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Iron Chelation by Clinically Relevant Anthracyclines: Alteration in Expression of Iron-Regulated Genes and Atypical Changes in Intracellular Iron Distribution and Trafficking

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ABSTRACT

Anthracyclines are effective anticancer agents. However, their use is limited by cardiotoxicity, an effect linked to their ability to chelate iron and to perturb iron metabolism (*Mol Pharmacol* **68:**261–271, 2005). These effects on iron-trafficking remain poorly understood, but they are important to decipher because treatment for anthracycline cardiotoxicity uses the chelator, dexrazoxane. Incubation of cells with doxorubicin (DOX) up-regulated mRNA levels of the iron-regulated genes *transferrin receptor-1* (*TfR1*) and *N-myc downstream-regulated gene-1* (*Ndrg1*). This effect was mediated by iron depletion, because it was reversed by adding iron and it was prevented by saturating the anthracycline metal binding site with iron. However, DOX did not act like a typical chelator, because it did not induce cellular iron mobilization. In the presence of DOX and ^{59}Fe-transferrin, iron-trafficking studies demonstrated ferritin-^{59}Fe accumulation and decreased cytosolic-^{59}Fe incorporation. This could induce cytosolic iron deficiency and increase TfR1 and Ndrg1 mRNA. Up-regulation of TfR1 and Ndrg1 by DOX was independent of anthracycline-mediated radical generation and occurred via hypoxia-inducible factor-1α-independent mechanisms. Despite increased TfR1 and Ndrg1 mRNA after DOX treatment, this agent decreased TfR1 and Ndrg1 protein expression. Hence, the effects of DOX on iron metabolism were complex because of its multiple effector mechanisms.

Anthracyclines are known iron chelators (Fig. 1A), but their effects on cellular iron metabolism are poorly understood (Xu et al., 2005). These compounds have high activity against hematological malignancies and a variety of other tumors (Xu et al., 2005). However, a major problem is their cardiotoxic effect at high cumulative doses that limit their clinical use (Gianni and Myers, 1992). The mechanism of anthracycline-mediated cardiotoxicity is unclear (Kaiserová et al., 2007), probably because of the multiple effects of these agents, including DNA binding, intercalation, alkylation, inhibition of topoisomerase II, and the generation of reactive oxygen species (ROS) (Gianni and Myers, 1992).

Previous studies have indicated that interactions of anthracyclines with cellular iron pools are of great importance in their cardiotoxic effects and in their ability to induce apoptosis (Hershko et al., 1993; Kotamraju et al., 2002). Anthracyclines such as doxorubicin (DOX) can directly chelate Fe(III), forming an iron complex with an overall association constant of 10^{33} (May et al., 1980; Beraldo et al., 1985). Hershko and associates demonstrated that iron loading potentiates the cardiotoxic effects of anthracyclines (Hershko et al., 1993; Link et al., 1996), and some chelators can prevent this (Kaiserová et al., 2007). In fact, the clinical intervention for anthracycline cardiotoxicity involves the chelator dexrazoxane (Xu et al., 2005). Hence, understanding the mechanisms of how anthracyclines interfere with iron metabolism is a key for preventing cardiotoxicity.

Iron is transported by its binding to transferrin (Tf), and it is delivered to cells via binding to the transferrin receptor-1 (TfR1) (Xu et al., 2005). After this, Tf is internalized by receptor-mediated endocytosis, and the iron is released. Iron is then transported into the cell, and it becomes part of the intracellular iron pool. Iron that is not used for metabolic

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ABBREVIATIONS: ROS, reactive oxygen species; DOX, doxorubicin; Tf, transferrin; TfR1, transferrin receptor-1; ferritin-H, ferritin heavy chain; ferritin-L, ferritin light chain; IRP, iron regulatory protein; DAU, daunorubicin; EPI, epirubicin; Ndrg1, *N*-myc downstream-regulated gene-1; DFO, desferrioxamine; PIH, pyridoxal isonicotinoyl hydrazone; MEF, murine embryo fibroblast; HIF-1α, hypoxia inducible factor-1α; apo-Tf, apotransferrin; PAGE, polyacrylamide gel electrophoresis; FPLC, fast pressure liquid chromatography; RT, reverse transcriptase; PCR, polymerase chain reaction; FAC, ferric ammonium citrate; CON, control medium; F, fraction; VEGF, vascular endothelial growth factor; SOD, superoxide dismutase; RS, radical scavenger(s); 5-i-DAU, 5-imino-daunorubicin.

requirements is stored in ferritin, a polymeric protein consisting of -H- and -L subunits (Minotti et al., 2004a).

The translation of TfR1 and ferritin is regulated by the binding of iron regulatory proteins (IRPs) to iron-responsive elements present in the 5′- or 3′-untranslated regions of TfR1 and ferritin mRNAs (Xu et al., 2005). There are two IRPs, IRP1 and IRP2, and anthracyclines have been shown to decrease their mRNA binding activity in most cell types (Minotti et al., 2001; Kwok and Richardson, 2002).

Apart from the effect of anthracyclines on IRP mRNA binding activity, these agents have been shown to affect a variety of molecules and metabolic pathways involved in iron metabolism (Minotti et al., 2004a). For example, DOX is known to directly bind iron, and it has been reported to remove iron from isolated ferritin, Tf, and microsomal membranes (Xu et al., 2005). However, using intact cells, we showed that incubation of many cell types with anthracyclines (Fig. 1A), such as DOX, daunorubicin (DAU), or epirubicin (EPI), induced ferritin iron loading as a result of their ability to prevent iron release from this protein (Kwok and Richardson, 2003, 2004). The precise mechanism by which anthracyclines prevent ferritin-iron mobilization was not clear, but inhibition of protein synthesis, proteasomal/lysosomal activity, or both were suggested to be involved (Kwok and Richardson, 2004). Incubation of cells with DOX also increased ferritin expression (Kwok and Richardson, 2003; Corna et al., 2004) and this was suggested to act as a protective response against the ability of DOX to generate ROS (Corna et al., 2004).

In the present study, we demonstrate for the first time that anthracyclines act as atypical chelators, having a number of effects on cellular iron metabolism and the expression of iron-regulated genes, including *TfR1*, *N-myc downstream regulated gene-1* (*Ndrg1*), and *ferritin*. Although iron chelation mediated by anthracyclines increased TfR1 and Ndrg1 mRNA expression, the protein levels of these molecules were decreased. Paradoxically, ferritin protein expression increased after incubation with DOX, as did ferritin iron accumulation, suggesting that anthracyclines have a selective effect on gene expression. The effects of anthracyclines on cellular iron metabolism were complex, probably because they act on multiple molecular targets.

Materials and Methods

Reagents. Desferrioxamine (DFO) was from Novartis (Basel, Switzerland). DOX, DAU, and EPI were from Pharmacia (Sydney, Australia). All other reagents were from Sigma-Aldrich (St. Louis, MO). Pyridoxal isonicotinoyl hydrazone (PIH) was synthesized and characterized by standard methods (Ponka et al., 1979; Richardson et al., 1995).

Cell Culture. Cell lines were obtained from the American Type Culture Collection (Manassas, VA). Murine embryonic fibroblasts

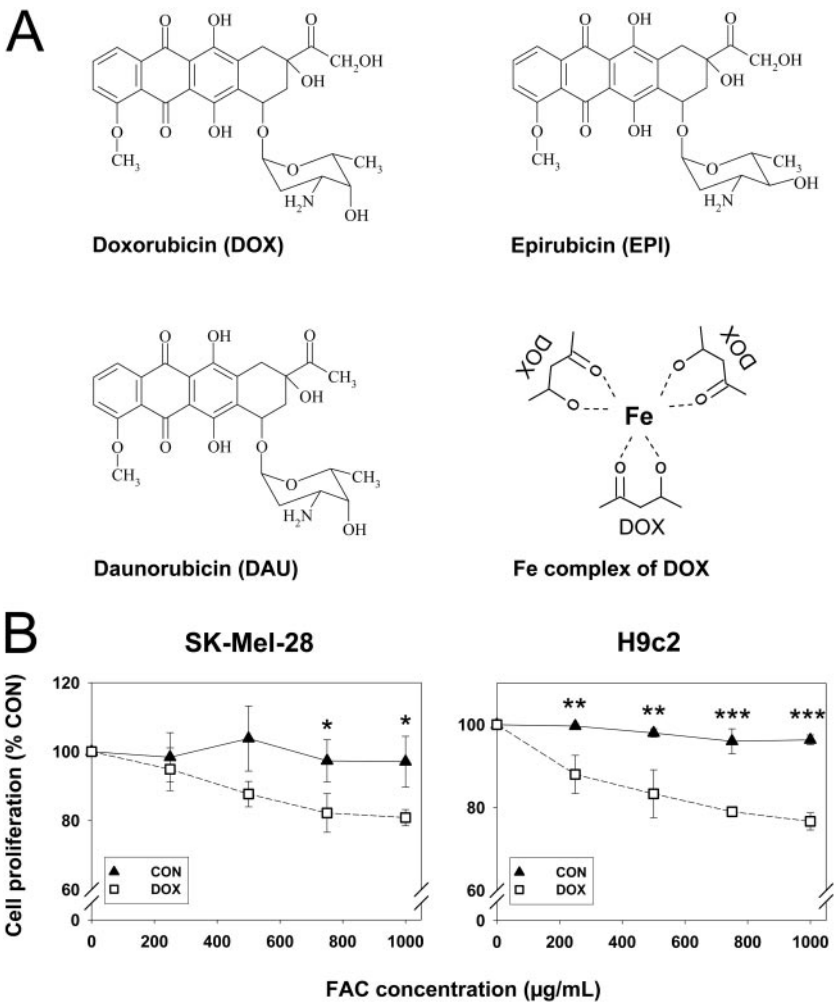

Fig. 1. A, schematic illustration of DOX, EPI, DAU, and the iron complex of DOX. B, preincubation with DOX could not protect cells from the toxicity of subsequent iron loading by FAC. Human SK-Mel-28 melanoma cells or rat H9c2 cardiomyocytes were preincubated with CON or 5 μM DOX for 24 h at 37°C, and then they were washed. The cells were then reincubated for 16 h at 37°C with CON containing increasing concentrations of FAC (250, 500, 750, and 1000 μg/ml). Cell viability was examined using trypan blue staining. The percentage of viable cells in control or DOX-pretreated group was plotted compared with the relative control, which was set as 100%. Results are mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus control values (Student's t test).

(MEFs) from wild-type and homozygous *hypoxia inducible factor-1α* (*HIF-1α*) knockout mice were obtained from Dr. R. Johnson (University of California San Diego, La Jolla, CA). Cells were grown using standard conditions (Kwok and Richardson, 2003; Le and Richardson, 2004).

^{59}Fe-Transferrin. Human apotransferrin (apo-Tf; Sigma-Aldrich) was labeled with ^{59}Fe (PerkinElmer Life and Analytical Sciences, Waltham, MA) to produce $^{59}Fe_2$-transferrin (^{59}Fe-Tf), as described previously (Kwok and Richardson, 2003). In brief, apo-Tf was labeled with iron using the ferric-nitrilotriacetate complex at a ratio of 1 iron to 10 nitrilotriacetate. This complex was prepared in 0.1 M HCl, and then this solution adjusted to pH 7.4 using 1.4% $NaHCO_3$. This solution was added to apo-Tf, and then the complex was incubated for 1 h at 37°C. Unbound iron was removed by exhaustive vacuum dialysis against 0.15 M NaCl adjusted to pH 7.4 using 1.4% $NaHCO_3$. The saturation of Tf with iron was monitored by UV-Vis spectrophotometry, with the absorbance at 280 nm (protein) being compared with that at 465 nm (iron binding site). In all studies, fully saturated diferric Tf was used.

Effect of Anthracyclines on ^{59}Fe Efflux from Intact Prelabeled Cells. Experiments examining the ability of agents to mobilize cellular ^{59}Fe were performed using standard techniques (Richardson et al., 1995). In brief, cells were prelabeled with 0.75 μM ^{59}Fe-Tf for 3 h at 37°C. This medium was aspirated, and the cell monolayer was washed four times with ice-cold phosphate-buffered saline. The cells were then reincubated for 24 h at 37°C with medium in the presence or absence of the agents to be tested. After this incubation, the overlying media containing released ^{59}Fe were collected in gamma-counting tubes. The cells were removed from the petri dishes and placed in a separate set of tubes. Radioactivity was measured in both the cell pellet and supernatant using a Wallac WIZARD 1480 3″ gamma-counter (PerkinElmer Wallac, Turku, Finland).

Assay for Examining the Ability of Anthracyclines to Bind ^{59}Fe from Cell Lysates. Established methods (Watts and Richardson, 2002) using ultrafiltration through a 5-kDa cut-off filter were used to determine the efficacy of anthracyclines at mobilizing ^{59}Fe from SK-Mel-28 cell lysates. In brief, cells were labeled with 0.75 μM ^{59}Fe-Tf for 3 h at 37°C and placed on a tray of ice. The medium was then decanted, and the cell monolayer was washed four times with ice-cold phosphate-buffered saline. The cells were lysed by one freeze-thaw cycle, and then they were detached from the flask using a Teflon spatula in the presence of the nonionic detergent Triton X-100 (1.5%) at 4°C. The supernatant was obtained by centrifugation at 16,500*g* for 30 min at 4°C, and then it was incubated for 3 h at 37°C with the agents of interest. After this incubation, the samples were subjected to centrifugation at 4°C through a 5-kDa exclusion filter (Millipore Corporation, Billerica, MA). After centrifugation, the eluate was taken to estimate ^{59}Fe levels.

Determination of Intracellular Iron Distribution Using Native PAGE-^{59}Fe Autoradiography. Native gradient PAGE-^{59}Fe autoradiography was performed using established techniques (Babusiak et al., 2005). In brief, cells labeled with 0.75 μM ^{59}Fe-Tf were lysed at 4°C in buffer containing 1.5% Triton X-100, 0.14 M NaCl, and 20 mM HEPES, pH 8, supplemented with an EDTA-free protease inhibitor cocktail (Roche, Penzberg, Germany). Samples were then vortexed and centrifuged at 16,000*g* for 45 min at 4°C. The supernatants were loaded onto a native (3–12%) gradient PAGE gel (100 μg of protein per lane), and electrophoresis was performed at 20 mA/gel overnight at 4°C. Gels were subsequently dried, and autoradiography was performed. Bands on X-ray film were quantified by scanning densitometry, and they were analyzed using the Quantity One program (Bio-Rad Laboratories, Hercules, CA).

Fast Pressure Liquid Chromatography and Native Gradient PAGE. SK-Mel-28 cells were incubated with or without 2 μM DOX in the presence of 0.75 μM ^{59}Fe-Tf. Cells were then washed four times and lysed on ice in 20 mM HEPES, 140 mM NaCl, and 1.5% Triton X-100, pH 8.0. Cell lysates were centrifuged at 16,500*g*, and the supernatant was loaded onto a Superdex 200 10/300 GL column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and proteins were eluted with 20 mM HEPES and 140 mM NaCl, pH 8.0, using FPLC (Bio-Rad Laboratories). Fractions (1 ml) were collected, and radioactivity was examined using the gamma-counter as described above.

Fractions were concentrated and desalted using the microfilter units described above, with a 5-kDa cut-off. Concentrated fractions were then separated and examined via native gradient PAGE-^{59}Fe autoradiography (Babusiak et al., 2005).

RNA Isolation, Reverse Transcriptase-PCR, and Western Analysis. RNA isolation and reverse transcriptase (RT)-PCR were performed by published procedures (Le and Richardson, 2004) using the primers in Table 1. Western blot analysis was done as described previously (Le and Richardson, 2004).

[^{3}H]Leucine Incorporation Assay. To assess protein synthesis, [^{3}H]leucine assays were performed using standard procedures (Kwok and Richardson, 2004).

Statistical Analysis. Data were compared using the Student's *t* test. Results were considered statistically significant when $p < 0.05$.

Results

Challenge of DOX-Treated Cells with Iron Leads to Decreased Viability. Incubation of cells with anthracyclines leads to alterations in iron metabolism (Kwok and Richardson, 2003; Corna et al., 2004; Xu et al., 2005). Initial experiments examined whether DOX altered the ability of cells to protect against a challenge with excess iron. In these studies, SK-Mel-28 melanoma cells or H9c2 cardiomyocytes were preincubated for 24 h at 37°C with 5 μM DOX, and then they were reincubated for 16 h at 37°C with increasing concentrations of ferric ammonium citrate (FAC; 250-1000 μg/

TABLE 1
Primers for amplification of human and mouse mRNA

Organism and Pair No.	Target Gene	GenBank Accession No.	Oligonucleotides (5′-3′)		Product Size
			Forward	Reverse	
					bp
Homo sapiens					
1	*β-Actin Ndrg1*	NM_001101	CCCGCCGCCAGCTCACCATGG	AAGGTCTCAAACATGATCTGGGTC	397
2	*TfR1*	NM_006096	CCCTCGCGTTAGGCAGGTGA	AGGGGTACATGTACCCTGCG	370
3		NM_003234	GCTCGGCAAGTAGATGGC	TTGATGGTGCTGGTGAAG	359
Mus musculus					
4	*β-Actin*	NM_007393	CCCGCCACCAGTTCGCCATGG	AAGGTCTCAAACATGATCTGGGTC	397
5	*HIF-1α*	NM_010431	CTGGATGCCGGTGGTCTAGACAGT	CGAGAAGAAAAAGATGAGTTCTGAACGTCG	217
6	*Ndrg1*	NM_008681	TGCTTGCTCATTAGGTGTGTGATAGC	CCATCCTGAGATCTTAGAGGCAGC	581
7	*TfR1*	NM_011638	TCCCGAGGGTTATGTGGC	GGCGGAAACTGAGTATGATTGA	324
8	*VEGF1*	NM_009505	CCATGCCAAGTGGTCCCAG	GTCTTTCTTTGGTCTGCATTCACAT	346

ml) that donates iron to cells (Corna et al., 2004). Direct cell counts and viability were then assessed using trypan blue staining. These incubation conditions were identical to those used by others to demonstrate the protective effect against an iron challenge of preincubating H9c2 cells with DOX (Corna et al., 2004).

In contrast to results by others (Corna et al., 2004), preincubation with DOX did not protect against an iron challenge. In fact, it resulted in significantly decreased viability of H9c2 and SK-Mel-28 cells at FAC concentrations >500 μg/ml (Fig. 1B). Hence, DOX decreased the ability of cells to appropriately accommodate the iron load and to prevent its cytotoxic effects.

DOX Increases mRNA Expression of the Iron-Responsive Genes *TfR1* and *Ndrg1*. To further understand how DOX affects iron metabolism, we investigated the effect of DOX on TfR1 expression (Fig. 2). To examine this, SK-Mel-28 cells were initially used (Fig. 2A) because their iron metabolism is well characterized, and these cells were previously used to assess the effects of DOX on iron trafficking (Kwok and Richardson, 2002, 2003, 2004).

Incubation of cells for 24 h at 37°C with the iron chelator DFO at 100 μM was used as a positive control because it increases TfR1 mRNA and protein expression (Hentze and Kuhn, 1996). Incubation of SK-Mel-28 cells with DFO increased TfR1 expression >6-fold compared with the control (Fig. 2A). DOX (0.5–5 μM) induced a dose-dependent increase in TfR1 mRNA up to 2 μM, at which point its expression was 3-fold greater than the control (Fig. 2A). The up-regulation of TfR1 mRNA after incubation with DOX was relatively marked considering the dose maximally up-regulating its expression (2 μM) was 50-fold lower than that of DFO (100 μM; Fig. 2A). At 5 μM DOX, TfR1 expression then decreased, and this down-regulation may be related to the drug acting as a transcriptional inhibitor (Tarr and van Helden, 1990).

The increase in TfR1 mRNA after incubation of SK-Mel-28 cells with DOX may be mediated via its ability to act as an iron chelator (May et al., 1980; Gianni and Myers, 1992). Examination of four other cell types also demonstrated that DOX increased TfR1 mRNA, although the dose dependence and extent of up-regulation was different for each cell type (Fig. 2, B–E). In general, maximum TfR1 mRNA expression was found at 1 to 2 μM DOX.

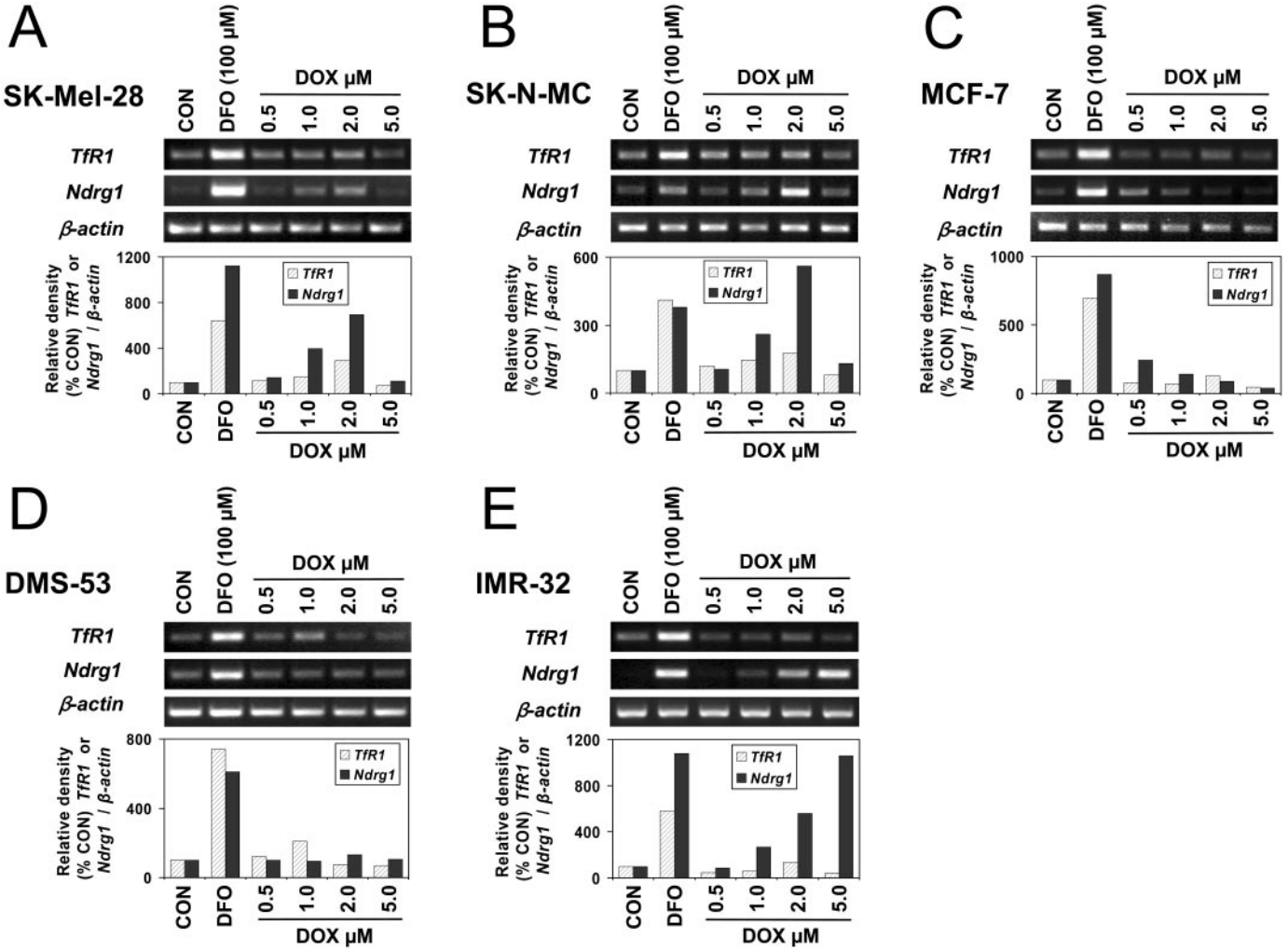

Fig. 2. DOX up-regulates TfR1 and Ndrg1 mRNA levels in a concentration-dependent manner in a variety of tumor cell lines. SK-Mel-28 melanoma cells (A), SK-N-MC neuroepithelioma cells (B), MCF-7 breast cancer cells (C), DMS-53 lung carcinoma cells (D), and IMR-32 neuroblastoma cells (E) were incubated with CON, 100 μM DFO, or DOX at increasing concentrations (0.5, 1, 2, and 5 μM) for 24 h at 37°C. The mRNA was then extracted, and the expression of TfR1 and Ndrg1 mRNA levels was evaluated using RT-PCR. Densitometry was performed, and gene expression was then calculated relative to the β-actin control. Results are a typical experiment from three experiments performed.

Iron chelation is known to typically up-regulate TfR1 mRNA by the IRP-iron-responsive element mechanism (Hentze and Kuhn, 1996). However, the lower DOX concentrations (1–2 μM) used in this study have little effect on IRP mRNA binding activity in SK-Mel-28 cells (Kwok and Richardson, 2002). Thus, it was unclear whether this mechanism was responsible for DOX-mediated up-regulation of TfR1 mRNA (Fig. 2A). Apart from the IRPs, other iron-sensing mechanisms could be responsible for altering TfR1 mRNA expression. Considering this, HIF-1α protein expression is known to increase after iron chelation or hypoxia, and it can transcriptionally up-regulate TfR1 and other genes (Beerepoot et al., 1996; Bianchi et al., 1999; Lok and Ponka, 1999; Le and Richardson, 2004).

To determine whether HIF-1α activity is affected by anthracyclines, we examined the effect of DOX on HIF-1α target gene expression. These studies investigated the metastasis suppressor *Ndrg1* that is known to be up-regulated after iron chelation by HIF-1α (Le and Richardson, 2004). This gene was important to assess as its antiproliferative and -metastatic effects could be relevant to DOX activity (Kovacevic and Richardson, 2006). Similarly to TfR1, DOX also increased Ndrg1 mRNA expression in SK-Mel-28 cells (Fig. 2A). Assessment of four other cell types demonstrated that as for SK-Mel-28 cells, DFO increased Ndrg1 mRNA (Fig. 2, B–E). Again, the response of Ndrg1 mRNA levels to increasing DOX concentrations was variable in terms of dose-response and the extent of up-regulation between cell types (Fig. 2, A–E). The differences in gene expression between these cell types may relate to variation in the uptake and metabolism of DOX. Of interest, the mRNA levels of another HIF-1α target gene, namely, *Nip3* (Bruick, 2000), was also up-regulated by DOX in a similar way to TfR1 and Ndrg1 (data not shown). Further studies then examined the effect of other anthracyclines on iron metabolism using the SK-Mel-28 cell type.

Daunorubicin and Epirubicin Also Increase TfR1 and Ndrg1 mRNA Expression. DAU and EPI are structurally related to DOX (Fig. 1A), and they also increased TfR1 and Ndrg1 mRNA in a dose-dependent manner (Fig. 3A). However, the response of SK-Mel-28 melanoma cells to each of the anthracyclines was different (Fig. 3A). Daunorubicin gradually increased TfR1 and Ndrg1 mRNA up to 5 μM, with the effect at this latter concentration being similar to 2 μM DOX (Fig. 3A). As found for DOX, EPI increased TfR1 and Ndrg1 mRNA up to 2 μM, and then at the highest EPI concentration assessed (i.e., 5 μM), the expression of these genes decreased (Fig. 3A). In general, these results demonstrated that DOX, DAU, and EPI increased TfR1 and Ndrg1 mRNA up to a concentration of 1 to 2 μM.

Anthracyclines Increase TfR1 and Ndrg1 Expression as a Function of Time. The effect of anthracyclines on TfR1 and Ndrg1 mRNA was then assessed as a function of incubation time. The optimal anthracycline concentration that up-regulated gene expression in SK-Mel-28, namely, 2 μM (Fig. 2A), was incubated with this cell type for 3 to 24 h at 37°C. The effect of DOX was compared with the positive

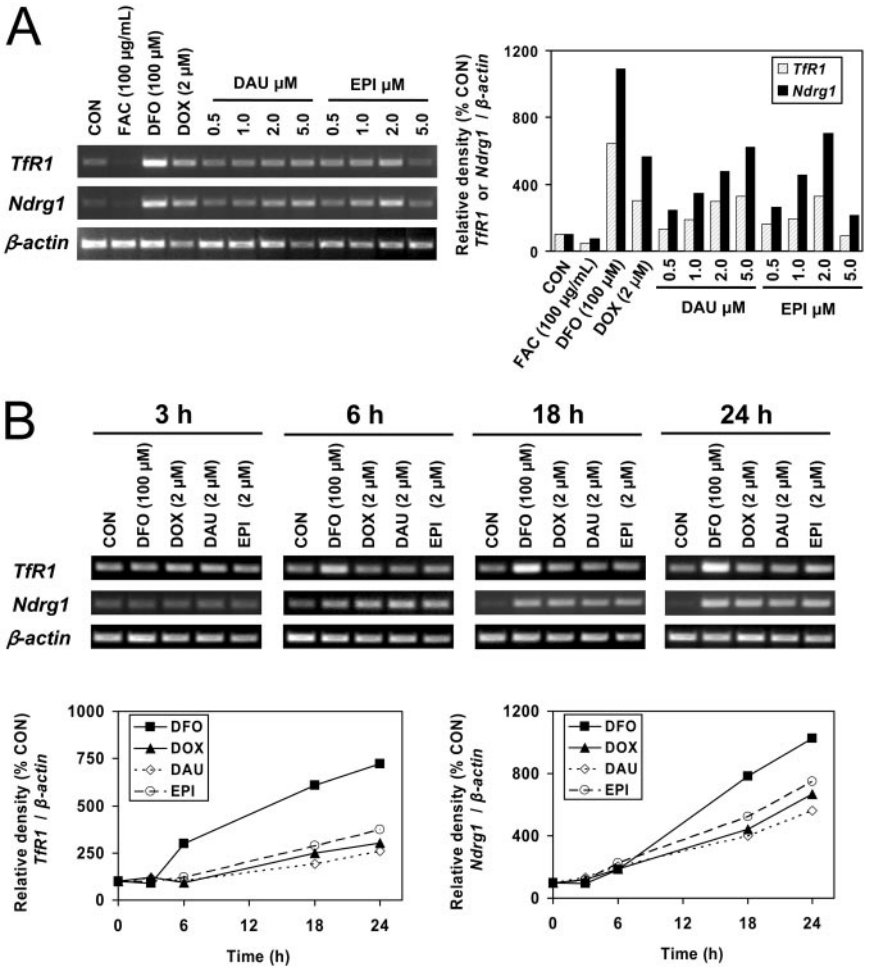

Fig. 3. Anthracyclines increase TfR1 and Ndrg1 mRNA expression in a dose-dependent (A) and time-dependent (B) manner in SK-Mel-28 melanoma cells. A, cells were incubated for 24 h at 37°C with CON, 100 μg/m FAC, 100 μM DFO, 2 μM DOX, or DAU or EPI at 0.5, 1, 2, and 5 μM. The expression of TfR1 and Ndrg1 mRNA levels were evaluated using RT-PCR. B, cells were incubated with CON, 100 μM DFO, 2 μM DOX, 2 μM DAU, or 2 μM EPI for 3, 6, 18, and 24 h. The expression of TfR1 and Ndrg1 mRNA levels were evaluated using RT-PCR. Gene expression was then calculated relative to the β-actin control. Results are typical from three separate experiments performed.

control DFO at 100 μM. In these studies, DFO increased TfR1 and Ndrg1 mRNA expression after 6 h (Fig. 3B). This was in agreement with previous studies using DFO and other cell types (Le and Richardson, 2004). A significant ($p < 0.05$) increase in TfR1 mRNA expression after incubation with the anthracyclines was evident after 18 h. However, the anthracyclines increased Ndrg1 mRNA expression after only 6 h to a comparable or greater extent than DFO (Fig. 3B).

The DOX-Mediated Increase in TfR1 and Ndrg1 mRNA Is Iron-Dependent. DOX, DAU, and EPI possess the same iron binding sites (carbonyl and hydroxyl moieties) that are necessary for iron chelation (Fig. 1A). The ability of these agents to bind iron was shown in the "test tube" (May et al., 1980), but not in intact cells. Certainly, these compounds may be acting as chelators to deplete iron pools and to increase TfR1 and Ndrg1 mRNA. To examine this, the effect of 2 μM DFO was compared with the anthracyclines at the same concentration. Furthermore, the efficacy of the anthracyclines at increasing Ndrg1 and TfR1 was also compared with their preformed 3:1 ligand-Fe(III) complexes (Fig. 4A).

After a 24-h incubation, DFO clearly increased TfR1 and Ndrg1 mRNA expression (Fig. 4A). The 1:1 DFO-iron complex largely prevented TfR1 and Ndrg1 up-regulation that was observed with DFO. The anthracyclines DOX, DAU, and EPI all increased both TfR1 and Ndrg1 mRNA expression, whereas their iron complexes were significantly ($p < 0.001$) less effective over three experiments. Hence, this suggested that up-regulation of TfR1 and Ndrg1 was due to anthracyclines binding cellular iron (Fig. 4A). It is noteworthy that the effect of the anthracyclines at inducing Ndrg1 expression was more pronounced than that observed with TfR1 (Fig. 4A).

Further experiments assessed whether DOX-mediated up-regulation of TfR1 and Ndrg1 could be reversed by iron added as FAC (100 μg/ml; Fig. 4, B and C). SK-Mel-28 cells were preincubated with control medium (CON), 100 μM DFO, or 2 μM DOX for 20 h (primary incubation), and then they were reincubated for another 20 h (secondary incubation) with CON, 100 μg/ml FAC, 100 μM DFO, or 2 μM DOX.

After primary incubation with CON, secondary incubation with FAC (Fig. 4, B and C, lane 2) decreased TfR1 and Ndrg1 mRNA levels compared with cells treated with CON (Fig. 4, B and C, lane 1). The treatment with FAC acted as a positive control to demonstrate both genes are iron-regulated. Cells treated with DFO or DOX followed by CON (Fig. 4, B and C, lanes 4 and 7) led to increased TfR1 and Ndrg1 mRNA expression compared with the control (Fig. 4, B and C, lane 1). Depletion of cellular iron by primary and secondary incubation with DFO resulted in more pronounced up-regulation of TfR1 and Ndrg1 levels (Fig. 4, B and C, lane 6) in comparison with DFO followed by CON (Fig. 4B, lane 4). Primary and secondary incubation with DOX caused similar up-regulation of TfR1 and Ndrg1 (Fig. 4, B and C, lane 9) as DOX followed by CON (Fig. 4, B and C, lane 7). It is noteworthy that primary incubation with DFO or DOX and reincubation with FAC (Fig. 4, B and C, lanes 5 and 8) significantly ($p < 0.01$) decreased TfR1 and Ndrg1 up-regulation compared with the relative control (Fig. 4, B and C, lanes 4 and 7). This

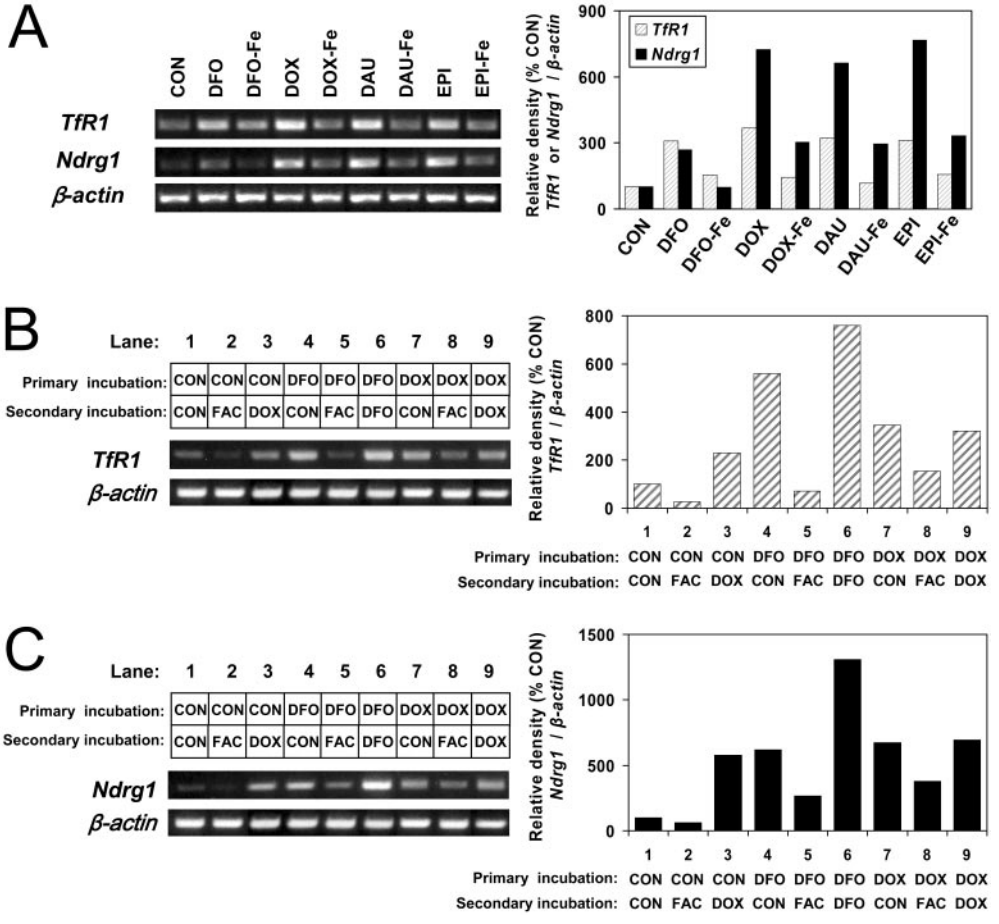

Fig. 4. Anthracyclines up-regulate TfR1 and Ndrg1 mRNA levels by iron deprivation. A, anthracycline-iron complexes are far less active than their parent ligands at increasing gene expression. B and C, soluble iron salt FAC decreases TfR1 (B) and Ndrg1 (C) mRNA expression after incubation with anthracyclines. A, SK-Mel-28 cells were incubated for 24 h at 37°C with CON, 2 μM DFO, the 1:1 DFO-iron complex at 2 μM, 2 μM DOX, the 3:1 DOX-iron complex at 2 μM, 2 μM DAU, the 3:1 DAU-iron complex at 2 μM, 2 μM EPI, or the 3:1 EPI-iron complex at 2 μM. B and C, SK-Mel-28 cells were preincubated with CON, 100 μM DFO, or 2 μM DOX for 20 h at 37°C (primary incubation), followed by a 20-h reincubation at 37°C with CON, 100 μg/ml FAC, 100 μM DFO, or 2 μM DOX (secondary incubation). The expression of TfR1 and Ndrg1 mRNA levels were evaluated using RT-PCR. Densitometric analysis was performed, and gene expression was then calculated relative to the β-actin control. Results are typical of three experiments performed.

further confirmed that anthracyclines increased TfR1 and Ndrg1 mRNA via iron chelation, and this up-regulation was reversible upon adding iron.

DOX Does Not Induce Cellular Iron Mobilization but It Causes Intracellular Iron Redistribution. To understand how DOX affected iron metabolism to up-regulate TfR1 and Ndrg1 mRNA, studies examined its effects on cellular ^{59}Fe mobilization. The ability of 0.1 to 5 μM DOX at mobilizing ^{59}Fe was compared with the chelators DFO at 100 μM and PIH at 25 μM, over 24 h at 37°C (Fig. 5A). These DOX concentrations were chosen because they were used to examine TfR1 and Ndrg1 mRNA expression (Figs. 2–4). Both DFO and PIH increased cellular ^{59}Fe mobilization to 225 and 270% of the control, whereas DOX had no effect (Fig. 5A). Further studies examined the ability of DOX to mobilize ^{59}Fe from cell lysates. In contrast to the positive control 100 μM DFO, which caused marked ^{59}Fe mobilization from lysates, 0.5 to 5 μM DOX had no effect (Fig. 5B). Collectively, despite DOX having high iron binding affinity (May et al., 1980) and its ability to up-regulate TfR1 and Ndrg1 mRNA by iron depletion (Fig. 4), it does not act like a typical chelator to induce iron efflux.

Further studies were performed using FPLC to examine alterations in intracellular ^{59}Fe distribution (Fig. 5C). Cells were labeled with 0.75 μM ^{59}Fe-Tf in the presence or absence of 2 μM DOX for 24 h at 37°C, and then they were washed, lysed, and centrifuged. The supernatant was fractionated on a size exclusion column, and the fractions were measured for ^{59}Fe. In control cells, two major high relative molecular mass peaks were detected (Fig. 5C). According to the column calibration, the first peak at fraction 12 (F12) represented ^{59}Fe-containing molecules of ≈700 kDa. A second peak at fraction 15 (F15) comigrated with horse spleen ferritin (≈400 kDa; Fig. 5C). After incubation with DOX, ^{59}Fe in F12 was significantly ($p < 0.01$) decreased over three experiments. In contrast, in the ferritin fraction (F15) there was a significant increase in ^{59}Fe incorporation. There were two other lower relative molecular mass peaks eluting at fractions 20 and 27, although there was no significant difference between them comparing control and DOX-treated cells (Fig. 5C).

To further elucidate the nature of the ^{59}Fe-containing molecules, F12 and F15 were concentrated and separated using native gradient PAGE (Fig. 5D). These studies showed that DOX decreased ^{59}Fe incorporation into high relative molecular mass proteins (F12), whereas there was ferritin-^{59}Fe accumulation (F15). The ferritin-^{59}Fe loading was confirmed by addition of anti-ferritin antibody to the latter fraction leading to a supershifted ferritin band (Fig. 5D). These data demonstrated redistribution of ^{59}Fe between ferritin and other ^{59}Fe-containing proteins, extending our previous observations (Kwok and Richardson, 2003, 2004). This ferritin-^{59}Fe accumulation leads to cytosolic iron deficiency that may up-regulate TfR1 and Ndrg1 mRNA (Figs. 2 and 3).

HIF-1α-Independent Mechanisms Are Involved in Up-Regulation of TfR1 and Ndrg1 after Incubation with DOX. The up-regulation of TfR1 mRNA by anthracy-

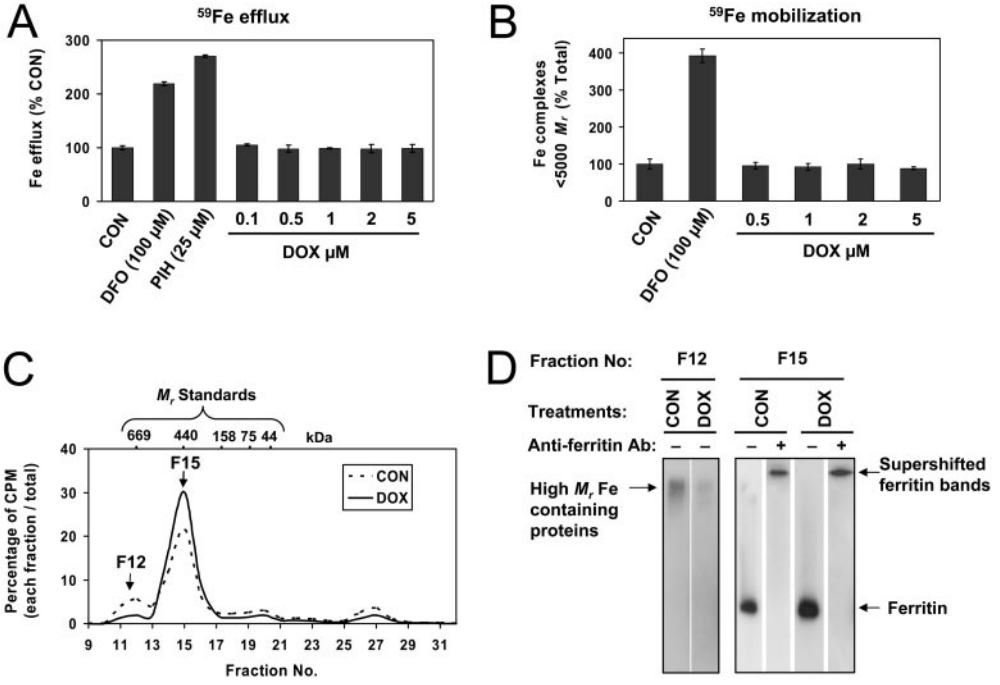

Fig. 5. DOX does not act like a typical iron chelator, and it cannot induce ^{59}Fe efflux from intact cells (A) or effect ^{59}Fe mobilization from cellular lysates (B). However, DOX prevents ^{59}Fe mobilization from ferritin to other cellular compartments as shown by FPLC (C) and native PAGE-^{59}Fe autoradiography (D). SK-Mel-28 cells were labeled for 3 h at 37°C with 0.75 μM ^{59}Fe-Tf, washed, and then reincubated for 24 h at 37°C with CON, 100 μM DFO, 25 μM PIH, or 0.1 to 5 μM PIH. The overlying media and cells were collected, and the ^{59}Fe levels were examined. B, SK-Mel-28 cells were labeled for 3 h at 37°C with 0.75 μM ^{59}Fe-Tf, and the cells were lysed, centrifuged, and then the supernatant was isolated. The supernatant was incubated with 100 μM DFO or 0.5 to 5 μM DOX for 3 h at 37°C, and then it was subjected to ultrafiltration through a 5-kDa cut-off filter. The eluted fraction was collected, and the radioactivity was examined. C, SK-Mel-28 cells were labeled for 24 h at 37°C with 0.75 μM ^{59}Fe-Tf in the presence or absence of 2 μM DOX, and the cellular lysates were isolated as described under *Materials and Methods*. The samples were then separated using a Superdex 200 10/300 GL size exclusion column. The radioactivity in each fraction (1 ml) was examined by a gamma-counter. D, fraction 12 (F12) and 15 (F15) from C were assessed using 3 to 12% native gradient PAGE-^{59}Fe autoradiography. F15 contained ferritin, which was confirmed by a supershift experiment using an anti-ferritin antibody. Data in A and B are mean ± S.D. (three experiments), whereas data in C and D are a typical experiment from three performed.

clines could occur by the classic IRP mechanism (Hentze and Kuhn, 1996) and/or also via HIF-1α because the *TfR1* promoter contains a hypoxia response element (Bianchi et al., 1999; Lok and Ponka, 1999). The increase in Ndrg1 mRNA expression after iron chelation by DFO was previously shown to occur by HIF-1α-dependent and -independent mechanisms (Le and Richardson, 2004).

To examine the role of HIF-1α in TfR1 and Ndrg1 up-regulation after incubation with DOX, we used *HIF-1α* knockout (*HIF-1$\alpha^{-/-}$*) MEFs in comparison with wild-type (*HIF-1$\alpha^{+/+}$*) MEFs (Ryan et al., 2000) (Fig. 6A). Both *HIF-1$\alpha^{+/+}$* and *HIF-1$\alpha^{-/-}$* MEFs were incubated with 100 μM DFO (positive control) or 2 μM DOX for 8 h, and then TfR1, Ndrg1, and HIF-1α mRNA expression was assessed (Fig. 6A). Incubation of *HIF-1$\alpha^{+/+}$* or *HIF-1$\alpha^{-/-}$* cells with DFO or DOX increased TfR1 mRNA levels irrespective of *HIF-1α* status, suggesting another mechanism was responsible. For DFO, this could be mediated by the IRPs (Hentze and Kuhn, 1996). Previous studies examining SK-Mel-28 cells demonstrated that at high DOX concentrations (i.e., 20 μM), IRP mRNA binding activity was reduced (Kwok and Richardson, 2002). However, at low concentrations (1 μM), IRP binding was not markedly affected (Kwok and Richardson, 2002). This suggested the DOX-induced TfR1 mRNA up-regulation at 1 to 2 μM in SK-Mel-28 cells (Fig. 2A) may not be mediated by IRPs.

The expression of Ndrg1 mRNA was more significantly up-regulated ($p < 0.05$) by DFO in *HIF-1$\alpha^{+/+}$* cells than their *HIF-1$\alpha^{-/-}$* counterparts (Fig. 6A), in agreement with previous studies (Le and Richardson, 2004). This suggests that HIF-1α is important in up-regulating Ndrg1 mRNA after iron chelation but that a HIF-1α-independent mechanism was also present (Le and Richardson, 2004). The up-regulation of Ndrg1 mRNA after incubation of DOX occurred in *HIF-1$\alpha^{-/-}$* and *HIF-1$\alpha^{+/+}$* cells (Fig. 6A), suggesting the response was HIF-1α-independent. In fact, in three experiments, Ndrg1 mRNA up-regulation was significantly ($p < 0.045$) more marked in *HIF-1$\alpha^{-/-}$* than *HIF-1$\alpha^{+/+}$* cells (Fig. 6A).

The effect of DOX and DFO was also examined on the expression of vascular endothelial growth factor-1 (VEGF1) mRNA (Fig. 6A), which is a typical HIF-1α-regulated gene (Beerepoot et al., 1996). The ability of DFO at increasing

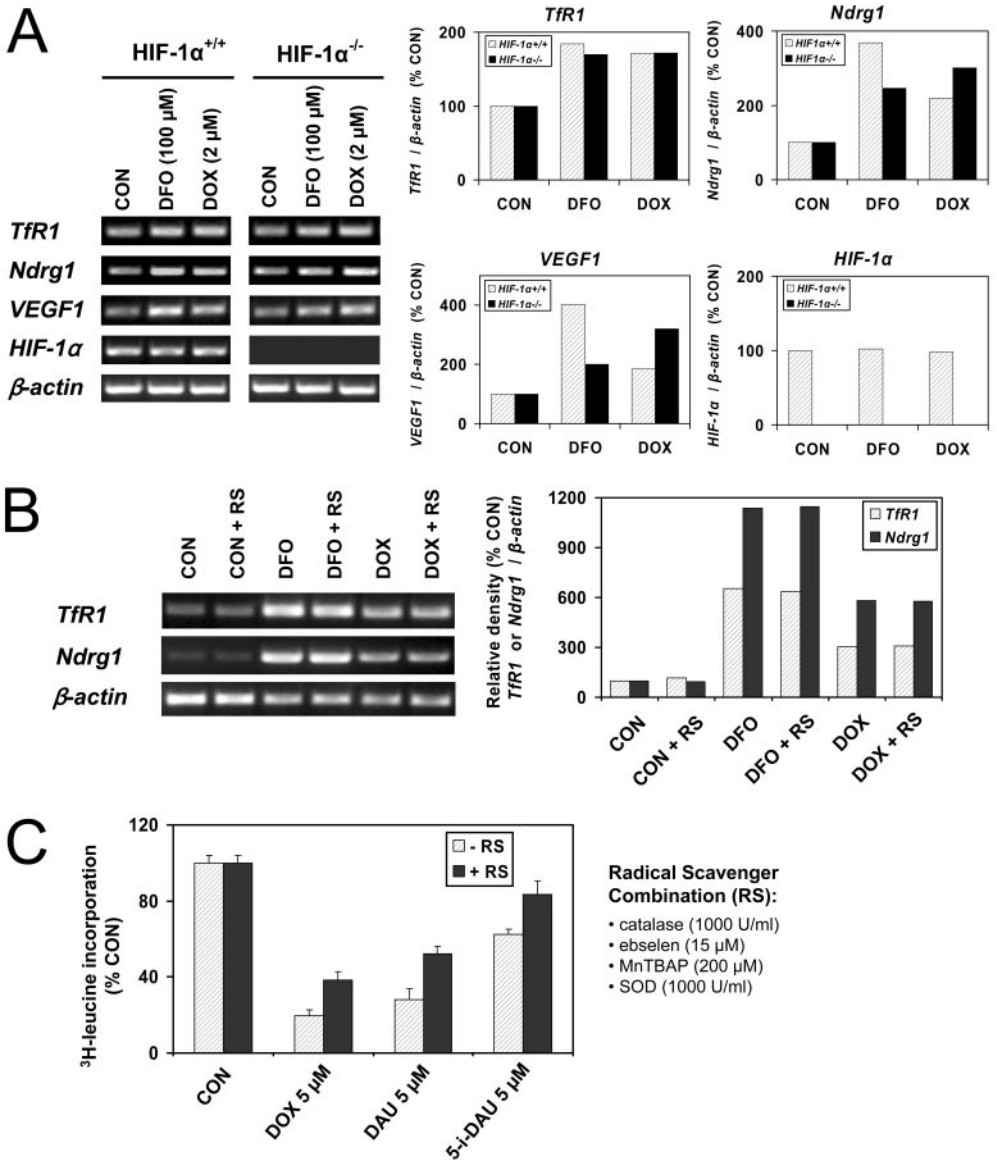

Fig. 6. A, DOX-mediated up-regulation of TfR1 and Ndrg1 mRNA occurs via a HIF-1α-independent mechanism. B, DOX-generated reactive oxygen species are not involved in TfR1 and Ndrg1 mRNA up-regulation, but they play a role in DOX-mediated protein synthesis inhibition (C). A, wild-type (*HIF-1$\alpha^{+/+}$*) and *HIF-1α*-knockout (KO; *HIF-1$\alpha^{-/-}$*) murine embryo fibroblasts were incubated with CON, 100 μM DFO, or 2 μM DOX for 8 h at 37°C. The expression of TfR1 and Ndrg1 mRNA levels was then examined by RT-PCR. Densitometry was performed, and gene expression was then calculated relative to the β-actin control. B, SK-Mel-28 cells were incubated with CON, 100 μM DFO, or 2 μM DOX in the presence or absence of a combination of RS for 24 h at 37°C. The RS included membrane impermeable agents SOD (1000 U/ml) and catalase (1000 U/ml), the cell-permeable SOD mimetic Mn(III) tetrakis (4-benzoic acid)-porphyrin (200 μM), and the cell-permeable glutathione peroxidase mimetic ebselen (15 μM). The expression of TfR1 and Ndrg1 mRNA was examined using RT-PCR. Densitometry was performed as described in A. C, cells were incubated with CON, 5 μM DOX, 5 μM DAU, or 5 μM 5-i-D for 22 h at 37°C, and then [^{3}H]leucine (1 μCi/plate) was added into the media for 2 h at 37°C. The data in A and B are typical from three experiments, whereas the data in C are mean ± S.D. (three experiments).

VEGF1 mRNA was more pronounced in *HIF-1α*$^{+/+}$ than *HIF-1α*$^{-/-}$ cells. Hence, similarly to Ndrg1, this indicates that HIF-1α is important in up-regulating VEGF1 mRNA after DFO but that a HIF-1α-independent mechanism was also present. After incubation with DOX, VEGF1 mRNA was more highly expressed in *HIF-1α*$^{-/-}$ cells than *HIF-1α*$^{+/+}$ cells, indicating the anthracycline was up-regulating this gene via a HIF-1α-independent mechanism.

As an appropriate control, HIF-1α status was examined in *HIF-1α*$^{+/+}$ and *HIF-1α*$^{-/-}$ cell types. In these studies, HIF-1α mRNA expression was clearly evident in *HIF-1α*$^{+/+}$ cells and not markedly affected by the incubation with DFO or DOX. In contrast, and as expected, no transcript was detected in *HIF-1α*$^{-/-}$ cells (Fig. 6A).

Activity of Free Radical Scavengers on Ndrg1 and TfR1 Expression after Incubation with Anthracyclines. Anthracyclines are well known to generate radicals (Corna et al., 2004), and increased TfR1 protein expression occurs after oxidant stress, at least in part, through IRP activation (Pantopoulos and Hentze, 1995). To determine the role of anthracycline-induced oxidant stress in TfR1 and Ndrg1 mRNA expression, we assessed the effect of radical scavengers (RS) on DOX-induced TfR1 and Ndrg1 mRNA expression (Fig. 6B) and also the ability of DOX to inhibit protein synthesis (Fig. 6C). In these experiments, we combined superoxide dismutase (SOD; 1000 U/ml) and catalase (1000 U/ml) with the cell-permeable glutathione peroxidase mimetic ebselen (15 μM) and cell-permeable SOD mimetic Mn(III) tetrakis (4-benzoic acid)-porphyrin (200 μM), because these agents alone and in combination are effective RS (Kotamraju et al., 2002; Kwok and Richardson, 2002). The addition of the RS had no significant effect on the up-regulation of either TfR1 or Ndrg1 mRNA by either DOX or DFO over three experiments (Fig. 6B). This suggested TfR1 and Ndrg1 mRNA up-regulation was not due to anthracycline-induced oxidant stress.

As a positive control to demonstrate that RS reduced ROS generation and the cytotoxic effects of anthracyclines, experiments were performed with various anthracyclines to assess their ability to inhibit protein synthesis ([^{3}H]leucine incorporation) in the presence and absence of the same combination of RS (Fig. 6C). In these studies, DOX and DAU were compared with 5-imino-daunorubicin (5-i-DAU) that generates less ROS than the former anthracyclines (Corna et al., 2004).

All anthracyclines were effective at reducing [^{3}H]leucine incorporation (Fig. 6C). From the anthracyclines examined, DOX was the most effective, whereas 5-i-DAU demonstrated the least ability to inhibit [^{3}H]leucine incorporation (Fig. 6C). This could be because 5-i-DAU is less redox active than DOX (Corna et al., 2004). For all anthracyclines, the combination with RS significantly ($p < 0.05$) increased [^{3}H]leucine incorporation compared with their relative controls (Fig. 6C). Hence, the RS could partially rescue the effects of anthracyclines at depressing [^{3}H]leucine incorporation.

DOX Inhibits the Translation of TfR1 and Ndrg1 mRNA into Protein, whereas Ferritin Protein Expression Increases. The ability of DOX to prevent [^{3}H]leucine incorporation into protein suggested mRNA translation could be inhibited. These data agree with our earlier studies using SK-Mel-28 cells where DOX markedly inhibited [^{3}H]leucine incorporation (Kwok and Richardson, 2004). Hence, it was important to investigate whether up-regulation of TfR1 and Ndrg1 mRNA after incubation with DOX (Figs. 2 and 3) leads to increased protein expression.

At the lowest DOX concentration, 0.5 μM, a slight but not significant increase in TfR1 protein expression occurred in SK-Mel-28 melanoma cells relative to the control (Fig. 7A). At the same concentration, a more pronounced and significant ($p < 0.04$) increase in Ndrg1 protein expression was found relative to the control (Fig. 7B). However, at higher DOX concentrations, 5 and 7.5 μM, TfR1 and Ndrg1 protein expression decreased, potentially because of inhibition of protein synthesis (Fig. 6C). In contrast, ferritin-H and -L protein levels increased in the presence of DOX (Fig. 7C), in agreement with previous studies (Kwok and Richardson, 2003; Corna et al., 2004). It is also of interest that ferritin-H and -L mRNA increased as a function of DOX concentration up to 5 μM (Fig. 7D), which is in contrast to TfR1 and Ndrg1 mRNA, which decreased at this latter concentration (Fig. 2A). This indicated differential effects of DOX on gene expression.

Preincubation with DOX followed by Labeling with ^{59}Fe-Transferrin Decreases Cellular ^{59}Fe Uptake. Considering the decreased TfR1 protein expression at higher DOX concentrations (Fig. 7A), studies were performed to examine the effect of DOX on ^{59}Fe uptake from ^{59}Fe-Tf (Fig. 7E). After a 24-h preincubation with 2 μM DOX, cells were incubated with 0.75 μM ^{59}Fe-Tf for 0.5 to 4 h. There was a significant ($p < 0.05$) decrease in ^{59}Fe uptake after 1 to 4 h in cells preincubated with DOX compared with control medium (Fig. 7E). The intracellular distribution of ^{59}Fe was then assessed using native gradient PAGE-^{59}Fe autoradiography (Babusiak et al., 2005) (Fig. 7F). Again, cells were preincubated for 24 h at 37°C with control medium or 2 μM DOX, washed, and then incubated with 0.75 μM ^{59}Fe-Tf for up to 4 h at 37°C. Most ^{59}Fe was incorporated into a band in the middle of the gel, which was shown to be ferritin by supershift studies with an anti-ferritin antibody (Fig. 7F, lanes 9 and 10). Transferrin migrated below ferritin as demonstrated using purified ^{59}Fe-Tf (Fig. 7F, lane 11). The ferritin-^{59}Fe uptake was linear up to 4 h, with less ^{59}Fe being incorporated into cells preincubated with DOX.

Preincubation with DOX Decreased both ^{59}Fe-Tf Uptake (Fig. 7E) and ^{59}Fe Incorporation into Ferritin (Fig. 7F). This was in contrast to studies with no preincubation period, where DOX and ^{59}Fe-Tf were incubated together for 24 h, leading to ferritin-^{59}Fe accumulation (Fig. 5, C and D). Preincubation with DOX before the addition of ^{59}Fe-Tf clearly inhibits protein synthesis (Fig. 6C), which is a crucial secondary event that decreases TfR1 and thus ^{59}Fe uptake.

Discussion

Anthracyclines bind iron and act as bidentate chelators via their carbonyl and hydroxyl groups (Fig. 1A) (May et al., 1980). The same ligating sites are involved in iron chelation by the effective chelator deferiprone (Kalinowski and Richardson, 2005); thus, the effects of anthracyclines on iron metabolism are important to dissect. However, the effects of anthracyclines on metabolism are complex because these agents have multiple molecular targets (Minotti et al., 2004a; Xu et al., 2005). In this study, we demonstrate for the first time that anthracyclines act as atypical chelators, having a

number of effects on iron metabolism and the expression of iron-regulated genes.

Previous work suggested that preincubation with DOX protected cells from an iron challenge as a result of increased ferritin expression (Corna et al., 2004). In this investigation, we repeated this experiment and demonstrated that preincubation with DOX followed by an iron challenge did not protect cells. In fact, it was detrimental, resulting in decreased cellular viability (Fig. 1B). The reason for this observation is probably related to several factors. First, it was shown by Link et al. (1996) that iron loading potentiates the cytotoxic effect of DOX, which is probably through the generation of a redox-active DOX-iron complex (Gianni and Myers, 1992). Second, we previously demonstrated that incubation of cells with DOX prevented ferritin iron release (Kwok and Richardson, 2004), which may be related to its ability to act as a protein synthesis inhibitor, inhibit lysosomal and proteasomal activity, or a combination (Kwok and Richardson, 2004). The inability of ferritin to release iron for essential metabolic processes would not be beneficial and could play a role in the cytotoxicity of anthracyclines. Third, in combination with the other well characterized cytotoxic effects of anthracyclines [e.g., inhibition of topoisomerase II and DNA intercalation (Minotti et al., 2004b)], the multiple effects of preincubating cells with DOX markedly affects cellular metabolism, leading to an ineffective response to an iron challenge.

Although chemical studies have shown that anthracyclines

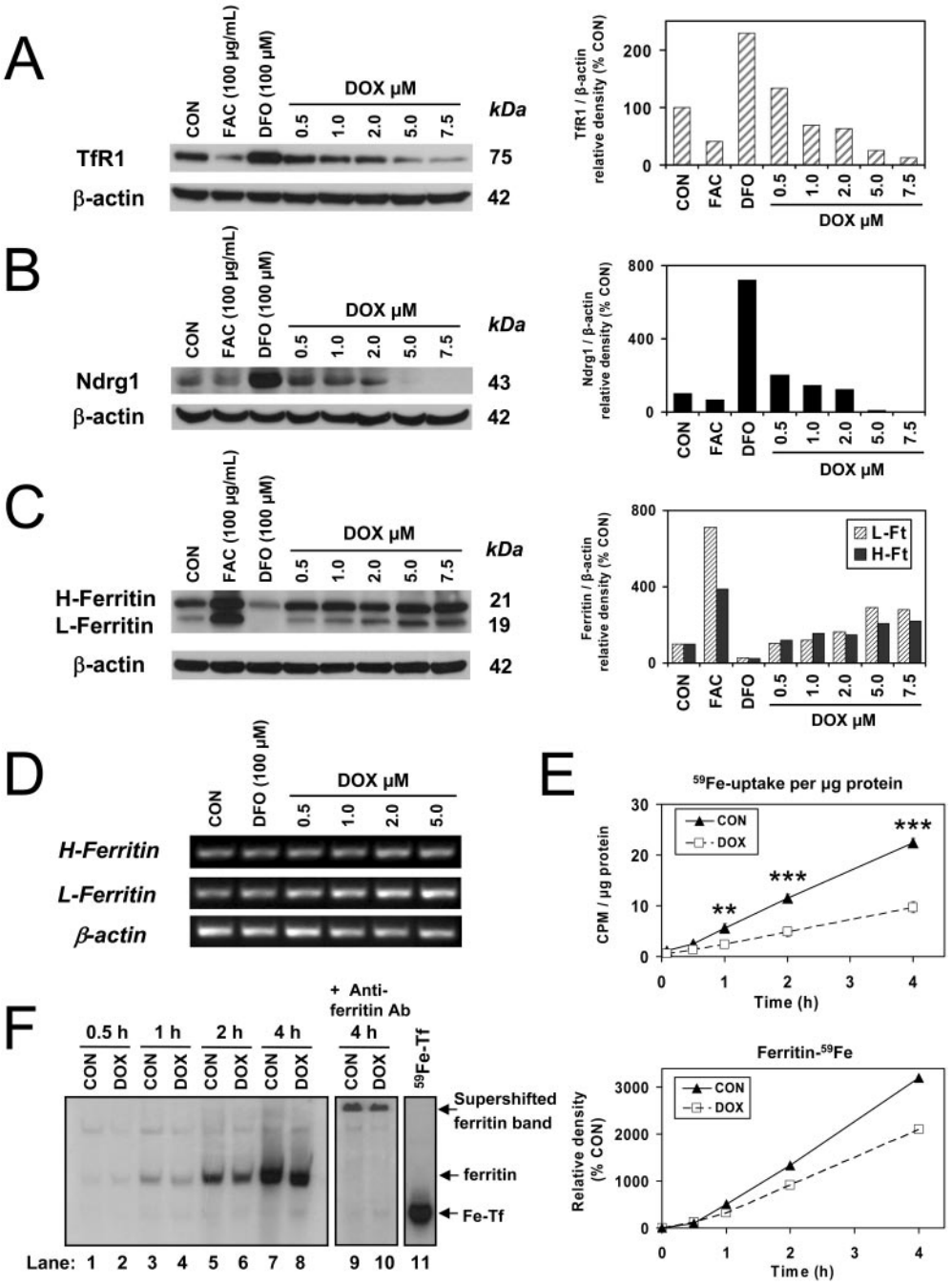

Fig. 7. A to C, DOX induces a dose-dependent reduction on both TfR1 and Ndrg1 protein levels, whereas ferritin-H and -L protein expression increases. D, DOX increases ferritin-H- and -L mRNA levels as a function of dose. E, preincubation with DOX results in decreased TfR1 protein expression that leads to depressed ^{59}Fe uptake from ^{59}Fe-transferrin, and reduced incorporation of ^{59}Fe into ferritin protein (F). A to C, SK-Mel-28 cells were incubated with CON, 100 μg/ml FAC, 100 μM DFO, or increasing concentrations of DOX (0.5–7.5 μM) for 24 h at 37°C. Western blot was performed using anti-TfR1, anti-Ndrg1, anti-ferritin, or anti-β-actin antibodies. D, SK-Mel-28 cells were incubated with CON, 100 μM DFO, or DOX at increasing concentrations (0.5–5 μM) for 24 h at 37°C. The mRNA was then extracted, and the expression of ferritin-H and -L mRNA levels were evaluated using RT-PCR. E, SK-Mel-28 cells were preincubated with CON or 2 μM DOX for 24 h at 37°C. These media were then removed, and the cells were reincubated for 0.5, 1, 2, and 4 h at 37°C with control media in the presence 0.75 μM ^{59}Fe-Tf. F, cell samples from E were lysed, and native gradient PAGE-^{59}Fe autoradiography was then performed. The incorporation of ^{59}Fe into ferritin was confirmed by supershift experiments using an anti-ferritin antibody. Data in A to D and F are typical from three experiments performed. Data in E are mean ± S.D. (three experiments). **, $p < 0.01$ and ***, $p < 0.001$ versus control values (Student's t test).

directly bind iron (May et al., 1980), the intracellular consequences of this iron depletion have not been established. This is probably due to the complexity of their cellular interactions (Minotti et al., 2004a; Xu et al., 2005). In this study, we demonstrated that DOX, DAU, and EPI could act like the well known chelator DFO, increasing mRNA expression of the iron-regulated genes *TfR1* (Hentze and Kuhn, 1996) and *Ndrg1* (Le and Richardson, 2004). This effect was marked, because at an equimolar concentration to DFO (2 μM), all the anthracyclines were as, or more effective at increasing TfR1 and Ndrg1 mRNA (Fig. 4A). The high iron chelation efficacy of the anthracyclines is probably related to their marked lipophilicity (Miura et al., 1991), which enables rapid intracellular access in comparison with DFO, which is hydrophilic and poorly penetrates cells (Richardson and Milnes, 1997).

In the current investigation, increased expression of TfR1 and Ndrg1 mRNA acted as a sensitive index of intracellular iron chelation and could be inhibited by presaturating the iron binding site of anthracyclines with iron (Fig. 4A). These iron complexes still entered cells because they are highly hydrophobic (Miura et al., 1991), and this was obvious from the red color of the cell pellets that are usually white. Hence, the formation of the iron complex prevented intracellular iron chelation, but it did not stop cellular access.

The nature of the iron pools that regulate TfR1 and Ndrg1 expression remains unknown. However, these iron pools influence IRP mRNA binding activity, which post-transcriptionally regulates TfR1 mRNA (Hentze and Kuhn, 1996) and HIF-1α that transcriptionally up-regulates TfR1, Ndrg1, and VEGF1 (Beerepoot et al., 1996; Bianchi et al., 1999; Lok and Ponka, 1999; Le and Richardson, 2004; Kalinowski and Richardson, 2005). The DOX concentrations that up-regulate TfR1 mRNA in SK-Mel-28 cells (i.e., 1–2 μM; Fig. 2A) were previously shown not to markedly affect IRP mRNA binding activity in this cell type (Kwok and Richardson, 2002), suggesting it was not an IRP response. Considering this, we also assessed the role of HIF-1α in regulating gene expression using HIF-1α knockout (*HIF-1*$\alpha^{-/-}$) MEFs compared with their wild-type counterparts (*HIF-1*$\alpha^{+/+}$). These studies suggested up-regulation of TfR1, Ndrg1, and VEGF1 mRNA by DOX occurred via an HIF-1α-independent mechanism, as regulation was comparable in the presence or absence of this transcription factor. Other studies examining HIF-1α activation by hypoxia also demonstrated that regulation of its target genes occurred irrespective of HIF-1α status in MEFs (Helton et al., 2005). Moreover, we showed using MEFs that DFO increased Ndrg1 mRNA expression by HIF-1α-dependent and -independent mechanisms (Le and Richardson, 2004). Collectively, the current work and previous studies (Le and Richardson, 2004; Helton et al., 2005) indicated functional redundancy in the control of HIF-1α target gene expression, with a HIF-1α-independent mechanism responding to iron chelation. This is of interest, as HIF-1α-independent pathways have been identified to be involved in the up-regulation of genes by hypoxia (Wood et al., 1998) and may also respond to iron depletion. Such pathways could be mediated by molecules related to HIF-1α, such as HIF-2α (Hu et al., 2003) and HIF-3α (Gu et al., 1998).

Although anthracyclines could act like typical chelators such as DFO to bind iron and induce up-regulation of iron-responsive genes, the effect on cellular ^{59}Fe mobilization and intracellular ^{59}Fe distribution were atypical compared with other ligands. For example, in contrast to DFO and PIH that induce cellular iron efflux (Ponka et al., 1979; Richardson and Milnes, 1997), DOX had no effect on ^{59}Fe release from cells or cellular lysates at the same concentrations that up-regulated TfR1 and Ndrg1 mRNA. This suggests the high lipophilicity of DOX and its ^{59}Fe complex leads to marked retention in membranes and organelles, as shown by others (Miura et al., 1991; Hurwitz et al., 1997; Jung and Reszka, 2001).

The multifunctional activity of DOX was shown by FPLC to lead to ferritin-^{59}Fe accumulation and prevent ^{59}Fe incorporation into high relative molecular mass compartments. This work confirmed and extended our previous observations demonstrating anthracyclines inhibit ferritin-iron mobilization, which is probably mediated through inhibition of protein synthesis (Kwok and Richardson, 2003, 2004). Moreover, considering the alteration in ^{59}Fe distribution, it can be suggested that anthracycline-mediated iron deprivation, which up-regulates TfR1 and Ndrg1 mRNA, could not only be due to direct iron chelation but also to inhibition of ferritin-iron mobilization.

An interesting observation that also demonstrated the multifunctional effect of DOX was that it acted as an effective protein synthesis inhibitor. This potentially could be responsible for the observed decrease in TfR1 and Ndrg1 protein as a function of DOX concentration. However, it was paradoxical that increasing DOX concentrations led to elevated ferritin protein expression, suggesting selective targeting of gene expression. This finding was surprising, but it was in accordance with previous studies demonstrating the effect of DOX at differentially targeting the expression of other genes (Ito et al., 1990; Chen et al., 1999). This selective activity of DOX has not been reported for genes involved or modulated by iron metabolism. At present, it remains uncertain what precise molecular mechanism leads to DOX inhibiting TfR1 and Ndrg1 protein expression and increasing ferritin protein synthesis. The apparent selectivity in altering gene expression could be important for understanding the complex pharmacological effects of DOX.

As discussed above, the marked inhibition of TfR1 protein expression by DOX in SK-Mel-28 cells may be due to the depression of protein synthesis. Hence, this seemed to be a secondary response unrelated to iron chelation that occurred after long preincubations with DOX that led to decreased iron uptake from Tf. Certainly, the decreased TfR1 and increased ferritin protein expression observed after incubation with DOX is opposite to that found with typical iron chelators such as DFO (Hentze and Kuhn, 1996) that are not potent protein synthesis inhibitors (Richardson and Milnes, 1997). Our current observations with neoplastic cells were in contrast to results using endothelial cells, where anthracyclines induced iron uptake via increasing TfR1 protein (Kotamraju et al., 2002). These latter authors suggested that DOX-mediated apoptosis was accompanied by increased iron uptake via TfR1 that was responsible for inducing apoptosis (Kotamraju et al., 2002). This result is controversial, because decreased intracellular iron is generally associated with apoptosis and inhibiting proliferation (Kalinowski and Richardson, 2005).

In summary, anthracyclines act as atypical chelators up-regulating the mRNA expression of the iron-regulated genes *TfR1* and *Ndrg1* by their chelation of intracellular iron. However, this complexation of iron did not lead to increased TfR1

or Ndrg1 protein levels, and DOX did not induce cellular iron mobilization. The lack of anthracycline-mediated iron efflux was probably because of the high lipophilicity of the so-formed iron complexes that remained intracellular. Considering the effect of anthracyclines on TfR1 and Ndrg1 expression, it was surprising and paradoxical that DOX increased ferritin protein expression and led to ferritin iron accumulation. Hence, the effect of anthracyclines on iron metabolism was multifaceted, probably because of their complicated chemical properties, which leads to multiple mechanisms of action.

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