E2F6 Inhibits Cobalt Chloride-Mimetic Hypoxia-induced Apoptosis through E2F1

Wei-Wei Yang,* Bo Shu,* Yi Zhu,[†] and Huang-Tian Yang*

*Laboratory of Molecular Cardiology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China; and [†]Department of Cardiology, Ruijin Hospital, Institute of Cardiovascular Diseases, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Submitted February 19, 2008; Revised May 13, 2008; Accepted June 9, 2008
Monitoring Editor: Kunxin Luo

E2F6, a potent transcriptional repressor, plays important roles in cell cycle regulation. However, roles of E2F6 in hypoxia-induced apoptosis are unknown. Here, we demonstrated biological functions of E2F6 in hypoxia-induced apoptosis and regulatory pathways. During hypoxia (CoCl₂, 800 μ M)-induced human embryonic kidney 293 cell apoptosis, E2F6 expression was down-regulated with concurrent increases in E2F1 expression and transactivation. E2F6 overexpression abrogated hypoxia-induced apoptosis and alteration of E2F1. Conversely, specific knockdown of E2F6 by small interfering RNA had opposite effects. Chromatin immunoprecipitation assay confirmed that E2F6 regulated E2F1 expression through the transrepression of E2F1 promoter. Interestingly, E2F1 transactivation and apoptosis induced by hypoxia in cells stably expressing E2F1 were inhibited by E2F6 overexpression, suggesting that the inhibitory effects of E2F6 are not only mediated by the repression of E2F1 promoter. This was confirmed by E2F6-inhibited transactivation of E2F1 and apoptosis via competing with E2F1 for DNA binding sites evidenced by the different behaviors of E2F6 Δ C (C-terminal deletion) and E2F6.E68 (mutant DNA binding site) and by the lack of association of E2f6 with E2F1 protein. Moreover, hypoxia up-regulated expression of E2F1-responsive proapoptotic gene apoptosis protease-activating factor 1 was repressed by E2F6 overexpression. Together, these findings demonstrate a novel role of E2F6 in control of hypoxia-induced apoptosis through regulation of E2F1.

INTRODUCTION

Apoptotic cell death is a universal mechanism for multicellular organisms to regulate appropriate growth during development, tissue homeostasis, and toxic stress through the elimination of cells (Harbour and Dean 2000). Apoptosis can be initiated by many extracellular and intracellular signal molecules or physiological and pathological inducers, including hypoxia that occurs during acute and chronic vascular diseases, pulmonary diseases, cancer, and others (Harris 2002; Nagarajah *et al.*, 2004; Weinmann *et al.*, 2004). It has been reported that severe hypoxia can induce widespread tumor cell apoptosis (Gee *et al.*, 1999). During hypoxia, cells initiate apoptosis to prevent the accumulation of cells with hypoxia-induced mutation (Huang *et al.*, 2007), presumably because hypoxia induces genetic instability by the induction of fragile sites causing gene amplification (Coquelle *et al.*, 1998) and reduces DNA mismatch repair activity (Perou *et al.*, 2000). Therefore, to understand the mechanisms involved in hypoxia-induced apoptosis would have significant clinic and therapeutic values.

E2F transcription factors play crucial roles in the regulation of cellular proliferation, differentiation, and cell fate (Muller *et al.*, 2001). E2F family contains 10 genes that encode E2F (1–8) and DP (1–2) proteins (DeGregori 2002). E2F6, a recently identified E2F family member, is believed to repress E2F-responsive genes (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). The structural feature of E2F6 compared with other E2Fs is that it lacks the sequences required for transactivative activity and the pocket protein binding domain (Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). E2F6 shares significant homology with other E2Fs and forms heterodimers with DP proteins that recognize E2F consensus sites (TTTCGCGC) but present a preference for the TTTC-CCGC E2F recognition site (Cartwright *et al.*, 1998). Thus, it may compete with activating E2Fs for downstream target promoters. However, the biological roles of E2F6 have not yet been fully understood. E2F6 actively represses the transcription of E2F1-responsive genes through its ability to recruit the polycomb transcriptional repressor complex via the C-terminal repression domain (Trimarchi *et al.*, 2001). It has also been shown that E2F6 increases the percentage of cells in S phase and, when overexpressed, it inhibits S phase entry of quiescent cells and induces subsequent proliferate arrest (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Kherrouche *et al.*, 2001). We demonstrated recently that E2F6 can negatively regulate DNA damage-induced apoptosis via modulation of BRCA1 through its C terminus (Yang *et al.*, 2007), whereas the implication of E2F6 in hypoxia-induced apoptosis remains to be identified.

E2F1, the founding member of E2F family, has an apparently unique ability to induce apoptosis. Ectopic expression

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-02-0171>) on June 18, 2008.

Address correspondence to: Huang-Tian Yang (htyang@sibs.ac.cn).

Abbreviations used: Apaf-1, apoptosis protease-activating factor 1; ChIP, chromatin immunoprecipitation; DHFR, dihydrofolate reductase; EGFP, enhanced green fluorescent protein; NS, nonspecific; PCR, polymerase chain reaction.

of E2F1 leads to apoptosis in culture cells (Qin *et al.*, 1994; Kowalik *et al.*, 1998) and transgenic mice (Guy *et al.*, 1996; Holmberg *et al.*, 1998). The observation of pRb-mediated protection from hypoxia-induced apoptosis in cardiomyocytes through neutralization of E2F1 transactivation potential (Hauck *et al.*, 2002) suggests that transrepression of E2F1-controlled genes is required for cell survival. E2F1-induced apoptosis occurs via multiple pathways. Among them, E2F1/apoptosis protease-activating factor 1 (Apaf-1)/caspases pathway plays an important role in E2F1-induced apoptosis because E2F1-induced apoptosis is significantly reduced by inhibitors of caspase activity or by gene disruption of Apaf-1 (Yang *et al.*, 2000; Furukawa *et al.*, 2002). However, the importance of this pathway in E2F6-regulated apoptosis remains unknown.

Cobalt chloride (CoCl₂) can mimic hypoxic responses in many respects, including the induction of apoptosis in different types of cells (Zou *et al.*, 2002; Jung and Kim 2004; Guo *et al.*, 2006). Therefore, in the present study, we used CoCl₂-treated human embryonic kidney (HEK) 293 cells as a model to investigate the biological role of E2F6 in regulation of hypoxia-induced apoptosis and molecular pathways involved. Dose- and time-dependent relationships between expression of E2F6 and E2F1 during CoCl₂-induced apoptosis were examined. Furthermore, the functional regulation between E2F6 and E2F1 and the regulatory region of E2F6 in CoCl₂-induced apoptosis were identified by overexpression of wild-type and mutant E2F6 and RNA interference-mediated knockdown of E2F6 expression, combined with the cells stably expressing E2F1 and luciferase reporter assay. We demonstrate the important roles of E2F6 in hypoxia-induced apoptosis and provide evidence for the regulatory mechanisms of functional link between E2F6 and hypoxia-induced apoptosis via its modulation of E2F1/Apaf-1 pathway.

MATERIALS AND METHODS

Cell Culture and Hypoxia Treatment

A human embryonic kidney cell line, HEK293, was cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂ (Fan *et al.*, 2005). For hypoxia treatment, CoCl₂ at 200–1000 µM was added into media or incubated for 3–48 h, respectively. All cultivation medium and other substances for cell cultures were purchased from Invitrogen (Paisley, United Kingdom).

Plasmid Construction and Transfection

The plasmids pcDNA3-HA-E2F6 (E2F6 cDNA construct), pcDNA3-HA-E2F6ΔC (Δ220–281, lacking the repression domain), and pcDNA3-HA-E2F6.E68 (with a point mutant of amino acid in position 68) were provided by Prof. David M. Livingston (Harvard Medical School, Boston, MA; Gaubatz *et al.*, 1998). The plasmids pcDNA3.1-E2F1 and pcDNA3.1-E2F1 (1–374, lacking transactivation domain) were obtained from Prof. Ludger Hauck (Humboldt University, Berlin, Germany; Hauck *et al.*, 2002). To generate enhanced green fluorescent protein (EGFP)-containing E2F6wt, E2F6ΔC and E2F6.E68 expression vectors, an EcoR I-Xba I cytomegalovirus (CMV)-EGFP-containing fragment from pAdtrack-CMV (Qbiogene, Irvine, CA) was subcloned into pcDNA3-HA-E2F6, pcDNA3-HA-E2F6ΔC, and pcDNA3-HA-E2F6.E68, respectively. At 24 h before transfection, cells were seeded onto six-well culture plates and transfected at a 70–80% confluence with 1 µg of plasmid DNA each by using Polyfect transfection reagent (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Transfection efficiency was evaluated by the percentage of green fluorescent protein-positive cells. To generate stable clones expressing E2F1, cells were transfected with pcDNA3.1-E2F1 and pcDNA3.1 empty vector by using Polyfect transfection reagent (QIAGEN) after linearization. Transfected cells were selected by G418 (neomycin) as described previously (Lyons *et al.*, 2005). Neo-resistant clones were picked after 8–10 d of G418 selection and propagated. Overexpression of E2F1 in cells stably expressing E2F1 was confirmed by Western blot using antibody for E2F1 (Supplemental Figure 2).

Small Interfering RNA (siRNA) Transfection

At 24 h before transfection, HEK293 cells were plated on six-well culture plates and transfected with siRNAs at 70–80% confluence. Double-stranded siRNAs were purchased from GenePharma and included siE2F6 (AAG-GAUUGUCACAGCAGCUG-custom order), or scrambled siE2F6 (GGUU-GUGUGUACCAACCGGAA-custom order) as nonspecific control siRNA (NS). Transfections were performed with RNAiFect reagent (QIAGEN) according to manufacturers' instructions. RT-PCR and Western analysis were performed as described below to verify the efficiency of the siRNA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total cell RNA was extracted from monolayer cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (0.5 µg) was converted to cDNA by using Superscript II reverse transcriptase (Invitrogen) in a final volume of 20 µl, and 0.4 µl of this was used for each PCR reaction. Semiquantitative PCR was performed using Taq DNA polymerase (Promega, Madison, WI) in a Mastercycler gradient (Eppendorf, Hamburg, Germany) under the following conditions: 5 min at 95°C followed by 35 cycles. Each cycle consisted of denaturation at 95°C for 45 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. After completion of the last cycle, there was an autoextension for 5 min at 72°C. PCR products were visualized on a 1% agarose gel containing ethidium bromide. The primers of Apaf-1 (forward, 5'-AGCCCACTCAACAGCAAA-3' and reverse, 5'-ACCCATCCTGGTTCA-CCT-3') and the housekeeping gene m28s (forward, 5'-AGCAGCCGACTTA-GAATGG-3' and reverse, 5'-TAGGGACAGTGGGAATCTCG-3') were used.

DNA Fragmentation Assay

DNA fragmentation was determined according to previous methods, with slight modifications (Wyllie and Morris 1982; Yang *et al.*, 2007). In brief, floating and attached cells (3–4 × 10⁶) were lysed in 500 µl of TTE buffer (containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% Triton X-100). Lysates were centrifuged at 13,000 × g for 15 min, and the supernatants containing low-molecular-weight fragmented DNA were collected. RNA was removed by addition of RNase A (0.25 µg/µl) and incubated at 37°C for 1 h. The DNA was deproteinized by one extraction in phenol:chloroform:isoamylalcohol (25:24:1) and two extractions in chloroform:isoamylalcohol (24:1), followed by precipitation at –20°C in 50% isopropanol and 130 mM NaCl. DNA was visualized on a 2% agarose gel containing ethidium bromide.

Flow Cytometry Analysis

Samples were prepared for flow cytometry as described previously (Zhang *et al.*, 2005). Briefly, 1 × 10⁶ treated cells were fixed in cold 70% ethanol for 30 min, treated with 100 µg/ml DNase-free RNase A (Sigma Chemie, Deisenhofen, Germany), and labeled with 50 µg/ml propidium iodide (Sigma Chemie). Cells were then analyzed by fluorescence-activated cell sorting (FACS) (FACStar Plus flow cytometer; BD Biosciences, San Jose, CA).

Measurement of Caspase-3 Activity

Caspase-3 activity was measured using the CaspACETM colorimetric assay system (Promega) according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in cell lysis buffer (2 × 10⁷/ml). Lysates were centrifuged (16,000 × g) for 10 min at 4°C. Then, 10 µl of supernatant was mixed with 80 µl of assay buffer and 10 µl of 2 mM Asp-Glu-Val-Asp-p-nitroanilide substrate. After incubation at 37°C for 2–4 h, absorbance was measured using a microplate reader at 405 nm. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank from that of the sample.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

Apoptosis-induced nuclear DNA fragmentation was detected by TUNEL technique using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells were fixed with a freshly prepared paraformaldehyde solution (4% in phosphate-buffered saline [PBS], pH 7.4) at 15–25°C for 1 h. The samples were washed three times with PBS and permeabilized by 0.2% Triton X-100 in PBS for 2 min on ice. After being washed twice, cells were incubated in the presence of TUNEL reaction mixture at 37°C for 60 min in the dark. The samples were washed three times with PBS and analyzed by flow cytometry analysis.

Western Blotting

Cells were lysed as described previously (Scully *et al.*, 1997). Cell lysates or immunoprecipitates were separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4°C with antibodies: mouse anti-E2F1 (1:1000; BD Biosciences), goat anti-E2F6 (1:1000;

Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-actin (1:1000; Sigma Chemie), and then incubated with horseradish peroxidase-linked secondary anti-mouse (1:4000 for E2F1 and 1:8000 for actin; Sigma Chemie) and anti-goat (1:2000 for E2F6; Sigma Chemie), respectively.

Coimmunoprecipitation Assay

Cells were lysed in lysis buffer containing 25 mM Tris, pH 7.6, 150 mM NaCl, 1 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (Sigma Chemie), 1 mM sodium orthovanadate, and 1 μ g/ml proteinase inhibitor mixture (Sigma Chemie), as described previously (Zhan *et al.*, 2002). Immunoprecipitations were performed as described previously (Wu *et al.*, 2005) by using 1 μ g of goat anti-E2F6 (Santa Cruz Biotechnology), mouse anti-E2F1 (BD Biosciences), normal mouse immunoglobulin G (IgG), or normal goat IgG antibodies (Santa Cruz Biotechnology), respectively, with end-over-end mixing at 4°C for 6 h. Protein G-agarose beads (20 μ l; Santa Cruz Biotechnology) were then added, and the reaction mixtures were mixed further at 4°C for 1 h. Immunoprecipitates were separated from the supernatant by centrifugation and washed with PBS containing 0.05% Nonidet P-40. Proteins eluted from the agarose beads were boiled in 1 $\times$ SDS gel-loading buffer and resolved on 12% SDS-PAGE.

Chromatin Immunoprecipitation (ChIP)

The ChIP assay was performed as described previously (Hsieh *et al.*, 2002) on HEK293 cells with the following exceptions. The chromatin was sheared to an average size of 500–2000 base pairs. After cross-linking reversal and proteinase K digestion, individual immunoprecipitate was purified to isolate DNA using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. The ChIP-enriched DNA was amplified by PCR with specific primers for E2F1 promoter (forward, 5'-GCAGCAGTGGGCAATAGA-3' and reverse, 5'-CACCGGAATCCCTGTAAT-3') and Apaf-1 promoter (forward, 5'-GCCCCGACTTCTCCGGCTCTTCA-3' and reverse, 5'-GAGCTGGCAGCTGAAAGACTCA-3'; Furukawa *et al.*, 2002). E2F6 (Santa Cruz Biotechnology) or E2F1 (BD Biosciences) antibodies are used in ChIP assays.

Reporter Gene Assays

To create E2F-SV40 luciferase reporter construct, E2F-SV40-Luc (firefly), the oligonucleotide 5'-CTAGCAGCTGCTGCGATTTCGCGCCAACTTGACG-3', which contains an E2F site (–20 to +9 from the dihydrofolate reductase [DHFR] promoter) was inserted into the vector pGL3-promoter containing simian virus 40 (SV40) promoter in the upstream of the luciferase cDNA (Promega; Slansky *et al.*, 1993). All transfections were performed with Polyfect transfection reagent (QIAGEN) according to manufacturer's instructions. HEK293 cells were seeded at a density of 3×10^5 cells on six-well plates and incubated for one night. The next day, cells were transfected with 600 ng of E2F-SV40-Luc (firefly) and 40 ng of SV40-Luc (*Renilla*). 24 h later, cells were treated with CoCl_2 at different doses and incubation times. After 24 h, luciferase activity was determined as the protocol of dual-luciferase reporter assay (Promega).

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed using one-way analysis of variance and Fisher's protected least significant difference test for multiple comparisons (SPSS 10.0; SPSS, Chicago, IL). $p < 0.05$ was considered to be statistically significant.

RESULTS

Differential Expression Patterns of E2F6 and E2F1 in CoCl_2 -induced Apoptosis

To evaluate the roles of E2F6 and E2F1 in hypoxia-induced apoptosis, the expression patterns of E2F6 and E2F1 were examined in HEK293 cells treated with different doses of CoCl_2 (0, 200, 400, 600, 800, and 1000 μ M) for 24 h or with different time (0, 3, 6, 12, 24, and 48 h) at 800 μ M CoCl_2 . Significant apoptosis was detected in the cells treated with $\geq 600 \mu\text{M}$ CoCl_2 for 24 h in a dose-dependent manner and in cells treated with 800 μM CoCl_2 for ≥ 12 h in a time-dependent manner, as measured by nuclear morphology (condensed and wizened chromatin or apoptotic bodies; Figure 1A). In the same set of experiments, CoCl_2 treatment resulted in a significant down-regulation of E2F6 expression at 600 μ M or higher doses, whereas E2F1 expression was up-regulated with increased doses of CoCl_2 . Similarly, E2F6 expression decreased greatly with a concurrent increase in E2F1 expression after the increased CoCl_2 treatment time

Figure 1. Dose- and time-dependent changes of endogenous E2F6 and E2F1 protein expression during CoCl_2 -induced apoptosis in HEK293 cells. (A) Representative images (top) and average percentage (bottom) of apoptotic cells induced by CoCl_2 at indicated doses and treatment time. Nuclei were stained with Hoechst 33342. Condensed chromatin or apoptotic bodies were detected in apoptotic cells. More than 200 cells were scored per experiment. (B) Representative images (top) and averaged results of immunoblotting (bottom) of E2F6, E2F1, and actin in parallel-treated cell extracts. Similar results were obtained from at least three individual experiments.

≥ 12 h at 800 μM (Figure 1B). Together, these results suggest that the down-regulation of E2F6 expression may be associated with hypoxia-induced apoptosis via deregulation of E2F1.

E2F6 Negatively Regulates CoCl_2 -induced Apoptosis

To confirm the role of E2F6 in the regulation of hypoxia-induced apoptosis, we examined apoptotic responses after overexpression of wild-type E2F6 or knockdown of E2F6 by using siRNA. HEK293 cells were transfected with constructs that express EGFP (vector) or full-length wild-type E2F6-EGFP (E2F6wt), or transfected with nonspecific siRNA (NS) or E2F6-specific siRNA (siE2F6) with or without CoCl_2 treatment. RT-PCR and Western blot analysis confirmed the efficiency of specific knockdown of endogenous E2F6 by siE2F6 (Supplemental Figure 1). Fewer E2F6wt-expressing cells demonstrated apoptotic morphology and nuclear changes than did vector-expressing cells at 24 h after CoCl_2 treatment (800 μM), whereas siE2F6-transfected cells showed more apoptotic changes than did cells transfected with NS (Figure 2, A and B). Consistently, ectopic E2F6 significantly reduced the percentage of cells in hypodiploid DNA peak (sub-G1 population) analyzed by flow cytometry (fluorescence-activated cell sorting [FACS]; Figure 2C) and the fragmentation of genomic DNA, a typical pattern of apoptosis (Figure 2D), seen in CoCl_2 -treated vector-transfected cells. In contrast, siE2F6 transfection accumulated more cells in the sub-G1 population (Figure 2C) and enhanced DNA fragmentation after CoCl_2 treatment (Figure

Figure 2. Effects of E2F6wt and siE2F6 on CoCl₂-induced apoptosis. (A) Cell morphology and percentage of apoptotic cells (showing round and distorted morphology) with or without CoCl₂ treatment after transfection of vector or E2F6wt (left), or NS or siE2F6 (right). (B) Nuclear morphology and percentage of apoptotic cells treated as described in A. Nuclei were stained with Hoechst 33342 and apoptotic cells showed condensed chromatin or apoptotic bodies. (C) Flow cytometric analysis of propidium iodide-stained cells treated as described in A. (D) DNA fragmentation. DNA of $\sim 4 \times 10^6$ cells was loaded, and DNA fragmentation analyzed by 2% agarose gel and visualized with ethidium bromide under UV light. $n = 4$ each. More than 200 cells were scored per experiment in A and B. * $p < 0.05$, ** $p < 0.01$ compared with the CoCl₂-treated group expressing control vector or to the CoCl₂-treated group transfected with NS.

2D). These results reveal a functional role of E2F6 in the regulation of the apoptotic response to hypoxia, i.e., down-regulation of endogenous E2F6 facilitates the progress of hypoxia-induced apoptosis.

E2F6 Abolishes CoCl₂-induced Increases of E2F1 Expression and Transactivation

Activation of E2F1-dependent gene transcription is required for apoptosis in cardiomyocytes (Hauck *et al.*, 2002). This suggests that transrepression of E2F1-controlled genes is beneficial in cell survival. To demonstrate the functional relationship between E2F6 and E2F1 during hypoxia-induced apoptosis, we then examined the activity of E2F1 transactivation in HEK293 cells treated with different doses of CoCl₂ for 24 h or 800 μ M of CoCl₂ for different times. To detect such activity of E2F1, we constructed a luciferase reporter driven by SV40. Because E2F1 up-regulates DHFR transcription via binding to E2F site within its promoter (Slansky *et al.*, 1993), the oligonucleotide containing an E2F site (-20 to $+9$ from the DHFR promoter) was inserted into

the upstream of SV40 to control luciferase activity (E2F-SV40). As shown in Figure 3A, CoCl₂ at 600 μ M and higher doses led to a dose-dependent increase in luciferase activity driven by E2F-SV40. Similarly, CoCl₂ at 800 μ M increased luciferase activity in a time-dependent manner. Next, we examined the effects of E2F6 overexpression or specific knockdown on the expression and transactivation of E2F1. As shown in Figure 3, B and C, CoCl₂-induced increases in E2F1 expression and E2F-SV40-driven luciferase activity were abrogated in E2F6wt cells, whereas cells with siE2F6 treatment enhanced E2F1 protein level and luciferase activity with or without CoCl₂ treatment. To further determine whether E2F1 transactivation is required for CoCl₂-induced apoptosis, HEK293 cells were transfected with or without control vector, wild-type E2F1 (E2F1wt), or mutant E2F1 lacking transactivation domain [E2F1 (1-374)] and then treated with 800 μ M CoCl₂ for 24 h. Cells expressing E2F1wt had more apoptotic nucleus and TUNEL-positive cells as well as higher caspase-3 activity compared with the cells without

Figure 3. E2F6 inhibits CoCl₂-induced increases of the expression and transactivation of E2F1 and apoptosis. (A) Luciferase activities were examined in HEK293 cells transfected with E2F-SV40 luciferase reporter construct (E2F-SV40-Luc) after CoCl₂ treatment at different doses for 24 h or 800 μM CoCl₂ for 3–48 h. (B) Western blotting analysis for E2F1 and actin to control for protein loading in cells transfected with vector, E2Fwt, NS, or siE2F6 with or without CoCl₂ treatment (800 μM; 24 h). (C) Analysis of luciferase activity driven by E2F-SV40 in cells treated as described above. (D) Apoptosis evaluated by nuclear morphology stained with Hoechst 33342 (left), by TUNEL assay (middle; TUNEL-positive cells were identified as apoptotic cells), and by caspase-3 activation assay (right). Cells transfected with or without vector, E2F1wt or E2F1 (1-374) were treated with or without CoCl₂ at 800 μM for 24 h. n = 3 each. More than 200 cells were scored per experiment in chromatin condensation assay, and 30,000 cells per experiment were scored in TUNEL assay. *p < 0.05, **p < 0.01 compared with corresponding control values.

(control) or with the transfection of vector after CoCl₂ treatment. Conversely, overexpression of transcriptionally inactive E2F1 (1-374) partially rescued HEK293 cells from apoptosis (Figure 3D). Thus, E2F6 may negatively regulate apoptosis by modulation of hypoxia-induced E2F1 expression and transactivation.

E2F6 Transcriptionally Regulates E2F1 Expression in CoCl₂-induced Apoptosis

Because E2F6 can transcriptionally repress E2F1 transcription (Gaubatz *et al.*, 1998), we then asked whether increased E2F1 expression by hypoxia is due to the decreased repression of E2F6 on E2F1 promoter. To determine this, we performed ChIP assay to examine the recruitment of E2F6 to the E2F1 promoter. We found that E2F1 promoter was greatly enriched by the E2F6 antibody without CoCl₂ treatment. Such binding was significantly decreased after CoCl₂ treatment and decreased further by siE2F6 treatment. However, more recruitment of E2F6 to E2F1 promoter was detected in cells overexpressing E2F6wt (Figure 4). These data indicate that E2F6 can bind to E2F1 promoter, but the recruitment of E2F6 to the E2F1 promoter is mitigated by hypoxia treatment.

E2F1 Overexpression Attenuates Antiapoptotic Activity of E2F6

To further evaluate the contribution of E2F1 to E2F6-regulated apoptosis, we examined the effects of E2F6 overexpression on CoCl₂-induced transactivation of E2F1 and apoptosis in HEK293 cells stably expressing E2F1. As shown in Figure 5, A and B, cells stably expressing E2F1 (HEK293-E2F1) had higher E2F-SV40-driven luciferase activity and more apoptosis than those expressing control vector (HEK293-

Treatment:	ChIP						Total Input					
	Control	Vector	E2F6wt	NS	siE2F6	siE2F6	Control	Vector	E2F6wt	NS	siE2F6	siE2F6
Antibody:	IgG						E2F6					
CoCl ₂ :	-	+	+	+	+	+	-	+	+	+	+	+
E2F1:	-											

Figure 4. Recruitment of E2F6 to E2F1 promoter is decreased by CoCl₂ and further by siE2F6 but increased by E2F6 overexpression. HEK293 cells transfected with or without control vector, E2F6wt, NS, or siE2F6 were subjected to ChIP analysis by using antibodies of E2F6 and normal rabbit IgG, 24 h after CoCl₂ treatment (800 μM). Then, 0.2% of the total input of each sample was subjected to PCR to show that equal amounts of starting input chromatin were used.

Figure 5. Overexpression of E2F1 partially inhibits antiapoptotic activity of E2F6. Control cells (HEK293-NEO) and cells stably expressing E2F1 (HEK293-E2F1) with or without transfection of vector or E2F6wt were treated with or without CoCl₂ (800 μ M; 24 h). (A) Analysis of luciferase activity driven by E2F-SV40 in cells treated as described above. (B) Parallel-treated cells were detected for apoptosis by apoptotic nuclear morphology (top), TUNEL assay (middle), and caspase-3 activation assay (bottom). $n = 3$. More than 200 cells were scored per experiment in chromatin condensation assay and 30,000 cells were scored in TUNEL assay.

NEO) with or without CoCl₂ treatment. These results confirmed that overexpression of E2F1 sensitizes cells to hypoxia-induced apoptosis. Interestingly, E2F6 overexpression could still partially reduce E2F-SV40-driven luciferase activity and apoptosis induced by CoCl₂ in stably E2F1-expressing cells. However, compared with HEK293-NEO, its inhibitory percentage significantly decreased from 90.4 ± 4.1 to $46.0 \pm 1.8\%$ on luciferase activity and from 52.0 ± 1.3 to $30.3 \pm 3.6\%$ on apoptosis evaluated by nuclear morphology; from 52.0 ± 1.9 to $35.9 \pm 8.8\%$ on apoptosis by TUNEL assay; and from 47.0 ± 0.8 to $33.8 \pm 1.9\%$ on apoptosis by caspase-3 activation assay (Figure 5, A and B). These results further confirmed that E2F6 inhibits hypoxia-induced E2F1 transactivation and subsequent apoptosis through the repression of E2F1 expression and other mechanisms in addition to its transcriptional repression of E2F1.

E2F6 Inhibits CoCl₂-induced Apoptosis via Competing with E2F1 for the Target Promoter

Because E2F6 can bind to E2F recognition site to repress the activation of target genes by other E2F members through mechanisms that involve promoter competition (Kherrouche *et al.*, 2001), we proposed that E2F6 might repress hypoxia-induced E2F1 transactivation by competing with

E2F1 for DNA binding sites, thereby inhibiting apoptosis. To test this, we performed ChIP assay to examine the effects of overexpression of E2F6 or its mutant E2F6 Δ C (deletion of C-terminal repressive domain, amino acids 220–281) and E2F6.E68 (a point mutant at DNA binding sites; Figure 6A) on the recruitment of E2F1 to its direct target promoter of Apaf-1 (Figure 6B). Western blot analysis showed that the cells transfected with E2F6 Δ C or E2F6.E68 had similar expression level of E2F6 as E2F6wt (Figure 6A). The Apaf-1 promoter was robustly enriched by the E2F1 antibody after CoCl₂ treatment, whereas the binding of E2F1 to Apaf-1 was significantly decreased by E2F6wt and partially by E2F6 Δ C transfection, but not by vector and E2F6.E68 transfection (Figure 6B).

To determine whether E2F6 attenuates the recruitment of E2F1 to Apaf-1 promoter by associating with E2F1 protein, we performed coimmunoprecipitation assay in cells expressing E2F6wt with or without CoCl₂ treatment by using normal mouse and goat IgG (control), or anti-E2F6 and anti-E2F1 antibodies. E2F6 could not be detected in the complexes with anti-E2F1, nor was E2F1 observed in the E2F6 immunoprecipitates with or without CoCl₂ treatment (Figure 6C).

To confirm whether E2F6 physically interacts with the promoter of Apaf-1, ChIP assay was performed in cells transfected with or without vector, E2F6wt, or its mutants with or without CoCl₂ treatment (800 μ M; 24 h). As shown in Figure 6D, the association of E2F6 with Apaf-1 promoter decreased in cells transfected with or without vector or E2F6.E68 after CoCl₂ treatment, whereas it was increased greatly in cells expressing E2F6wt or E2F6 Δ C. Furthermore, we examined the transcription of Apaf-1 during CoCl₂-induced apoptosis after transfection of E2F1wt, E2F6wt, or their mutants. Transcripts of Apaf-1 induced by CoCl₂ were further increased by E2F1wt transfection. Conversely, E2F6wt and transcriptionally inert E2F1 (1–374) greatly decreased CoCl₂-induced up-regulation of Apaf-1 mRNA level. However, such inhibitory effect of E2F6 lost in E2F6.E68-expressing cells and partially remained in E2F6 Δ C-expressing cells (Figure 6E). Meanwhile, we examined the effects of E2F6 and its mutants on CoCl₂-induced apoptosis. As shown in Figure 6F, E2F6 overexpression significantly inhibited CoCl₂-induced apoptosis and such inhibition totally disappeared in E2F6.E68-expressing cells, whereas partially reserved in E2F6 Δ C-expressing cells. Based on the different behaviors of E2F6, E2F6 Δ C, and E2F6.E68 in blocking the binding of E2F1 to its downstream promoter and in regulation of hypoxia-induced Apaf-1 transcription and apoptosis, we conclude that E2F6 represses E2F1 transactivation via competing with E2F1 for the DNA-binding site in proapoptotic Apaf-1 promoter, thereby inhibits hypoxia-induced apoptosis.

E2F6 Regulates E2F1 via its C-Terminal Repressive Domain and DNA Binding Activity

To further determine the importance of C-terminal repressive domain and DNA binding activity of E2F6 in the regulation of hypoxia-induced E2F1 activity and apoptosis, we examined the effects of two E2F6 mutants, E2F6 Δ C and E2F6.E68, on E2F1 expression and transactivation during CoCl₂-induced apoptosis. HEK293 cells with or without transfection of vector, E2F6wt, E2F6 Δ C, or E2F6.E68 were treated with or without CoCl₂ (800 μ M). CoCl₂-induced increase of E2F1 expression was significantly inhibited by E2F6wt, but not by E2F6 Δ C and E2F6.E68 (Figure 7A). Moreover, E2F-SV40-driven luciferase activity induced by CoCl₂ was repressed significantly by E2F6wt and partially by

Figure 6. E2F6 inhibits hypoxia-induced E2F1 transactivation and subsequent apoptosis via competing for E2F1-responsive promoter Apaf-1. (A) Schematic representation of the E2F6 mutants used (left) and expression of E2F6wt, E2F6ΔC, and E2F6.E68 detected by Western blotting using an anti-hemagglutinin (HA) antibody 24 h after transfection (right). (B) HEK293 cells with or without transfection of vector, E2F6wt, E2F6ΔC, or E2F6.E68 were treated with or without CoCl₂ (800 μM; 24 h). Treated cells were subjected to ChIP analysis, using antibody for E2F1 and normal mouse IgG, 24 h after CoCl₂ treatment (800 μM). Then, 0.2% of the total input of each sample was subjected to PCR to show that equal amounts of starting input chromatin were used. (C) Association of E2F6 and E2F1 in cells expressing E2F6wt with or without CoCl₂ treatment. Cell lysates were immunoprecipitated with normal mouse IgG, normal goat IgG, anti-E2F6, and anti-E2F1 followed by immunoblotting with indicated antibodies. Original lysates were used for positive immunoblotting analysis. (D) Cells treated as described in B were also subjected to ChIP analysis, using antibody for E2F6 and normal goat IgG. (E) Cells with or without transfection of vector, E2F1wt, E2F1 (1-374), E2F6wt, E2F6ΔC, or E2F6.E68 were treated with or without CoCl₂ (800 μM; 24 h). Transcripts of Apaf-1 were analyzed by RT-PCR. Similar results were obtained from three independent experiments. (F) Flow cytometric analysis (left) of propidium iodide-stained cells treated as described in B. Meanwhile, apoptosis was also evaluated by TUNEL assay (middle) and caspase-3 activation (right). n = 3 each.

E2F6ΔC, but not by E2F6.E68 (Figure 7B). These data confirm that DNA binding activity is essential for the inhibition of E2F1 by E2F6 during hypoxia-induced apoptosis, whereas the C terminus of E2F6 also contributes to the transcriptional repression of E2F1.

DISCUSSION

The present study demonstrates that 1) expression of E2F6 is down-regulated with a concurrent increase in E2F1 expression and transactivation during hypoxia-mimetic agent CoCl₂-induced apoptosis in HEK293 cells; 2) ectopic expression of E2F6 inhibits CoCl₂-induced apoptosis as well as up-regulation of E2F1 expression and transactivation, whereas RNA interference (RNAi)-mediated specific knockdown of E2F6 expression has opposite effects; 3) negative regulation of E2F6 on E2F1 expression is mediated by the transcriptional repression of E2F6 on E2F1 promoter; 4) the suppression of E2F6 on E2F1 transactivation involves the competition of E2F6 with E2F1 for the promoter of downstream proapoptotic gene Apaf-1; and 5) DNA binding activity is essential for the inhibitory effect of E2F6 on E2F1 and hypoxia-induced apoptosis, whereas C-terminal repressive domain also contributes to the inhibition of E2F6. These results confirm and extend our recent findings that E2F6 negatively regulates UV-induced apoptosis (Yang *et al.*,

2007), and they provide new insight into the functional role and regulatory mechanisms of E2F6 in hypoxia-induced apoptosis.

It has been shown that activation of E2F-dependent gene transcription is required for hypoxia-induced apoptosis (Hauck *et al.*, 2002). Because E2F6 is believed to repress E2F-responsive genes (Trimarchi and Lees 2002), it may be implicated in the regulation of hypoxia-induced apoptosis. In this study, we observed that CoCl₂-induced apoptosis is associated with down-regulated E2F6 expression in a dose- and time-dependent manner. Ectopic expression of E2F6 significantly represses hypoxia-induced apoptosis, whereas RNAi-mediated E2F6 knockdown sensitizes cells to hypoxia-induced apoptosis (Figure 2). These observations are consistent with our recent findings of negative regulation of E2F6 in UV-induced apoptosis (Yang *et al.*, 2007). Down-regulation of E2F6 may play an important role in tumor suppression and in radiotherapy by sensitizing damaged cells to death responses.

E2F1 mediates apoptosis caused by various stresses, including hypoxia (Field *et al.*, 1996; DeGregori *et al.*, 1997; Hauck *et al.*, 2002). Because E2F6 represses the activity of E2F1 promoter through recruiting the polycomb transcriptional repressor complex (Gaubatz *et al.*, 1998; Trimarchi *et al.*, 2001), E2F6 may regulate apoptosis through E2F1. However, no study so far has directly demonstrated the func-

Figure 7. Regulation of E2F1 by E2F6 during CoCl_2 -induced apoptosis requires its C-terminal domain and DNA binding ability. HEK293 cells with or without transfection of vector, E2F6wt, E2F6ΔC, or E2F6.E68 were treated with or without CoCl_2 (800 μM ; 24 h). (A) Western blot analysis for E2F1 and actin to control for protein loading. Representative image (top) and averaged data (bottom). (B) Analysis of luciferase activity driven by E2F-SV40 in cells treated as described above.

tional relationship between them during apoptosis. Here, we show that the expression of E2F6 was down-regulated with a concurrent increase in E2F1 expression during CoCl_2 -induced apoptosis. Combined with the results of overexpression and RNAi-mediated knockdown of E2F6, we conclude that the negative regulation of E2F6 on CoCl_2 -induced apoptosis correlates with its repression of E2F1 expression up-regulated by hypoxia. This is possibly mediated by the transcriptional repression of E2F6 on the E2F1 promoter (Gaubatz *et al.*, 1998). Such mechanism is confirmed by our observation that the recruitment of E2F6 to the E2F1 promoter is mitigated by CoCl_2 treatment and decreased further by siE2F6, but increased by E2F6 overexpression (Figure 4). Therefore, up-regulation of E2F1 expression by hypoxia results from the reduced transrepression of E2F6, i.e., E2F6 inhibits hypoxia-induced apoptosis through the transcriptional repression of E2F1. Moreover, the observation of decreased antiapoptotic ability of E2F6 in cells stably expressing E2F1 further confirms the regulation of apoptosis by E2F6 via alteration of E2F1 expression. In addition, the regulation of E2F6 on E2F1 expression during apoptosis requires its C-terminal repressive domain and DNA binding activity because both E2F6.E68 with mutant DNA binding sites and E2F6ΔC with deletion of C-terminal repressive domain are unable to repress E2F1 expression (Figure 7A). This result is consistent with the report that transcriptional repression of E2F6 depends on the integrity of a C-terminal repressive domain and on its DNA binding activity (Gaubatz *et al.*, 1998).

Transcriptional activation and derepression of E2F-regulated promoters are involved in hypoxia-induced apoptosis (Hauck *et al.*, 2002). Similar observation is obtained in the present study. We further observed that the antiapoptotic roles of E2F6 involve significant suppression of the transactivation of E2F1. Transrepression of E2F1-controlled genes is reported to be beneficial to cellular survival (Hauck *et al.*, 2002; Ma *et al.*, 2003; Rogoff *et al.*, 2004). In this regard, the repression of E2F1 transactivation by E2F6 would contribute to its antiapoptotic activity. Such repression could be a consequence of decreased expression of E2F1 by E2F6. In contrast, hypoxia-induced down-regulation of E2F6 would increase E2F1 expression and subsequent transactivation, and thereby promote the apoptotic response to hypoxia.

Interestingly, E2F6 could still partially repress hypoxia-induced E2F1 transactivation and subsequent apoptosis when E2F1 overexpressed. This suggests that E2F6-inhibition of E2F1 transactivation is not only a consequence of decreased expression of E2F1. As we know, E2F6 possesses high DNA binding activity, displaying a preference for a TTTCCCGC E2F recognition site, which is slightly different from the E2F consensus site derived from the E2 promoter (TTTCGCGC) (Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998). Thus, E2F6 can compete with other E2F proteins for the same DNA binding site and inhibit the activation of E2F-responsive promoter as a dominant-negative repressor (Gaubatz *et al.*, 1998). Here, we further confirmed that E2F6 regulates hypoxia-induced E2F1 transactivation and apoptosis by competing with E2F1 for E2F1-responsive proapoptotic promoters. The results of ChIP assay with anti-E2F6 antibody showed that E2F6 can physically interact with Apaf-1 promoter during hypoxia. This interpreted the observation that the recruitment of E2F1 to the promoter of downstream proapoptotic gene Apaf-1 is significantly mitigated by E2F6wt overexpression and partially by E2F6ΔC overexpression, but not by E2F6.E68 overexpression. No effect of E2F6.E68 on the binding of E2F1 to Apaf-1 promoter could be explained by the loss of E2F6 DNA binding activity that seems to be necessary for the competition with E2F1 at its recognition site and for transcriptional repression (Gaubatz *et al.*, 1998; Trimarchi *et al.*, 2001). The partial inhibition of E2F6ΔC on the binding of E2F1 to Apaf-1 promoter can be interpreted by lack of the C-terminal repressive domain that is required for the down-regulation of E2F1 expression by E2F6 (Gaubatz *et al.*, 1998; Trimarchi *et al.*, 2001; Kherrouche *et al.*, 2001). However, E2F6ΔC still preserves DNA binding activity that is essential for the competition with E2F proteins (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998). The decreased recruitment of E2F1 to the promoter by E2F6 might also be involved in the block of the binding sites of E2F1 with Apaf-1 promoter by E2F6 via its associating with E2F1 protein. However, our coimmunoprecipitation experiments indicate that E2F6 could not interact with E2F1 protein no matter with or without CoCl_2 treatment. Moreover, hypoxia-induced up-regulation of Apaf-1 transcription and subsequent apoptosis are reduced by E2F6wt overexpression and partially by E2F6ΔC overexpression, but not by E2F6.E68 overexpression, a consistent observation with the results from our ChIP assays. E2F1 directly activates the expression of the Apaf-1 gene (Furukawa *et al.*, 2002). During apoptosis, Apaf-1 assembles with cytochrome *c*, a mitochondrial protein released upon apoptotic signals, and activates procaspase-9, leading to the activation of downstream effector caspases, including caspase-3, caspase-6, and caspase-7 (Moroni *et al.*, 2001; Furukawa *et al.*, 2002). The observation of the alteration of Apaf-1 transcription provides functional evidence for the

Figure 8. Model of negative regulation of E2F6 on hypoxia-induced apoptosis via E2F1. E2F6 represses hypoxia-induced apoptosis by abrogating the increases of the expression and transactivation of E2F1. The action of E2F6 on E2F1 is related to the transrepression of E2F1 promoter via C-terminal repressive domain and the competition with E2F1 for DNA binding sites of downstream proapoptotic genes. Such regulation of E2F1 by E2F6 results in down-regulation of E2F1-responsive proapoptotic genes and subsequent inhibition of apoptosis.

competitive inhibition of E2F1 transactivation by E2F6 clarified by ChIP assay and also suggests that the down-regulation of Apaf-1 is also involved in the antiapoptotic roles of E2F6.

In conclusion, we demonstrate here that E2F6 negatively regulates hypoxia-induced apoptosis via control of E2F1 in HEK293 cells (Figure 8). Down-regulation of E2F6 sensitizes cells to hypoxia-induced apoptosis by increasing the expression and transactivation of E2F1 and subsequently up-regulating E2F1-dependent proapoptotic genes. The regulation of E2F6 to E2F1 transactivation is mediated not only by the transrepression of E2F1 expression via its C-terminal repressive domain but also by the competition with E2F1 for DNA binding sites. The potential roles of E2F6 in tumor suppression with severe tumor hypoxia via its modulation of E2F1 remain to be examined.

ACKNOWLEDGMENTS

We are grateful to Prof. David M. Livingston for providing plasmids pcDNA3-HA-E2F6 and pcDNA3-HA-E2F6ΔC and to Prof. Ludger Hauck for offering plasmids pcDNA3.1-E2F1 and pcDNA3.1-E2F1 (1-374). This study was supported in part by grants from National Basic Research Program of China (2006CB504100), Major and General programs of the National Natural Sciences Foundation of China (30393133 and 30370536), and Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-75).

REFERENCES

Cartwright, P., Muller, H., Wagener, C., Holm, K., and Helin, K. (1998). E2F-6, a novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* 17, 611–623.

Coquelle, A., Toledo, F., Stern, S., Bieth, A., and Debatisse, M. (1998). A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. *Mol. Cell* 2, 259–265.

DeGregori, J. (2002). The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochim. Biophys. Acta* 1602, 131–150.

DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc. Natl. Acad. Sci. USA* 94, 7245–7250.

Fan, Y., Wu, D., Jin, L., and Yin, Z. (2005). Human glutamylcysteine synthetase protects HEK293 cells against UV-induced cell death through inhibition of c-Jun NH2-terminal kinase. *Cell Biol. Int.* 29, 695–702.

Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* 85, 549–561.

Furukawa, Y., Nishimura, N., Furukawa, Y., Satoh, M., Endo, H., Iwase, S., Yamada, H., Matsuda, M., Kano, Y., and Nakamura, M. (2002). Apaf-1 is a mediator of E2F-1-induced apoptosis. *J. Biol. Chem.* 277, 39760–39768.

Gaubatz, S., Wood, J. G., and Livingston, D. M. (1998). Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6. *Proc. Natl. Acad. Sci. USA* 95, 9190–9195.

Gee, M. S. *et al.* (1999). Hypoxia-mediated apoptosis from angiogenesis inhibition underlies tumor control by recombinant interleukin 12. *Cancer Res.* 59, 4882–4889.

Guo, M., Song, L. P., Jiang, Y., Liu, W., Yu, Y., and Chen, G. Q. (2006). Hypoxia-mimetic agents desferrioxamine and cobalt chloride induce leukemic cell apoptosis through different hypoxia-inducible factor-1α independent mechanisms. *Apoptosis* 11, 67–77.

Guy, C. T., Zhou, W., Kaufman, S., and Robinson, M. O. (1996). E2F-1 blocks terminal differentiation and causes proliferation in transgenic megakaryocytes. *Mol. Cell Biol.* 16, 685–693.

Harbour, J. W., and Dean, D. C. (2000). Rb function in cell-cycle regulation and apoptosis. *Nat. Cell Biol.* 2, E65–E67.

Harris, A. L. (2002). Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer* 2, 38–47.

Hauck, L., Hansmann, G., Dietz, R., and von Harsdorf, R. (2002). Inhibition of hypoxia-induced apoptosis by modulation of retinoblastoma protein-dependent signaling in cardiomyocytes. *Circ. Res.* 91, 782–789.

Holmberg, C., Helin, K., Sehested, M., and Karlstrom, O. (1998). E2F-1-induced p53-independent apoptosis in transgenic mice. *Oncogene* 17, 143–155.

Hsieh, J. K., Yap, D., O'Connor, D. J., Fogal, V., Fallis, L., Chan, F., Zhong, S., and Lu, X. (2002). Novel function of the cyclin A binding site of E2F in regulating p53-induced apoptosis in response to DNA damage. *Mol. Cell Biol.* 22, 78–93.

Huang, L. E., Bindra, R. S., Glazer, P. M., and Harris, A. L. (2007). Hypoxia-induced genetic instability—a calculated mechanism underlying tumor progression. *J. Mol. Med.* 85, 139–148.

Jung, J. Y., and Kim, W. J. (2004). Involvement of mitochondrial- and Fas-mediated dual mechanism in CoCl₂-induced apoptosis of rat PC12 cells. *Neurosci. Lett.* 371, 85–90.

Kherrouche, Z., Begue, A., Stehelin, D., and Monte, D. (2001). Molecular cloning and characterization of the mouse E2F6 gene. *Biochem. Biophys. Res. Commun.* 288, 22–33.

Kowalik, T. F., DeGregori, J., Leone, G., Jakoi, L., and Nevins, J. R. (1998). E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2. *Cell Growth Differ.* 9, 113–118.

Lyons, T. E., Salih, M., and Tuana, B. S. (2005). Activating E2Fs mediate the transcriptional regulation of the human E2F6 repressor. *Am. J. Physiol. Cell Physiol.* 290, C189–C199.

Ma, Y., Cress, W. D., and Haura, E. B. (2003). Flavopiridol-induced apoptosis is mediated through up-regulation of E2F1 and repression of Mcl-1. *Mol. Cancer Ther.* 2, 73–81.

Moroni, M. C., Hickman, E. S., Lazzerini, D. E., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. (2001). Apaf-1 is a transcriptional target for E2F and p53. *Nat. Cell Biol.* 3, 552–558.

Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D., and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. *Genes Dev.* 15, 267–285.

Nagarajah, N. S., Vigneswaran, N., and Zacharias, W. (2004). Hypoxia-mediated apoptosis in oral carcinoma cells occurs via two independent pathways. *Mol. Cancer* 3, 38.

Perou, C. M. *et al.* (2000). Molecular portraits of human breast tumours. *Nature* 406, 747–752.

Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. USA* 91, 10918–10922.

Rogoff, H. A., Pickering, M. T., Frame, F. M., Debatis, M. E., Sanchez, Y., Jones, S., and Kowalik, T. F. (2004). Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. *Mol. Cell Biol.* 24, 2968–2977.

- Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* 90, 425–435.
- Slansky, J. E., Li, Y., Kaelin, W. G., and Farnham, P. J. (1993). A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol. Cell Biol.* 13, 1610–1618.
- Trimarchi, J. M., Fairchild, B., Verona, R., Moberg, K., Andon, N., and Lees, J. A. (1998). E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc. Natl. Acad. Sci. USA* 95, 2850–2855.
- Trimarchi, J. M., Fairchild, B., Wen, J., and Lees, J. A. (2001). The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proc. Natl. Acad. Sci. USA* 98, 1519–1524.
- Trimarchi, J. M., and Lees, J. A. (2002). Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* 3, 11–20.
- Weinmann, M., Jendrossek, V., Handrick, R., Guner, D., Goecke, B., and Belka, C. (2004). Molecular ordering of hypoxia-induced apoptosis: critical involvement of the mitochondrial death pathway in a FADD/caspase-8 independent manner. *Oncogene* 23, 3757–3769.
- Wu, X., Yang, Z., Liu, Y., and Zou, Y. (2005). Preferential localization of hyperphosphorylated replication protein A to double strand break repair and checkpoint complexes upon DNA damage. *Biochem. J.* 391, 473–480.
- Wyllie, A. H., and Morris, R. G. (1982). Hormone-induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am. J. Pathol.* 109, 78–87.
- Yang, H. L., Dong, Y. B., Elliott, M. J., Liu, T. J., and McMasters, K. M. (2000). Caspase activation and changes in Bcl-2 family member protein expression associated with E2F-1-mediated apoptosis in human esophageal cancer cells. *Clin. Cancer Res.* 6, 1579–1589.
- Yang, W. W., Wang, Z. H., Zhu, Y., and Yang, H. T. (2007). E2F6 negatively regulates ultraviolet-induced apoptosis via modulation of BRCA1. *Cell Death Differ.* 14, 807–817.
- Zhan, Q., Jin, S., Ng, B., Plisket, J., Shangary, S., Rathi, A., Brown, K. D., and Baskaran, R. (2002). Caspase-3 mediated cleavage of BRCA1 during UV-induced apoptosis. *Oncogene* 21, 5335–5345.
- Zhang, F. B., Li, L., Fang, B., Zhu, D. L., Yang, H. T., and Gao, P. J. (2005). Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells. *Biochem. Biophys. Res. Commun.* 336, 784–792.
- Zou, W., Zeng, J., Zhuo, M., Xu, W., Sun, L., Wang, J., and Liu, X. (2002). Involvement of caspase-3 and p38 mitogen-activated protein kinase in cobalt chloride-induced apoptosis in PC12 cells. *J. Neurosci. Res.* 67, 837–843.