually target DNA and microtubule, represent two different classes of chemotherapeutic agents. The IC_{50} of cisplatin-treated HeLa and SiHa cells were 36 and 80 μmol/L, respectively, in 24-h treatment (Fig. 6A), whereas it was decreased to 15 and 45 μmol/L, respectively, in 48-h treatment (Supplementary Fig. S1A).[4] Intriguingly, 2-fold reduction of IC_{50} was detected in HeLa- and SiHa-derived siRNA cells compared with their respective control cells (Fig. 6A; Supplementary Fig. S1A).[4] Restoration of Eps8 enabled siRNA cells more resistant to the toxicity of cisplatin in HeLa cells (Fig. 6A; Supplementary Fig. S1A).[4] At ~100 nmol/L, paclitaxel reached its maximal toxic effect (near 40% reduction of cell number) in

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

the 24-h treated HeLa and SiHa cells (Fig. 6B); longer incubation (48 h) did not seem to deteriorate the viability of these two cells further (Supplementary Fig. S1A).[4] However, Eps8-attenuated HeLa and SiHa cells not only became more sensitive to paclitaxel but also the inhibitory effect was time dependent (Fig. 6B; Supplementary Fig. S1A).[4] Again, ectopically expressed Eps8 reversed the sensitivity and maximal effect of paclitaxel to HeLa cells (Fig. 6B; Supplementary Fig. S1A).[4] Analysis of the cisplatin-mediated cytotoxicity, a significant increase of apoptosis (appeared in the sub-G_1 population) in Eps8-diminished HeLa (siRNA-1) or SiHa (siRNA-1 and siRNA-2) cells was observed and this cisplatin-induced cell death could be reversed by ectopic Eps8 in HeLa cells (Fig. 6C). Similar results were observed in another pair of HeLa-based siRNA and siRNA/Eps8 cells (Supplementary Fig. S1B).[4] Thus, in the circumstances of Eps8 attenuation, cisplatin and paclitaxel might exert better toxicity in cervical cancer cells.

Discussion

The possible involvement of Eps8 in cervical cancer was assessed in this study. We observed elevated expression of Eps8 in cervical carcinoma (Fig. 1A) as well as in human cervical cancer cell lines, HeLa and SiHa (Fig. 2A). Attenuation of Eps8 in HeLa and SiHa cells not only reduced their proliferation *in vitro* (Fig. 2B) but also impaired their tumorigenesis *in vivo* (Fig. 2C). This Eps8 attenuation-mediated growth inhibition could be attributable to the blockade of the cycling cells at G_1 stage (Fig. 3), which could be explained, at least in part, by increased levels of p53 and $p21^{Waf1/Cip1}$ and by decreased expression of cyclins D1, D3, and E manifested by the suppression of Rb hyperphosphorylation (Fig. 4). Reintroduced siRNA-resistant *eps8* could reverse these events and restore cell proliferation (Fig. 5). In addition, Eps8 also enabled HeLa and SiHa cells to be resistant to chemotherapeutic agents (Fig. 6). However, because the level of ectopic Eps8 did not approach that of endogenous in nonattenuated cells, only partial rescue effects were observed in proliferation and drug sensitivity assays. It was noteworthy that the expression level of Eps8 affected the prognosis of cervical cancer patients and an inverse correlation between augmented Eps8 and either DFS or OS was revealed (Fig. 1B and C). Furthermore, cervical cancer cells with high Eps8 expression tended to metastasize into parametrium and local lymph node (Table 1). With these findings, we confirm the participation of Eps8 in HPV-associated cervical cancer.

Unlike other inherited cancers, the mutations in *Rb* and *p53* genes that led to their inactivation and decreased expression were rarely reported in cervical cancer (33). Instead, down-regulation of p53 and Rb mediated by E6 and E7, respectively, in HPV-infected cells was well documented. Whereas p53 could associate with E6 and E6AP (an E3 ubiquitin ligase) and destined for degradation (34), Rb was also reported to undergo proteasome degradation via interaction with E7 (35). Eps8 attenuation in HeLa and SiHa cells in this study resulted in augmented expression of p53 but not Rb (Fig. 4). Interestingly, neither the amount of E6 (Fig. 4) nor the level of *p53* mRNA (data not shown) was altered in all HeLa- and SiHa-derived control and siRNA cells. Thus, it was plausible that, through the pathway mediated by the triple complex comprised of E6, p53, and E6AP, Eps8 facilitated the degradation of p53 and diminished Eps8 blocked this process and caused p53 accumulation. Indeed, the significantly increased half-life of p53 in HeLa cells in response to Eps8 attenuation supported this hypothesis (Fig. 5C).

As important transcription factors dedicated to G_1-S progression, E2F act as transactivators controlled by the phosphorylation status of its binding partner, Rb, whose

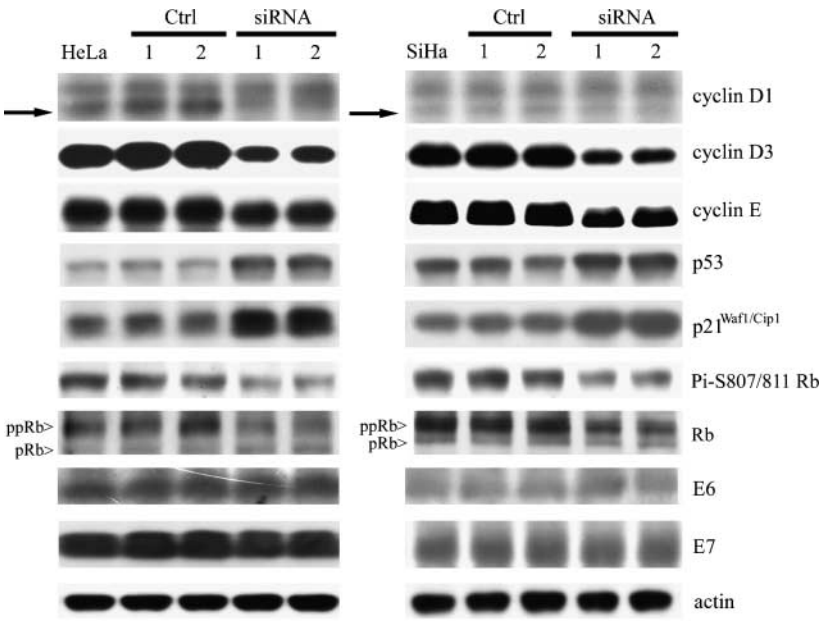

Figure 4. Eps8 attenuation increased the expression of p53 and $p21^{Waf1/Cip1}$ but decreased the expression of cyclins D1, D3, and E and the hyperphosphorylation of Rb. Lysates (100 μg) prepared from HeLa (*left*), SiHa (*right*), and their derived cells (Ctrl-1, Ctrl-2, siRNA-1, and siRNA-2) were resolved by SDS-PAGE and immunoblotted with antibodies as indicated. *Arrows,* cyclin D1. *ppRb,* hyperphosphorylated Rb; *pRb,* hypophosphorylated Rb.

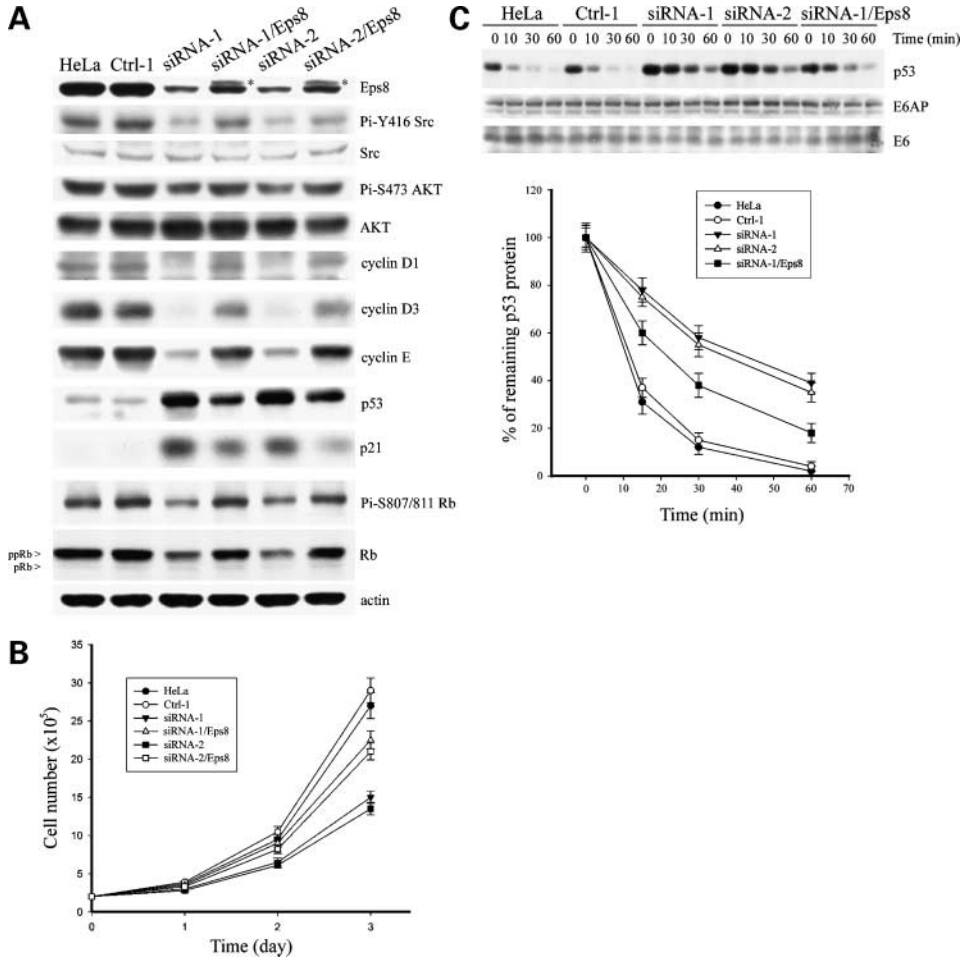

Figure 5. Ectopically expressed Eps8 decreased the expression of p53 and $p21^{Waf1/Cip1}$ but increased the activity of Src and AKT as well as the expression of cyclins D1, D3, and E and the hyperphosphorylation of Rb, leading to increased cell proliferation. **A,** lysates (100 μg) prepared from HeLa, SiHa, and their derived cells (Ctrl-1, siRNA-1, siRNA-1/Eps8, siRNA-2, and siRNA-2/Eps8) were resolved by SDS-PAGE and immunoblotted with antibodies as indicated. *Asterisks,* ectopically expressed Myc-Eps8. **B,** for cell proliferation, similar experiments were done as described in Fig. 2B. Cells were counted by hemocytometer after 1-, 2-, or 3-day culture and plotted. **C,** decreased turnover rate of p53 in Eps8-attenuated HeLa cells. One-day cultured HeLa and its derived cells were treated with cycloheximide (20 μg/mL) for various times as indicated. Lysates (100 μg) from each sample were resolved by SDS-PAGE and immunoblotted with antibodies as indicated (*top*). Densitometric quantification of p53 of three separate experiments was plotted as a percentage of p53 remaining as a function of time after cycloheximide treatment (*bottom*).

phosphorylation is mediated by G_1-specific cdk. Only hypophosphorylated Rb can prevent E2F from activating their target genes and inhibit cell proliferation. In Eps8-attenuated HeLa and SiHa cells, we observed decreased expression of cyclins D1, D3, and E, which are required for the activation of G_1-specific cdk. Consistently, Rb Pi-Ser^{807}/Ser^{811} and hyperphosphorylated Rb were greatly reduced (Fig. 4). However, the failure to detect the accordingly augmented hypophosphorylated Rb implicated that the degradation of Rb still occurred in *eps8* siRNA cells. In other words, Eps8 does not play a role in E7-mediated Rb degradation. In the absence of Rb, E2F were supposedly activated. However, this seems unlikely because the E7-mediated expression of E2F target genes (cyclins E and A; refs. 36–38) were not elevated in Eps8 knockdown cells (Fig. 4; data not shown). Thus, Eps8 does not affect E7-mediated Rb degradation but regulates the downstream E2F-mediated gene expressions.

For both HeLa and SiHa cells, Eps8 not only facilitated cell cycle progression but also increased their resistance to cisplatin and paclitaxel (Fig. 6; Supplementary Fig. S1).[4] This Eps8-mediated drug resistance might be attributable to its down-regulation of p53 in these cells. Previously, we have shown that Eps8 could elevate the activity of Src and AKT, two prosurvival proteins, in colon cancer cells (28). In HeLa and SiHa cells, similar results were also observed (Fig. 5A; data not shown). Thus, by virtue of p53 up-regulation and the suppression of Src and AKT activity, cells with diminished Eps8 were prone to death caused by these anticancer drugs. Indeed, more sub-G_1 population of Eps8-attenuated HeLa and SiHa cells was observed in the presence of cisplatin (Fig. 6C).

Because Src could up-regulate the expression of Eps8 (26), which in turn could promote its own activity (28), we therefore hypothesized that a mutual influence was present between Eps8 expression and Src activation. Indeed, Src activation as reflected by its Pi-Tyr^{416} was detected in HeLa and SiHa cells and was Eps8 dependent (Fig. 5A; data not shown). Although the prevalence of increased Src activity and the underlying mechanism(s) is still vague in cervical carcinoma, *cis*-activation of *src* promoter by integrated HPV DNA sequences (39) and E6-E6AP complex formation (29) have been postulated to account for the augmented Src level and its sustained activation respectively. Meanwhile, to elucidate the aberrant Eps8 expression in cervical carcinoma, we visualized that multiple mechanisms might be involved. In addition to Src-increased Eps8 expression, amplification of *eps8* gene reported in breast tumor

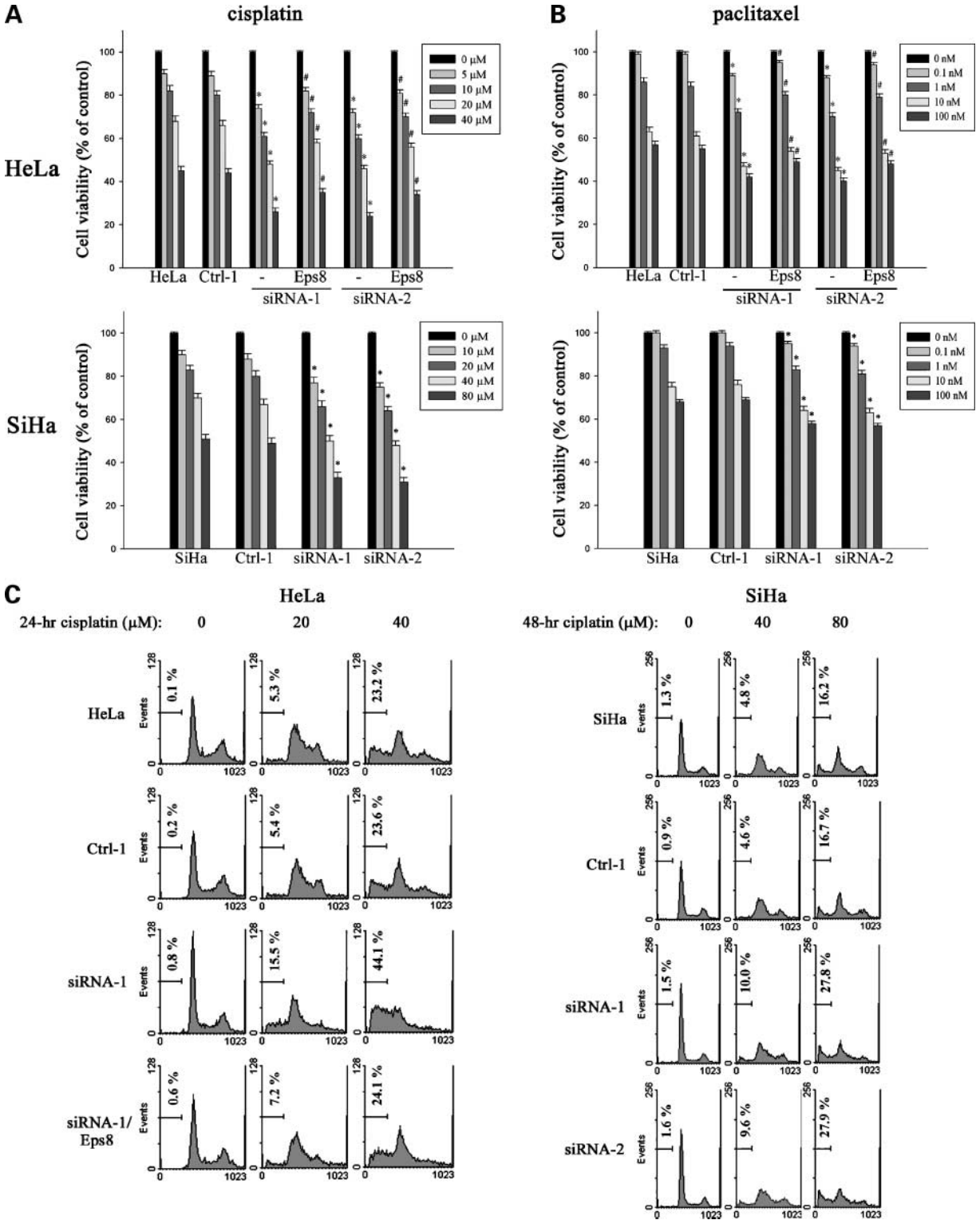

Figure 6. Eps8 attenuation increased the sensitivity of cervical cancer cells to the toxicity of cisplatin and paclitaxel. HeLa, SiHa, and their derived cells were treated with various concentrations of cisplatin (**A**) and paclitaxel (**B**) as indicated for 24 h, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. *Columns,* mean; *bars,* SD. *, $P < 0.05$, compared with parental and control cells; #, $P < 0.05$, compared with Eps8-attenuated cells. **C,** HeLa (*left*), SiHa (*right*), and their derived cells were treated with various concentration of cisplatin for 24 or 48 h as indicated. Then, these cells were trypsinized and analyzed by flow cytometric analysis. The sub-G_1 population was calculated as described in Materials and Methods and indicated. Similar results were repeated at least twice.

specimens (40) should not be excluded. To date, given that (*a*) Eps8 was overexpressed in breast (40), colon (28), and cervical carcinomas; (*b*) Eps8 contributed to the aggressiveness of colon (28) and cervical cancers (Table 1); and (*c*) Eps8 could regulate cell cycle progression and affected the survival of cervical cancer patients, a pivotal role of Eps8 in human neoplasm was unambiguously established.

In conclusion, through immunohistochemical staining and clinicopathologic examination, we showed an intimate correlation between the abundance of Eps8 and the aggressiveness (parametrium invasion or local lymph node metastasis) of early-stage cervical cancer. Consistently, Eps8 expression inversely correlated with the survival rate of cervical cancer patients. Attenuation of Eps8 in cultured cervical cancer cell lines reduced proliferation *in vitro* and xenograft growth *in vivo*. By virtue of reducing AKT activity and elevating the level of p53, Eps8 attenuation increased cellular sensitivity to chemotherapeutic agents and thus affected the response to chemotherapy. Our study unveiled and suggested a potential strategy for future cancer chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Wen-Hwa Lee and Wen-Ya Huang for providing antibody recognized Rb.

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Mol Cancer Ther 2008;7:1376-1385.