ARTICLE
Journal of Cellular Biochemistry 116:1755–1765 (2015)

Journal of Cellular Biochemistry

EGF Protects Cells Against Dox-Induced Growth Arrest Through Activating Cyclin D1 Expression

Chun-Xia Yao,[1] Jia-Chen Shi,[1] Cai-Xia Ma,[1] Cheng-Juan Xiong,[1] Yang-Liu Song,[1] Shu-Feng Zhang,[2] Shan-Feng Zhang,[1] Ming-Xi Zang,[1]* and Li-Xiang Xue[3]**

[1]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou City, Henan 450001, China*

[2]*The People's Hospital of Henan Province, Zhengzhou University, Zhengzhou, Henan 450001, China*

[3]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, Beijing 100191, China*

ABSTRACT

It has been reported that the antitumor drug doxorubicin (Dox) exerts its toxic effects via GATA-4 depletion and that over-expression of GATA-4 reverses Dox-induced toxicity and apoptosis; however, the precise mechanisms remain unclear. In this study, we observed, for the first time, that EGF protects cells against Dox-mediated growth arrest, G2/M-phase arrest, and apoptosis. Additionally, EGF expression was down-regulated in Dox-treated cells and up-regulated in GATA-4 over-expressing cells. Utilizing real-time PCR and western blotting analysis, we found that the expression of the cell cycle-associated protein cyclin D1 was inhibited in GATA-4-silenced cells and Dox-treated cells and was enhanced in GATA-4 over-expressing cells and EGF-treated cells. Furthermore, EGF treatment reversed the inhibited expression of cyclin D1 that was mediated by GATA-4 RNAi or Dox. Our results indicate that EGF, as a downstream target of Dox, may be involved in Dox-induced toxicity as well as in the protective role of GATA-4 against toxicity induced by Dox via regulating cyclin D1 expression, which elucidates a new molecular mechanism of Dox toxicity with important clinical implications. J. Cell. Biochem. 116: 1755–1765, 2015.

KEY WORDS: CELL GROWTH; DOX; EGF; GATA-4; CYCLIN D1

Although doxorubicin (Dox) is one of the most effective and widely used anticancer drugs, increased attention has been paid to its serious toxicity on many tissues, such as heart, kidney, and liver. Accumulated evidence has shown that Dox inhibits DNA synthesis and generates oxygen-free radicals, which eventually induce DNA damage, cell cycle arrest, and apoptosis [Mukhopadhyay et al., 2009; Gogolin et al., 2013; Fan et al., 2014] and may account for its toxicity. However, how to reverse the toxic effects of Dox and broaden its clinical use remains unknown. A previous study showed that Dox exerts a cardiotoxic effect via GATA-4 depletion, resulting in cardiomyocyte apoptosis. In addition, this study shows that Dox-induced cardiotoxicity and cardiomyocyte apoptosis are reversed by over-expressing GATA-4 [Aries et al., 2004], but the mechanism remains unknown.

GATA-4, a zinc finger transcription factor, has been shown to be involved in heart development and carcinogenesis through regulating the expression of specific genes, such as Bcl2, which is involved in apoptosis [Kobayashi et al., 2006; Kyronlahti et al., 2008]. Our previous studies showed that GATA-4 regulates cyclin D1 expression, which is involved in cell proliferation and the cell cycle; therefore, GATA-4 may exert its effects on cell growth [Yao et al., 2013]. Indeed, our previous studies showed that GATA-4 induces cell proliferation, promotes cell cycle progression, and inhibits cell apoptosis [Yao et al., 2013], but it is unknown whether GATA-4 reverses Dox toxicity via activating cyclin D1.

Cyclin D1 regulates cell cycle progression by associating with CDK4 and CDK6 to phosphorylate retinoblastoma tumor-suppressor protein (Rb) [Tamamori-Adachi et al., 2008]. Cyclin D1 expression is

Chun-Xia Yao and Jia-Chen Shi have contributed equally to this work.

Conflict of interest: The authors declare no competing financial interests.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81371895, 81141044, 31071206; Grant sponsor: Ministry of Science and Technology of China; Grant number: 2012AA022501.

*Correspondence to: Ming-Xi Zang, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Zhengzhou University, Ke Xue Da Dao 100, Zheng Zhou 450001, China. E-mail: mzang@zzu.edu.cn
**Correspondence to: Li-Xiang Xue, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, Beijing 100191, China. E-mail: lixiangxue@bjmu.edu.cn

Manuscript Received: 2 July 2014; Manuscript Accepted: 6 February 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 March 2015

DOI 10.1002/jcb.25134 • © 2015 Wiley Periodicals, Inc.

regulated by other proteins, such as the transcription factor KLF13 and the glycogen synthase kinase-3β (GSK-3β) as well as by GATA-4 [Nemer and Horb, 2007; Kerkela et al., 2008]. Most notably, cyclin D1 is induced by mitogens [Chen et al., 2012], but relatively little is known about the roles of mitogens and cyclin D1 in the protective role of GATA-4 against Dox-induced toxicity.

Epidermal growth factor (EGF) is a well-characterized mitogen that plays roles in proliferation, morphogenesis, and apoptosis [Fraguas et al., 2011; Fatimah et al., 2012; Feng et al., 2012]. Given that previous studies found that EGF [Poch et al., 2001] and GATA-4 induce cyclin D1 expression, cyclin D1 is thought to be a common downstream target of GATA-4 and EGF. On the other hand, when considering the protective role of GATA-4 against Dox-induced toxicity, we asked whether EGF exerts the same effects as GATA-4 and whether EGF accounts for the preventive roles of GATA-4 against Dox-induced toxicity.

To address the aforementioned issues, we investigated the effects of EGF on Dox-induced growth arrest, cell cycle arrest, and apoptosis. Here, we demonstrate that EGF protects cells against growth arrest and apoptosis mediated by Dox. Moreover, we show that EGF protects cells against growth arrest and apoptosis mediated by Dox via activating cyclin D1 expression. Utilizing real-time PCR analysis, we found that the expression of EGF was upregulated in GATA-4 overexpressing cells and downregulated upon Dox treatment. Our results indicate that EGF treatment may be involved in the protective role of GATA-4 against Dox-induced toxicity.

MATERIALS AND METHODS

ANTIBODIES AND PLASMIDS

The following antibodies were used: anti-GATA-4 (ab134057, Abcam, Cambridge, MA), anti-cyclin D1 (sc-753, Santa Cruz Biotechnology, Inc. Dallas, TX), and anti-α-tubulin (sc-32293, Santa Cruz). The RNAi GATA-4 constructs were designed according to the pSilencer neo™ Instruction Manual (Ambion, Foster, CA), as described previously [Zang et al., 2004]; the siRNA negative control was not homologous to any known mammalian gene. All constructs were confirmed by DNA sequencing. The sequences of the primers are listed in Table I.

CELL CULTURE AND TRANSFECTION

C2C12, human embryonic kidney 293 cells and A549 human lung adenocarcinoma epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% fetal bovine serum (FBS), and P19CL6 cells were grown in Minimum Essential Medium Alpha Medium (α-MEM, GIBCO) supplemented with 10% FBS. The empty pcDNA3.1(+) (G418-resistant), pcDNA3-GATA-4, or RNAi GATA-4 expression vectors were transfected into C2C12 and P19CL6 cells using Lipofectamine (Invitrogen, Carlsbad, CA) to select cells stably expressing GATA-4 or knocked down for GATA-4. After 24 h of transfection, the cells were treated with 400 μg/ml G418 (Amersham Pharmacia Biotech Co., Ltd., Shinjuku-ku, Tokyo, Japan), and G418-resistant colonies were selected after 2 weeks.

Transfections were carried out 24 h after plating cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the 1,000-bp human cyclin D1 promoter reporter construct was transiently transfected into C2C12 cells, P19CL6 cells, GATA-4-silenced cells, and cells stably expressing GATA-4. The cells were serum-starved for 12 h at 16 h posttransfection and then treated with EGF (500 or 20 ng/ml) for 24 h and/or 300 nM Dox for 12 h. Next, the cells were harvested, and luciferase activity was measured. The amount of the reporter was maintained at 1 μg per well of a 12-well plate, and the amount of DNA was kept constant by co-transfecting the empty expression vector.

REAL-TIME PCR

RNA was extracted from C2C12 cells, P19CL6 cells, GATA-4-silenced cells, GATA-4-expressing cells, or cells treated with EGF and/or Dox using the TRIzol method (Invitrogen). First-strand cDNA was used as the template in real-time PCR reactions, as described previously [Yao et al., 2013]. Comparative quantification and the 2(-delta delta C(T) method were used to quantify the mRNA, and the mRNA levels were normalized to those of ribosomal protein S16. The sequences of the primers are listed in Table I.

WESTERN BLOT ANALYSIS

Western blotting was performed using cell lysates from C2C12 cells, P19CL6 cells, GATA-4-silenced cells, or GATA-4-expressing cells; and the controls included pcDNA 3.1 empty vector- or siRNA negative-expressing cells or cells treated with EGF and/or Dox according to standard protocols, as previously described [Yao et al., 2012]. Briefly, proteins from the differently treated P19CL6 cells and C2C12 cells were separated by SDS–PAGE and subjected to immunoblotting using antibodies specific for GATA-4, cyclin D1, or α-tubulin. The bands were visualized using a standard ECL protocol (Pierce, Rockford, IL).

TABLE I. Primer Sequences

		Forward primer	Reverse primer
q-PCR	EGF (NM_010113)	TCGGAAGCAGCTATCAAACC	TATGTGGGGCTTCTGTCTCC
	GATA-4(NM_144730)	CAGCAGCAGTGAAGAGATGC	ATGTCCCCATGACTGTCAGC
	Cyclin D1 (NM_007631.2)	ATGAGAACAAGCAGACCATCCGCA	GCTTGACTCCAGAAGGGCTTCAAT
	40S ribosomal protein S16	TCTGGGCAAGGAGAGATTTG	CCGCCAAACTTCTTGGATTC
Cloning	RNAi GATA-4	GATCCG TCTCGATATGTTTGATGAC TTCAAGAGA GTCATCAAACATATCGAGATT TTTTGGAAA	AGCTTTTCCAAAA AATCTCGATATGTTTGATGAC TCTCTTGAA GTCATCAAACATATCGAGA CG
Transfection	siRNA negative control	GATCC ACAACATAGGGTGACTAGA TTCAAGAGA TCTAGTCACCCTATGTTGTTT TTTTGGAAA	AGCTTTTCCAAAA AAACAACATAGGGTGACTAGA TCTCTTGAA TCTAGTCACCCTATGTTGT G

CELL PROLIFERATION ASSAY

Murine C2C12 skeletal myoblasts are muscle precursor cells, and P19CL6 cells can efficiently differentiate into cardiomyocytes; therefore, these two cell lines were used to analyze Dox-induced cardiotoxicity. C2C12 cells, P19CL6 cells, GATA-4-silenced cells, or cells stably expressing GATA-4, and the controls included pcDNA 3.1 empty vector- or siRNA negative-expressing cells were seeded at 60,000 cells per well in a 24-well plate, 24 h later, the cells were serum-starved and then treated with EGF (500 or 20 ng/ml) for 24 h in the presence or absence of the anti-tumor drug Dox (300 nM, 12 h). Next, cell counting using Trypan blue staining was performed to determine the growth rate of the cells. All experiments were plated in sextuplicate and performed three times.

For MTT assays, 293 and A549 cells were seeded at 10,000 cells per well in a 96-well plate. Twenty-four hours later, the cells were serum-starved and then treated with EGF (500 ng/ml) for 24 h in the presence or absence of the anti-tumor drug Dox. Next, an MTT assay was performed to determine the growth rate of these cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Corporate, Louis, MO), as previously described [Xiong et al., 2013]. All experiments were plated in sextuplicate and were performed three times.

FLOW CYTOMETRIC ANALYSIS

P19CL6, 293, and A549 cells were seeded at 5×10^5 cells per well in a 6-well plate. Twenty-four hours later, cells were serum-starved and were then treated with EGF (20 ng/ml) for 24 h in the presence or absence of the anti-tumor drug Dox (5 μM or 300 nM, 12 h). The cells were then harvested, fixed with 70% ice-cold ethanol, and stained with propidium iodide (PI, Sigma) solution [Xiong et al., 2013]. The experiments were performed three times in duplicate.

ANNEXIN V-FITC/PI APOPTOSIS ASSAY

Apoptosis was quantified using an annexin V-FITC/PI apoptosis detection kit (Nanjing KeyGen Biotech. Co. Ltd, Nanjing, Jiangsu, China) according to the manufacturer's recommendations. In brief, P19CL6, 293, and A549 cells were seeded at 5×10^5 cells per well in a 6-well plate. Twenty-four hours later, the cells were serum-starved and then treated with EGF (20 ng/ml) for 24 h in the presence or absence of the anti-tumor drug Dox (5 μM, 12 h). Next, the cells were harvested and stained with annexin V-FITC and PI, and the percentage of apoptotic cells was determined by flow cytometric analysis using a BD FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). The experiments were performed three times in duplicate.

STATISTICS

The data are reported as the means ± SEMs. Analysis of variance (ANOVA) was used to compare differences between the differently treated cells. In all cases, differences were considered to be statistically significant when $P < 0.05$.

RESULTS

DOX INHIBITS THE EXPRESSION OF EGF, WHEREAS GATA-4 INDUCES EGF EXPRESSION

To determine the roles of EGF in the protective role of GATA-4 against Dox-induced toxicity, we constructed C2C12 cell lines expressing GATA-4 or knocked down for GATA-4 (RNAi GATA-4) and analyzed the mRNA level of EGF (Fig. 1A, left). Interestingly, the EGF mRNA level was up-regulated 1.7-fold in GATA-4-over-expressing C2C12 cells and was reduced by 43% in RNAi GATA-4 cells (Fig. 1A, left). Moreover, upon treatment of C2C12 cells with Dox, the EGF mRNA level was reduced by 46% (Fig. 1A, left). These data suggest that Dox inhibits, whereas GATA-4 induces, EGF expression.

On the other hand, the GATA-4 mRNA level was increased up to 2.0-fold in C2C12 cells treated with EGF and was reduced by 64% and 36% in Dox-treated cells and GATA-4-silenced cells, respectively (Fig. 1A, right). Moreover, in Dox-treated cells and GATA-4-silenced cells, the reduced expression of GATA-4 was rescued by EGF treatment (Fig. 1A, right). Together, given that GATA-4 induces EGF expression, these findings suggest that EGF treatment induces GATA-4 expression; therefore, there may be a regulatory loop between GATA-4 and EGF in C2C12 cells.

To further determine whether the above-described results could be reproduced, we performed the same experiments as in Figure 1B and C in P19CL6 cells. Consistent with the observations in C2C12 cells, GATA-4 induced EGF expression, while both GATA-4 RNAi and Dox treatment inhibited EGF expression. Moreover, both 500 and 20 ng/ml EGF treatments showed protective effects on the inhibition of GATA-4 expression mediated by GATA-4 RNAi and Dox (Fig. 1B and C). These results further showed that the regulatory loop between GATA-4 and EGF is not specific for C2C12 cells but also exists in P19CL6 cells.

EGF PROTECTS CELLS AGAINST DOX-MEDIATED GROWTH ARREST

A previous study showed that the antitumor drug Dox exerts a cardiotoxic effect via depleting GATA-4, resulting in cardiomyocyte apoptosis. In addition, this study showed that Dox-induced cardiotoxicity and cardiomyocyte apoptosis were reversed by over-expressing GATA-4 [Aries et al., 2004], suggesting that GATA-4 is essential for cell survival and growth. The fact that EGF expression was downregulated in concert with the inhibition of GATA-4 expression (Fig. 1A–C) suggests that lower EGF levels may account for Dox-induced cell death. To test this hypothesis, C2C12 cells were stably transfected with a GATA-4 or GATA-4 RNAi expression vector and treated with Dox and/or EGF. Next, cell viability was evaluated by cell counting using Trypan blue staining. As shown in Figure 2A and as expected, C2C12 cells stably expressing GATA-4 and treated with EGF demonstrated a 38% and 47% increase in cell number, respectively, whereas C2C12 cells knocked down for GATA-4 or treated with Dox showed 34% and 47% reductions in cell number, respectively (Fig. 2A). Moreover, EGF not only promoted cell growth but also reversed GATA-4 RNAi- and Dox-mediated growth arrest, implying that EGF exerts protective effects on Dox- or GATA-4 RNAi-mediated growth arrest.

On the other hand, the aforementioned results indicate that EGF upregulation is responsible for the observed GATA-4-induced increase in cell growth. Indeed, the mRNA levels of EGF were up-regulated in C2C12 cells over-expressing GATA-4 and down-regulated in C2C12 cells knocked down for GATA-4 (Fig. 1A, left). Furthermore, EGF mRNA levels were reduced in concert with the down-regulation of the GATA-4 mRNA level upon Dox treatment (Fig. 1), suggesting that the down-regulation of the EGF mRNA level

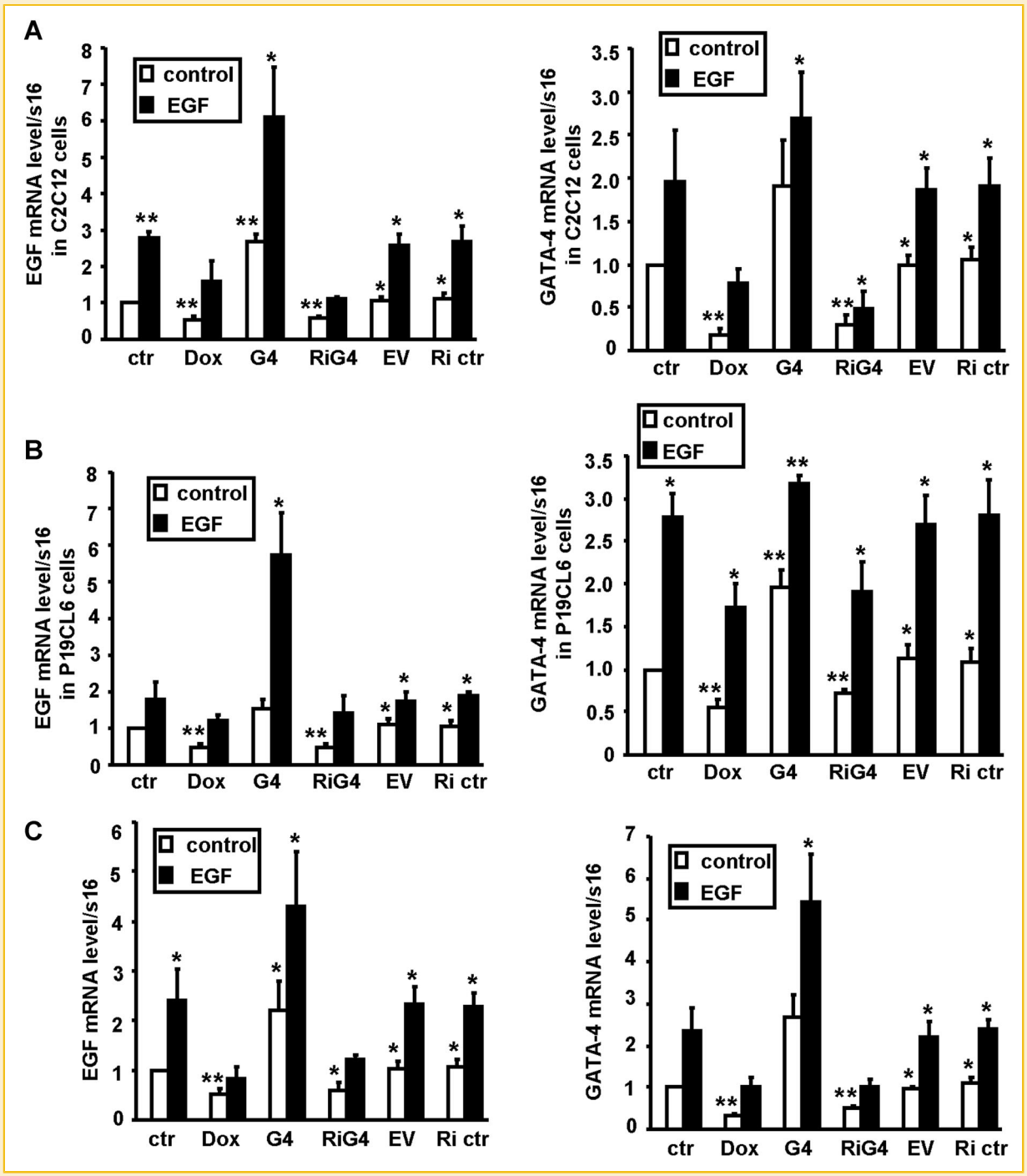

Fig. 1. EGF mRNA level was down-regulated in Dox-treated cells and up-regulated in stably expressing GATA-4 cells. (A) Real-time PCR analysis of the EGF (left) and GATA-4 (right) mRNA levels in C2C12 cells treated with 500 ng/ml EGF in the presence or absence of the anti-tumor drug Dox (300 nM) or stably transfected with pCDNA3.1 (+) empty vector (EV), the GATA-4 expression vector (G4), the GATA-4 knockdown vector (Ri G4), and Si RNA negative control vector (Ri ctr). ctr: control. (B) Real-time PCR analysis of the EGF (left) and GATA-4 (right) mRNA levels in P19CL6 cells treated as described in Figure 1A. (C) The EGF (left) and GATA-4 (right) mRNA levels were analyzed in P19CL6 cells treated with 20 ng/ml EGF in the presence or absence of the anti-tumor drug Dox (300 nM) or stably transfected with pCDNA3.1 (+) EV, the GATA-4 expression vector (G4), the GATA-4 knockdown vector (Ri G4), and Si RNA negative control vector (Ri ctr). ctr: control. The results are the means ± SEMs. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control.

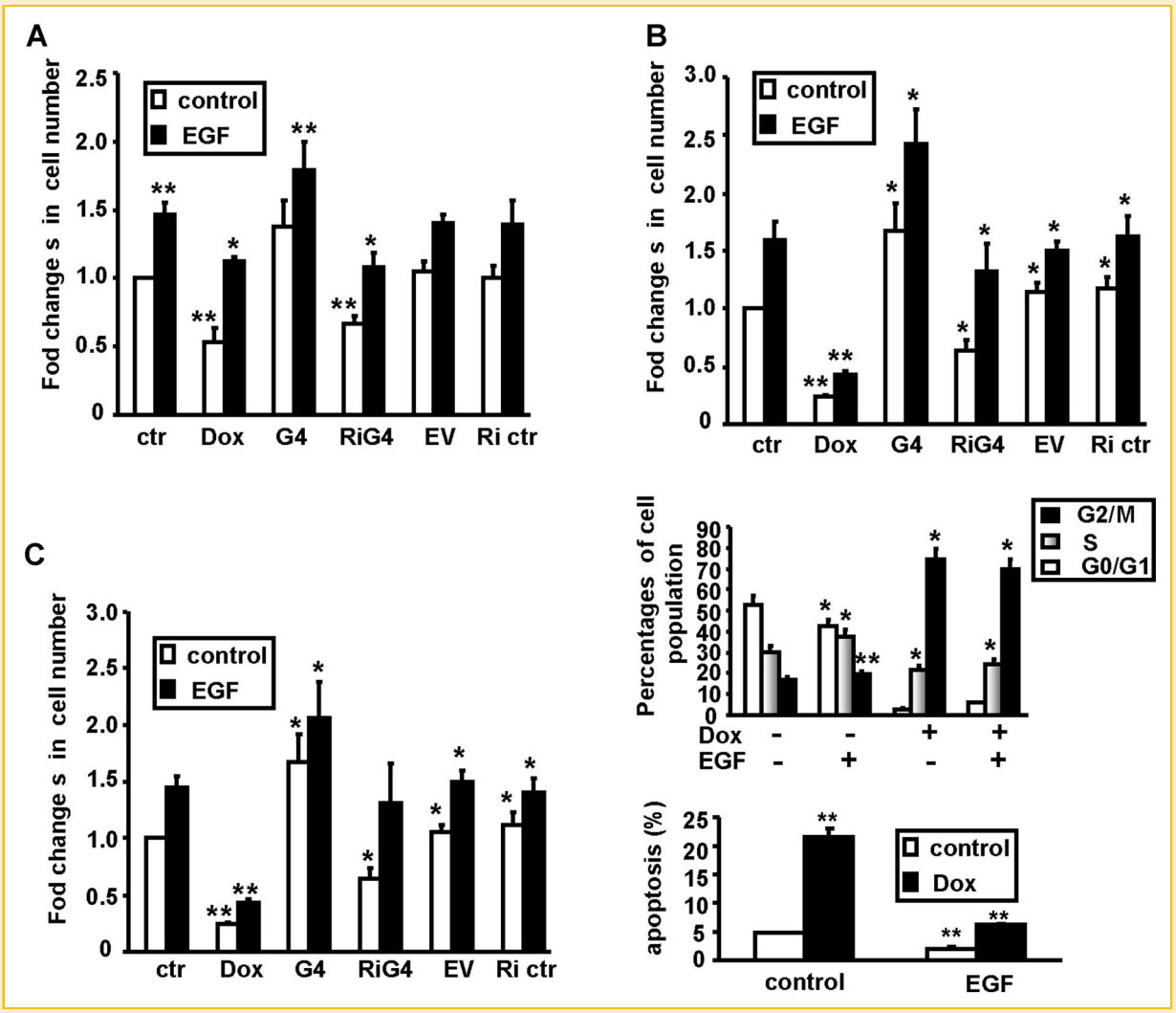

Fig. 2. EGF protects cells against growth arrest mediated by Dox and GATA-4 RNAi. (A and B) The effect of EGF on cell proliferation. C2C12 (A) and P19CL6 (B) cells were stably transfected with the empty vector (EV) or the GATA-4 (G4) or GATA-4 RNAi expression vectors (Ri G4) or Si RNA negative control vector (Ri ctr) and then seeded in 24-well plates and serum-starved after 24 h of growth. Next, the cells were treated with 500 ng/ml EGF for 24 h in the presence or absence of the anti-tumor drug Dox (300 nM, 12 h), and then cell counting using Trypan blue staining was performed to determine the growth rate of the cells. ctr: control. The data shown are the means ± SEMs of three independent experiments. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control. (C) Cell viability was determined in P19CL6 cells treated as described in Figure 2A, except that the dosage of EGF was 20 ng/ml, by cell counting using Trypan blue staining (left). P19CL6 cells were treated with 20 ng/ml EGF in the presence or absence of the anti-tumor drug Dox (300 nM) and subjected to FACS analysis (upper right) or annexin V-FITC/propidium iodide (PI) double staining to quantify the percentage of apoptotic cells (lower right). The data shown are the means ± SEMs of three independent experiments. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control (RiG4: GATA-4 knockdown).

may account for Dox-mediated growth arrest. Taken together, these data strongly suggest that GATA-4 and Dox accelerates and inhibits cell proliferation through, respectively, activating and inhibiting EGF expression.

To further determine whether the above-described results are specific for C2C12 cells, we performed the same experiments in P19CL6 cells. As shown in Figure 2B and C and consistent with the observations of C2C12 cells, GATA-4 and both 500 and 20 ng/ml EGF treatments induced cell growth, whereas knocking down GATA-4 or treating cells with Dox inhibited cell growth (Fig. 2B and C). Moreover, EGF not only promoted cell growth but also reversed GATA-4 RNAi- and Dox-mediated growth arrest, further indicating that EGF exerts protective effects on Dox- or GATA-4 RNAi-mediated growth arrest.

To determine whether the anti-proliferative effect of Dox is due to cell cycle arrest, we performed FACS analysis of untreated, Dox-treated and/or EGF-treated P19CL6 cells (Fig. 2C, upper right). In comparison to control cells, the G0/G1 population of the cells treated with Dox decreased by 94% in P19CL6 cells, whereas the G2/M population increased by 3.4-fold (Fig. 2C, upper right). These data suggest that Dox treatment leads to G2/M-phase arrest in P19CL6 cells. On the contrary, in comparison to control cells, EGF treatment resulted in a 19% decrease in the G0/G1 population and to a 15% increase in the G2/M population (Fig. 2C, upper right). Furthermore,

EGF protected cells against Dox-induced G2/M-phase arrest by decreasing the G2/M population and increasing the G0/G1 population (Fig. 2C, upper right), indicating that EGF promotes cell cycle progression.

To determine whether the anti-proliferative effect of Dox resulted from apoptosis, we examined the effect of Dox on apoptosis in P19CL6 cells using FACS analysis with annexin V-FITC/PI double staining. As shown in Figure 2C, Dox treatment resulted in a 4.4-fold increase in the percentage of apoptotic P19CL6 cells (Fig. 2C, lower right), suggesting that Dox induces apoptosis in P19CL6 cells. Furthermore, the percentage of apoptotic P19CL6 cells decreased to 1.3-fold in Dox-treated cells in the presence of 20 ng/ml EGF (Fig. 2C, lower right), suggesting that EGF protects cells against Dox-induced apoptosis.

Taken together, these results showed that Dox caused G2/M-phase arrest, induced cell apoptosis, and inhibited cell growth when compared to control cells, whereas EGF exerted a protective effect on Dox-induced growth arrest, cell cycle arrest, and apoptosis.

THE PROTECTIVE ROLES OF EGF ARE CELL-SPECIFIC

To further determine whether the above-described results could be reproduced in this system, we performed MTT analysis of 293 and A549 cells. As shown in Figure 3A, 293 cells treated with EGF demonstrated a 37% increase in cell number, whereas 293 cells treated with 5 μM Dox showed a 55% reduction in cell number. Moreover, EGF promoted cell growth and reversed Dox-mediated growth arrest, implying that EGF exerts protective effects on Dox-mediated growth arrest. However, although EGF promoted A549 cell growth, it could not reverse Dox-mediated growth arrest (Fig. 3A, right), suggesting that the protective roles of EGF are cell-specific.

Additional apoptosis and cell cycle assays further addressed the above-mentioned results. As shown in Figure 3B and C, EGF exerted a protective effect on Dox-induced apoptosis and cell cycle arrest in 293 cells, but not in A549 cells, which further indicated that the protective roles of EGF are cell-specific.

EGF PROTECTS CELLS AGAINST GROWTH ARREST MEDIATED BY DOX AND GATA-4 RNAi VIA ACTIVATING CYCLIN D1 EXPRESSION

Members of the D-type cyclin family, particularly cyclin D1, have important roles in regulating cell proliferation [Tamamori-Adachi et al., 2008; Nakajima et al., 2011]. In an attempt to determine the mechanism of GATA-4- and EGF-mediated cell growth, we employed transient transfection assays using the human cyclin D1 promoter in C2C12 cells to characterize the transcriptional activity of cyclin D1. The promoter activities of cyclin D1 are shown in Figure 4A. When C2C12 cells were transfected with GATA-4 or treated with EGF, we observed enhanced cyclin D1 promoter activity (Fig. 4A). In contrast, an siRNA stable knockdown of GATA-4 or Dox treatment in C2C12 cells inhibited the promoter activity of cyclin D1 (Fig. 4A). Moreover, this inhibition could be reversed by the addition of EGF, suggesting that cyclin D1 is a common target of GATA-4 and EGF. Consistent with these findings, we also observed increased expression of cyclin D1 mRNA and protein in C2C12 cells transfected with GATA-4 or treated with 500 ng/ml EGF as well as decreased mRNA and protein expression levels in C2C12 cells transfected with GATA-4 RNAi or treated with Dox (Fig. 4B and C). Additionally, EGF showed a protective effect on GATA-4 RNAi- and Dox-mediated inhibition of cyclin D1 expression (Fig. 4B and C). These results further confirmed that cyclin D1 is a common target of GATA-4 and EGF and may be involved in GATA-4- and EGF-mediated cell growth.

Consistent with the observations in C2C12 cells, we observed enhanced cyclin D1 promoter activity in P19CL6 cells transfected with GATA-4 or treated with 500 ng/ml EGF as well as inhibited cyclin D1 promoter activity in P19CL6 cells knocked down for GATA-4 or treated with Dox (Fig. 5A). Moreover, this inhibition could be reversed by the addition of 500 ng/ml EGF, suggesting that cyclin D1 is a common target of GATA-4 and EGF. Consistent with these findings, GATA-4 and EGF treatments induced cyclin D1 expression, whereas GATA-4 RNAi treatment and Dox treatment inhibited cyclin D1 expression, in P19CL6 cells. Moreover, EGF treatment showed a protective effect on GATA-4 RNAi- and Dox-mediated inhibition of cyclin D1 expression (Fig. 5A). These results further confirmed that cyclin D1 is a target of EGF in P19CL6 cells.

Given that the concentration of EGF (20 ng/ml) is within the range of physiological EGF levels [Sigismund et al., 2005], to further determine whether the above-described results could be reproduced at physiological concentrations of EGF, we repeated the experiments presented in Figure 5A using a dose of 20 ng/ml of EGF. Interestingly, as shown in Figure 5B, a similar result was obtained when 20 ng/ml of EGF was used, while over-expression of the pcDNA3.1 empty vector control or RNAi negative control did not result in a significant change in the protein level of cyclin D1 (Fig. 5C).

Taken together, these results show that Dox treatment and GATA-4 RNAi knockdown inhibit cyclin D1 expression, whereas GATA-4 expression and EGF treatment induce cyclin D1 expression both in C2C12 and P19CL6 cells. Moreover, EGF treatment reverses GATA-4 RNAi- and Dox-mediated inhibition of cyclin D1 expression.

DISCUSSION

Previous studies have shown that EGF is involved not only in proliferation, morphogenesis, and apoptosis but also in cell invasion, migration, and differentiation [Fraguas et al., 2011; Bogel et al., 2012; Cheng et al., 2012; Fatimah et al., 2012; Feng et al., 2012]. Here, we show that EGF protects cells against growth arrest mediated by Dox via activating Cyclin D1 expression. In cells over-expressing GATA-4 or treated with Dox, EGF expression was up-regulated and down-regulated, respectively. Our results indicate that EGF treatment may account for the protective role of GATA-4 against Dox-induced toxicity, which has very import clinical implications in the treatment of cancers using Dox.

Doxorubicin (Dox), an anthracycline antibiotic, has been widely used to treat various cancers in humans, such as cancers of the breast, liver, bladder, lung, and others. However, as one of the most effective chemotherapeutic agents, Dox usually causes serious toxicity in many tissues, especially in the heart, and can even lead to complications, such as heart failure. Due to its severe cardiotoxicity, the clinical use of Dox is limited; therefore, it is important to know how to reverse its toxic effects and broaden its clinical use. Here, we showed that EGF protects cells against growth arrest and apoptosis mediated by Dox, which, when combined with EGF, may be a superior strategy for the treatment of cancers.

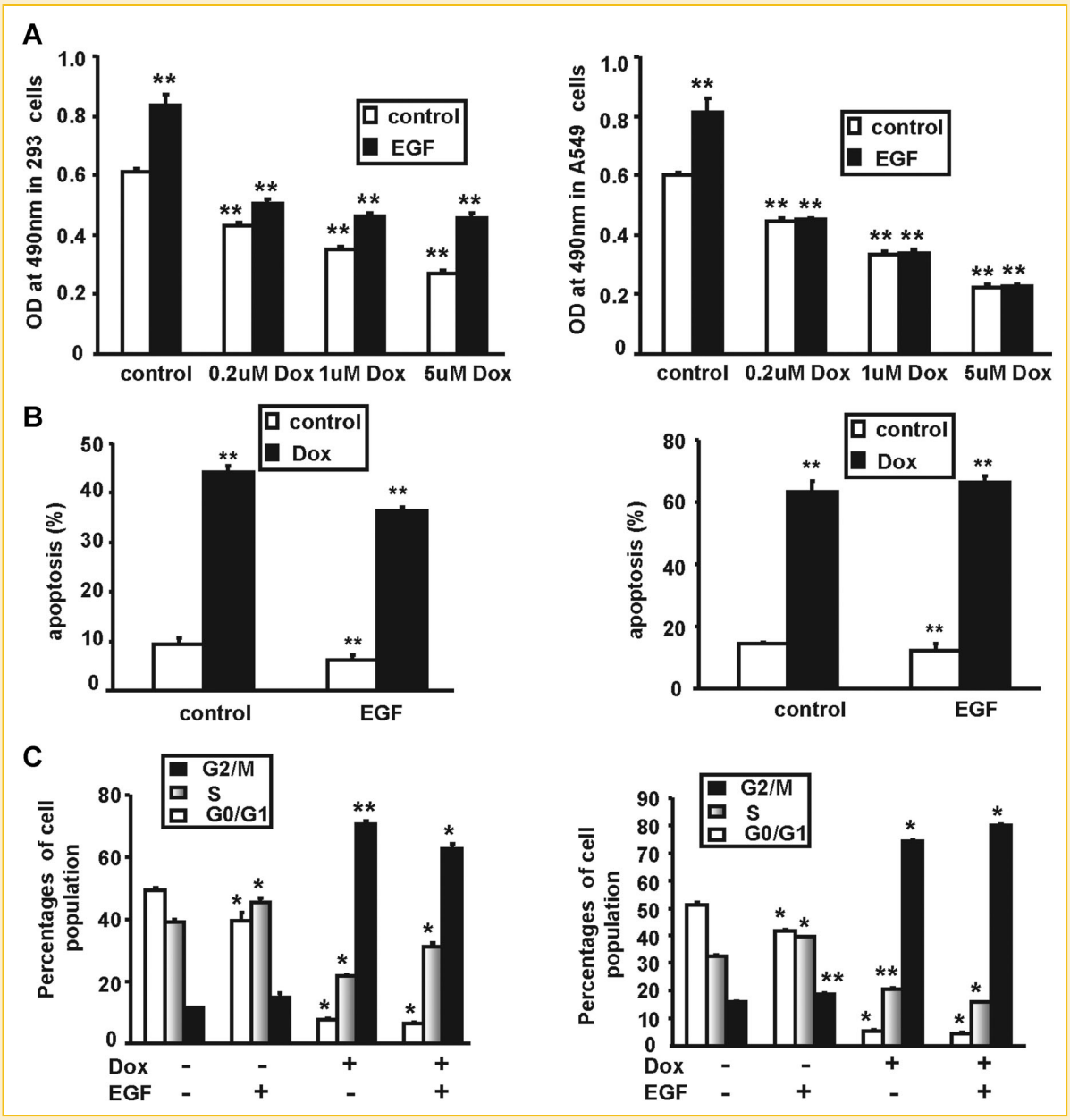

Fig. 3. The protective roles of EGF are cell-specific. (A) The effect of EGF on cell proliferation; 293 cells (left) and A549 cells (right) were seeded in 96-well plates and serum-starved after 24 h of growth. Next, the cells were treated with 500 ng/ml EGF for 24 h in the presence or absence of the anti-tumor drug Dox. Finally, cell viability was determined using MTT assays, as described in the "Materials and Methods" section. The data shown are the means ± SEMs of three independent experiments. "**" indicates $P < 0.01$ vs. control. (B) 293 cells (left) and A549 cells (right) were treated with 500 ng/ml EGF in the presence or absence of the anti-tumor drug Dox (5 μM) and subjected to annexin V-FITC/propidium iodide (PI) double staining to quantify the percentage of apoptotic cells. The data shown are the means ± SEMs of three independent experiments. "**" indicates $P < 0.01$ vs. control. (C) 293 cells (left) and A549 cells (right) were treated with 500 ng/ml EGF in the presence or absence of the anti-tumor drug Dox (5 μM) and subjected to FACS analysis. The data shown are the means ± SEMs of three independent experiments. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control.

Three lines of evidence indicated that EGF protects cells against growth arrest mediated by Dox. First, cell-counting experiments indicated that Dox treatment resulted in reduced C2C12 and P19CL6 cell growth, whereas the addition of EGF reversed this inhibition. Second, FACS analysis demonstrated that Dox treatment induces G2/M arrest, whereas the addition of EGF reverses this trend. Third, annexin V-FITC/PI double staining analysis showed that Dox induces cell apoptosis, whereas the addition of EGF results in a decreased percentage of apoptotic P19CL6 cells. Our findings determined that EGF has a protective role in cell growth, cell cycle, and apoptosis, which may help to reverse the toxic effects and broaden the clinical use of Dox.

Previous studies have shown that GATA-4 is involved in Dox-induced toxicity and apoptosis. These studies found that the

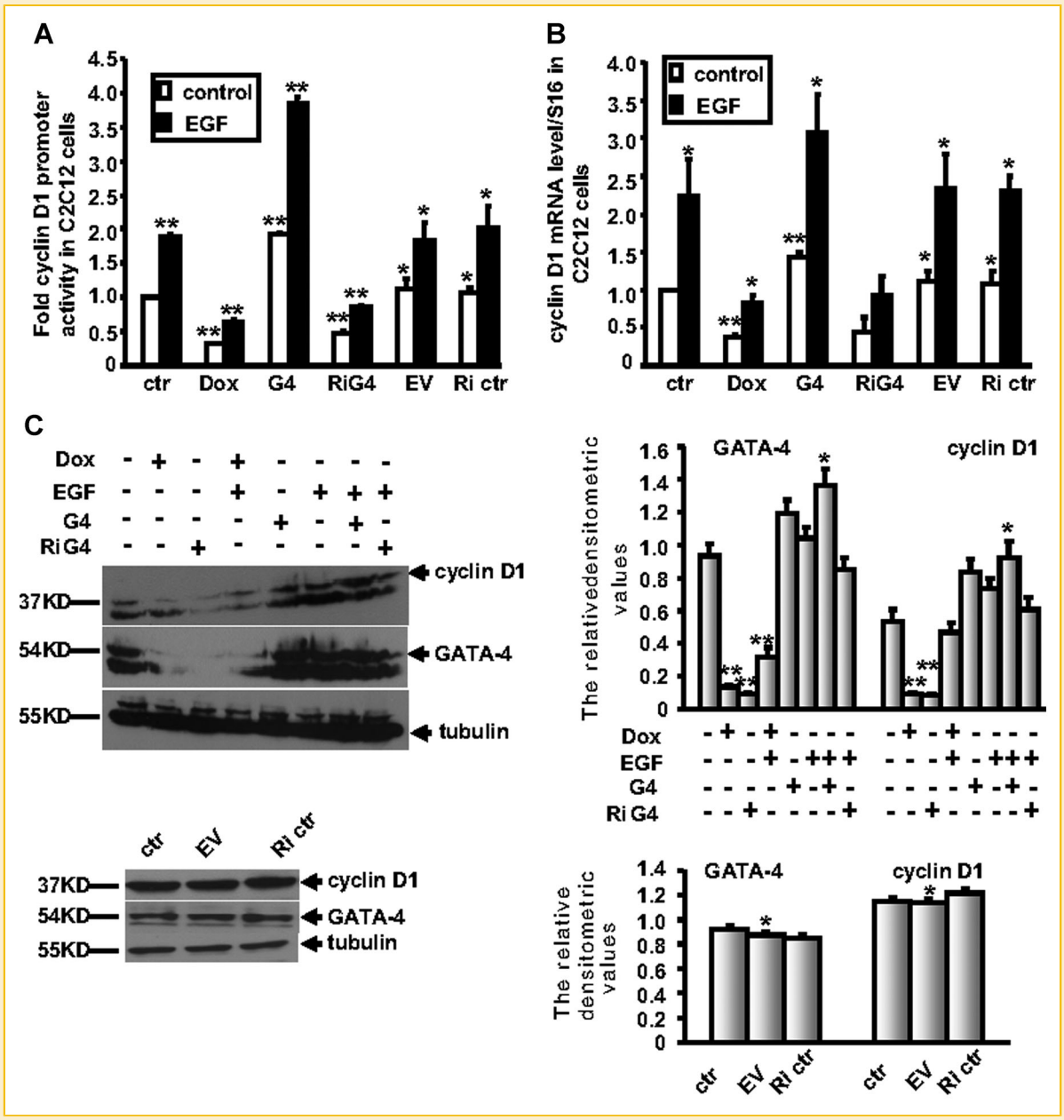

Fig. 4. EGF activates cyclin D1 expression. (A) Luciferase activity of reporter constructs harboring the 1,000-bp human cyclin D1 promoter sequence in C2C12 cells, which were treated as described in Figure 1A. (B) RNA from C2C12 cells, treated as described in Figure 1A, were analyzed by real-time PCR to confirm the induction of cyclin D1 mRNA expression by EGF. (C) Cyclin D1 protein expression in C2C12 cells, treated as described in Figure 1A. Densitometric analysis was performed to quantify the signal strength (right). G4, GATA-4; RiG4, GATA-4 knockdown; ctr, control; EV, the empty vector; Ri ctr, Si RNA negative control vector. The results are the means ± SEMs. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control.

expression of GATA-4 is inhibited in cardiomyocytes treated with Dox and that the over-expression of GATA-4 reverses Dox-induced cardiotoxicity and cardiomyocyte apoptosis [Aries et al., 2004], suggesting that GATA-4 may be involved in Dox-induced toxicity. Here, we show that Dox treatment results in reduced EGF expression and that the addition of EGF reverses Dox-induced growth arrest, cell cycle arrest, and apoptosis, indicating that EGF is involved in Dox-induced toxicity. To the best of our knowledge, we are the first to describe the protective roles of EGF against anti-growth and apoptosis induced by Dox, which is important for relieving the side effects of Dox.

In addition to showing that EGF protects cells against Dox-induced growth arrest and apoptosis, we have found that EGF is a downstream target of Dox. Previous studies demonstrated that EGF expression is regulated by high glucose levels in pancreatic cancer cells and by hypoxia in cardiomyocytes [Han et al., 2011; Munk et al.,

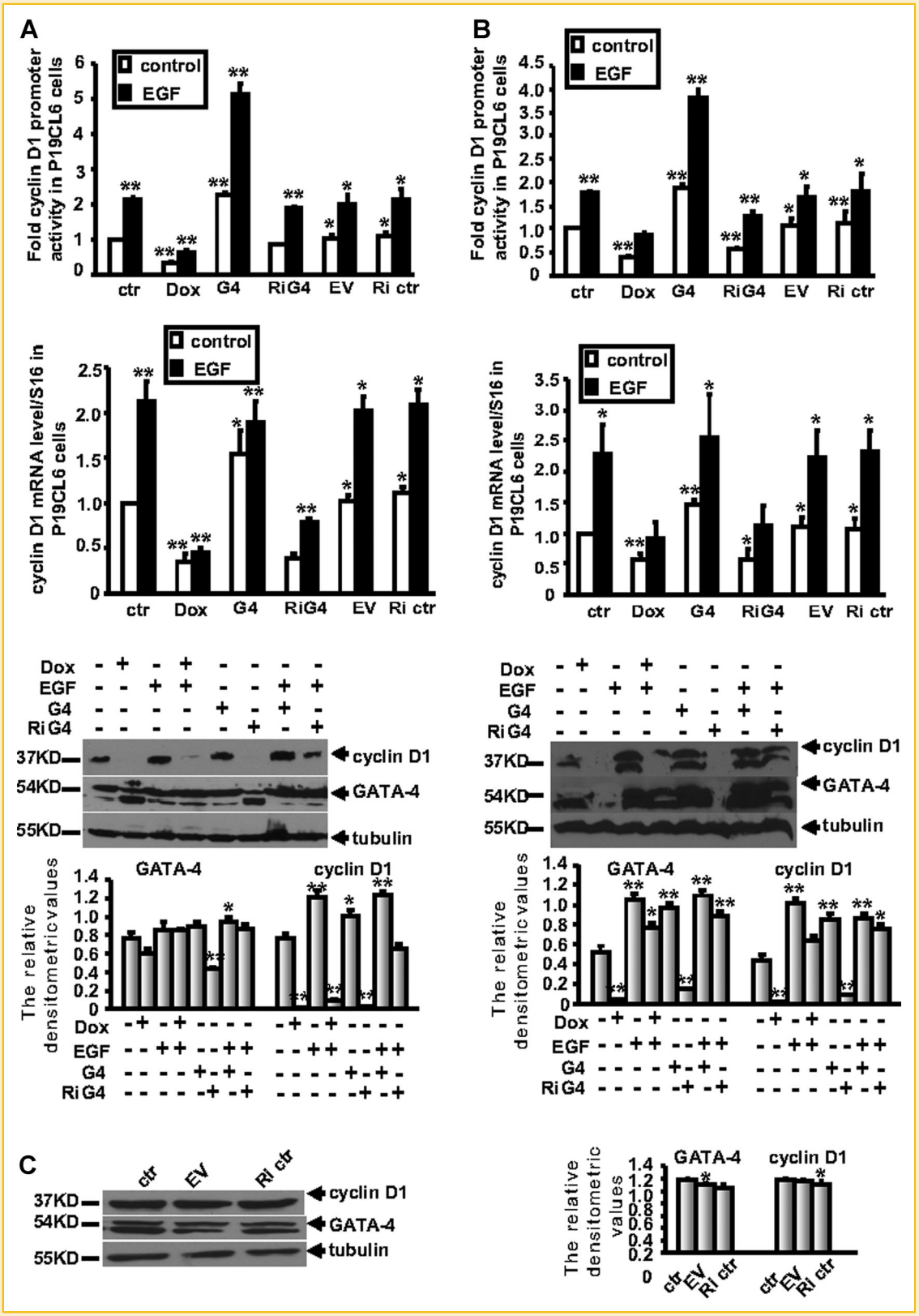

Fig. 5. Induction of cyclin D1 expression by EGF. (A) Luciferase activity of reporter constructs harboring the 1,000-bp human cyclin D1 promoter sequence in P19CL6 cells, which were treated as described in Figure 1A (upper). The cyclin D1 mRNA level (middle) and protein expression (lower) from P19CL6 cells treated as described in Figure 1A were analyzed by real-time PCR and western blotting, and the levels of protein expression were quantified by densitometric analysis. (B) Cyclin D1 promoter activity (upper), cyclin D1 mRNA level (middle), and protein expression (lower) were analyzed in P19CL6 cells treated as described in Figure 1A, except that the dosage of EGF was 20 ng/ml; the levels of protein expression were quantified by densitometric analysis. (C) Cyclin D1 protein expression in P19CL6 cells (ctr) or P19CL6 cells transfected with pcDNA3.1 empty vector or RNAi negative control (left), and densitometric analysis was performed to quantify the signal strength (right). G4, GATA-4; RiG4, GATA-4 knockdown; ctr, control; EV, the empty vector; Ri ctr, Si RNA negative control vector. The results are the means ± SEMs. "*" indicates $P < 0.05$ vs. control, and "**" indicates $P < 0.01$ vs. control.

2012]. Our studies further broaden our knowledge about the mechanisms by which EGF is regulated.

Our studies also describe the relationship between EGF and GATA-4. We found that the EGF mRNA level is down- and up-regulated in concert with the inhibition and enhancement of GATA-4 expression, respectively. On the other hand, the GATA-4 mRNA level is up-regulated upon EGF treatment. Taken together, these data strongly suggest that GATA-4 regulates EGF expression and that EGF regulates GATA-4 expression, indicating the presence of a regulatory loop between GATA-4 and EGF.

Our previous studies demonstrated that cyclin D1 is a down-stream target of GATA-4 [Yao et al., 2013]. Here, we found that the expression of cyclin D1 is regulated by EGF. As revealed by transient transfection, Q-PCR, and immunoblotting analysis, the expression of cyclin D1 is up-regulated upon treatment with 500 and 20 ng/ml EGF. In contrast, Dox treatment inhibited the expression of cyclin D1, while GATA-4 over-expression resulted in enhanced expression of cyclin D1. The fact that the expression of cyclin D1 is activated by EGF may account for the roles of EGF in cell growth and apoptosis.

Lastly, we found that both GATA-4 and EGF treatment could rescue the reduced expression of cyclin D1 mediated by Dox and GATA-4 RNAi. Because EGF exerts its effects via a signal transduction pathway [Brown et al., 2011], we speculate that both GATA-4 and cyclin D1 may be the downstream effectors of the EGF signaling pathway. It is also possible that Dox induces cell growth arrest, cell cycle arrest, and apoptosis via the EGF signaling pathway. Accordingly, further studies will be undertaken to investigate the association between EGF signaling, Dox, GATA-4, and cyclin D1.

In conclusion, the effects of Dox on C2C12 and P19CL6 cell growth and apoptosis are mediated through regulating EGF, GATA-4, and cyclin D1 expression. These findings represent a novel mechanism for the function of Dox in cell growth and apoptosis and have new clinical implications for the design of Dox therapies.

ACKNOWLEDGMENTS

We thank Dr Issei Komuro and Dr Yunzeng Zou for providing P19CL6 mouse embryonic carcinoma cells. This study was supported by grants from the National Natural Science Foundation of China (no. 81371895 and 81141044 to M.-X.Z, and no.31071206 to L-X.X), and the Ministry of Science and Technology of China (863 Program: 2012AA022501 to M.-X.Z.).

REFERENCES

Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M. 2004. Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. Proc Natl Acad Sci USA 101:6975–6980.

Bogel G, Gujdar A, Geiszt M, Lanyi A, Fekete A, Sipeki S, Downward J, Buday L. 2012. Frank-ter Haar syndrome protein Tks4 regulates epidermal growth factor-dependent cell migration. J Biol Chem 287:31321–31329.

Brown AC, Adams D, de Caestecker M, Yang X, Friesel R, Oxburgh L. 2011. FGF/EGF signaling regulates the renewal of early nephron progenitors during embryonic development. Development 138:5099–5112.

Chen CA, Cheng YC, Hwang JC, Chang JM, Guh JY, Chen HC. 2012. Cyclin D1 expression in podocytes: regulated by mitogens in collaboration with integrin-extracellular matrix interaction through extracellular signal-regulated kinase. Exp Biol Med (Maywood) 237:516–523.

Cheng JC, Chang HM, Leung PC. 2012. Epidermal growth factor induces human oviductal epithelial cell invasion by down-regulating E-cadherin expression. J Clin Endocrinol Metab 97:E1380–E1389.

Fan C, Zheng W, Fu X, Li X, Wong YS, Chen T. 2014. Strategy to enhance the therapeutic effect of doxorubicin in human hepatocellular carcinoma by selenocystine, a synergistic agent that regulates the ROS-mediated signaling. Oncotarget 5:2853–2863.

Fatimah SS, Tan GC, Chua KH, Tan AE, Hayati AR. 2012. Effects of epidermal growth factor on the proliferation and cell cycle regulation of cultured human amnion epithelial cells. J Biosci Bioeng 114:220–227.

Feng Y, Dai X, Li X, Wang H, Liu J, Zhang J, Du Y, Xia L. 2012. EGF signalling pathway regulates colon cancer stem cell proliferation and apoptosis. Cell Prolif 45:413–419.

Fraguas S, Barberan S, Cebria F. 2011. EGFR signaling regulates cell proliferation, differentiation and morphogenesis during planarian regeneration and homeostasis. Dev Biol 354:87–101.

Gogolin S, Ehemann V, Becker G, Brueckner LM, Dreidax D, Bannert S, Nolte I, Savelyeva L, Bell E, Westermann F. 2013. CDK4 inhibition restores G(1)-S arrest in MYCN-amplified neuroblastoma cells in the context of doxorubicin-induced DNA damage. Cell Cycle 12:1091–1104.

Han L, Ma Q, Li J, Liu H, Li W, Ma G, Xu Q, Zhou S, Wu E. 2011. High glucose promotes pancreatic cancer cell proliferation via the induction of EGF expression and transactivation of EGFR. PLoS ONE 6:e27074.

Kerkela R, Kockeritz L, Macaulay K, Zhou J, Doble BW, Beahm C, Greytak S, Woulfe K, Trivedi CM, Woodgett JR, Epstein JA, Force T, Huggins GS. 2008. Deletion of GSK-3beta in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. J Clin Invest 118:3609–3618.

Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM, Liang Q. 2006. Transcription factor GATA4 regulates cardiac BCL2 gene expression in vitro and in vivo. FASEB J 20:800–802.

Kyronlahti A, Ramo M, Tamminen M, Unkila-Kallio L, Butzow R, Leminen A, Nemer M, Rahman N, Huhtaniemi I, Heikinheimo M, Anttonen M. 2008. GATA-4 regulates Bcl-2 expression in ovarian granulosa cell tumors. Endocrinology 149:5635–5642.

Mukhopadhyay P, Rajesh M, Batkai S, Kashiwaya Y, Hasko G, Liaudet L, Szabo C, Pacher P. 2009. Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. Am J Physiol Heart Circ Physiol 296:H1466–H1483.

Munk M, Memon AA, Goetze JP, Nielsen LB, Nexo E, Sorensen BS. 2012. Hypoxia changes the expression of the epidermal growth factor (EGF) system in human hearts and cultured cardiomyocytes. PLoS ONE 7:e40243.

Nakajima K, Inagawa M, Uchida C, Okada K, Tane S, Kojima M, Kubota M, Noda M, Ogawa S, Shirato H, Sato M, Suzuki-Migishima R, Hino T, Satoh Y, Kitagawa M, Takeuchi T. 2011. Coordinated regulation of differentiation and proliferation of embryonic cardiomyocytes by a Jumonji (Jarid2)-cyclin D1 pathway. Development 138:1771–1782.

Nemer M, Horb ME. 2007. The KLF family of transcriptional regulators in cardiomyocyte proliferation and differentiation. Cell Cycle 6:117–121.

Poch B, Gansauge F, Schwarz A, Seufferlein T, Schnelldorfer T, Ramadani M, Beger HG, Gansauge S. 2001. Epidermal growth factor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression. Pancreas 23:280–287.

Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci USA 102:2760–2765.

Tamamori-Adachi M, Goto I, Yamada K, Kitajima S. 2008. Differential regulation of cyclin D1 and D2 in protecting against cardiomyocyte proliferation. Cell Cycle 7:3768–3774.

Xiong CJ, Li PF, Song YL, Xue LX, Jia ZQ, Yao CX, Wei QX, Zhang SF, Zhang YY, Zhao JM, Wang TQ, Guo MF, Zang MX. 2013. Insulin induces C2C12 cell proliferation and apoptosis through regulation of cyclin D1 and BAD expression. J Cell Biochem 114:2708–2717.

Yao CX, Xiong CJ, Wang WP, Yang F, Zhang SF, Wang TQ, Wang SL, Yu HL, Wei ZR, Zang MX. 2012. Transcription factor GATA-6 recruits PPARalpha to cooperatively activate Glut4 gene expression. J Mol Biol 415:143–158.

Yao CX, Wei QX, Zhang YY, Wang WP, Xue LX, Yang F, Zhang SF, Xiong CJ, Li WY, Wei ZR, Zou Y, Zang MX. 2013. miR-200b targets GATA-4 during cell growth and differentiation. RNA Biol 10:465–480.

Zang MX, Li Y, Wang H, Wang JB, Jia HT. 2004. Cooperative interaction between the basic helix-loop-helix transcription factor dHAND and myocyte enhancer factor 2C regulates myocardial gene expression. J Biol Chem 279: 54258–54263.