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ORIGINAL RESEARCH COMMUNICATION

Thioredoxin Regulates Cell Cycle *via* the ERK1/2-Cyclin D1 Pathway

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Abstract

Thioredoxin (TRX) is a key component of redox regulation and has been indicated to play an essential role in cell survival and growth. Here, we investigated the molecular mechanism of TRX in the regulation of cell survival and growth by using RNA interference (RNAi) in A549 lung cancer and MCF7 breast cancer cells. TRX knockdown did not significantly increase the basal level of cell death without exposure to stress, but CDDP-induced cell death was enhanced. Meanwhile, TRX knockdown resulted in significant cell-cycle arrest at the G_1 phase. Cyclin D1 expression was reduced by TRX knockdown at the protein and mRNA levels. TRX knockdown caused suppression of activation of the cyclin D1 promoter through elements including AP-1. TRX knockdown also reduced the levels of phosphorylated ERK1/2 and the nuclear translocation of ERK 1/2 induced by EGF. These results suggest that TRX is an important regulator of the cell cycle in the G_1 phase *via* cyclin D1 transcription and the ERK/AP-1 signaling pathways. *Antioxid. Redox Signal.* 11, 2957–2971.

Introduction

THIOREDOXIN (TRX) was identified in *Escherichia coli* as an electron donor for ribonucleotide reductase (RNR) (18) and acts as a key molecule of redox regulation by catalyzing protein disulfide reductions in combination with TRX reductase and NADPH (8). Human TRX was cloned from cells infected with human T-cell leukemia virus type I (38). Enhancement of the expression of TRX was shown in some cancer cells, tissues, and virus-infected cells. Members of the TRX system have been widely studied to understand tumor biology and to develop antitumor strategies (4). TRX enhances the binding of transcription factors such as activator protein-1 (AP-1), NF-κB, p53, and nuclear receptors to their cognate DNA sequences (6, 7, 11, 44, 48) by redox regulation. TRX is protective against hydrogen peroxide in collaboration with a family of TRX-dependent peroxidases, peroxiredoxins (32). Homozygous targeted mutant mice of TRX die shortly after implantation (21), indicating that TRX plays an essential role in cell survival or early development. TRX is reported to play a role as a safety lock of apoptosis signal-regulating kinase 1 (ASK1) (31, 33), and TRX is reported to inhibit the activity of phosphatase and tensin homologue deleted from chromosome 10 (PTEN), which is a tumor suppressor (19, 23). We previously reported that TRX interacts with thioredoxin-binding protein-2 (TBP-2)/thioredoxin interacting protein (Txnip) (30). However, the physiologic role of the interaction regulated by TRX in cell survival and growth remains to be elucidated.

TRX also is suggested to be required for cell-cycle progression in *E. coli* (18) or *Xenopus* (5). The disruption of yeast TRX genes induced cell-cycle arrest at G_1 to S phase (26, 27), and TRX is a physiologically relevant electron donor for RNR during DNA precursor synthesis in *E. coli*, *Saccharomyces cerevisiae*, and mammals (2, 15, 18). Meanwhile, TRX is reported to enhance cell growth in the human system (39, 45). The expression of human TRX is highest in early S phase (43). Although these previous reports collectively suggest that human TRX regulates the cell cycle at the G_1 to S phase, little is known about the mechanism of the regulation by TRX in the cell cycle.

Cell-cycle control is regulated through a wide range of signal cascades. Cyclin D1 serves as one of the key regulators and integrators of extracellular signals of cells in the G_1 phase. Cyclin D1 gene amplification or overexpression occurs with significant frequency in several types of human cancers (9, 25). Cyclin D1 is mainly regulated at the mRNA level by mitogenic stimulation (14) and many oncogenic signals (40, 46). In many cancer cells, receptor tyrosine kinases are constitutively activated, resulting in activation of downstream signaling,

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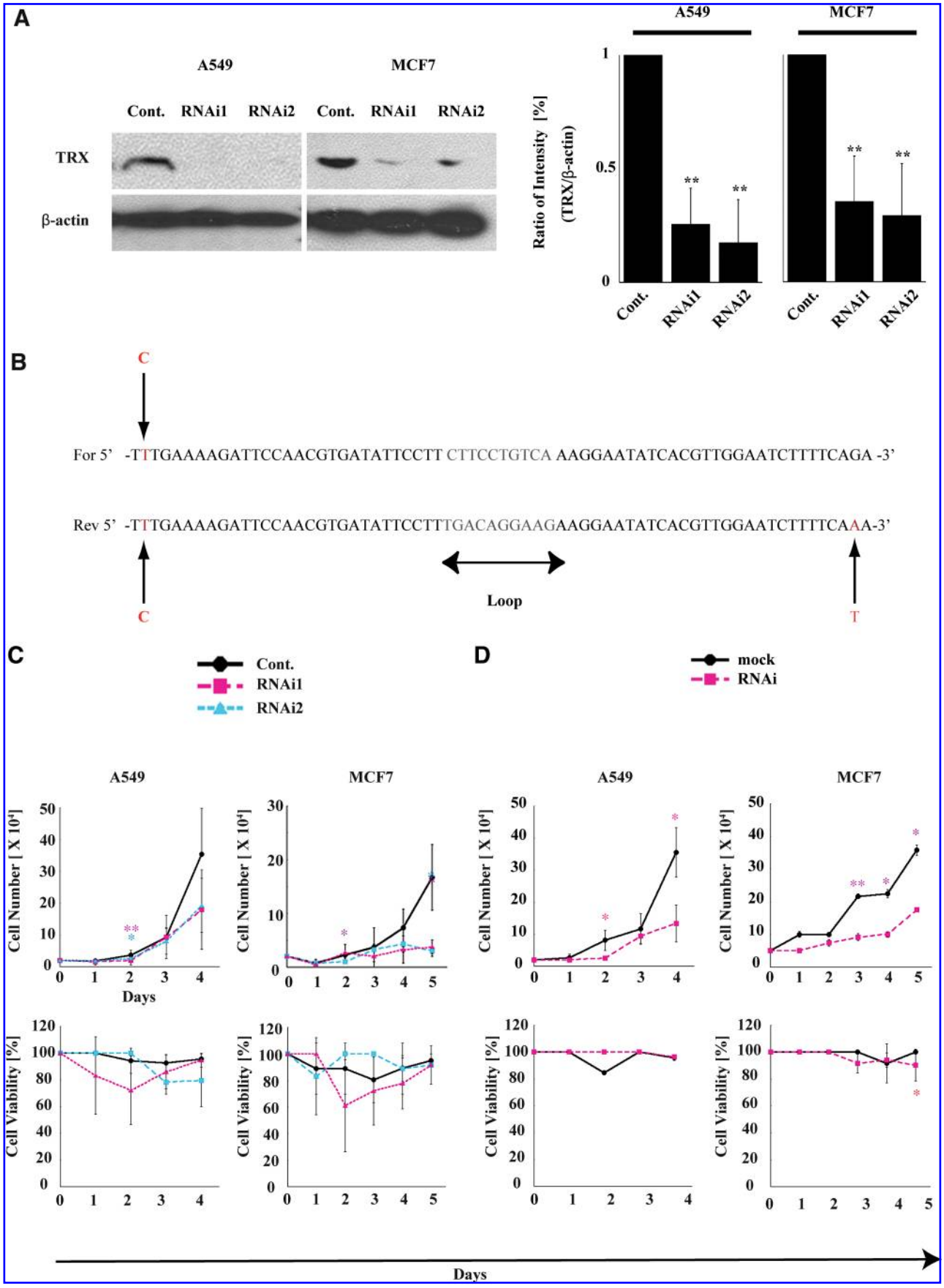
A
A549
MCF7
Cont. RNAi1 RNAi2 Cont. RNAi1 RNAi2
TRX
β-actin
Ratio of Intensity [%] (TRX/β-actin)
1
0.5
0
**
Cont.
RNAi1
RNAi2
B
C
For 5' -TTTGAAAAGATTCCAACGTGATATTCCTT CTTCCTGTCA AAGGAATATCACGTTGGAATCTTTTCAGA -3'
Rev 5' -TTTGAAAAGATTCCAACGTGATATTCCTTTGACAGGAAGAAGGAATATCACGTTGGAATCTTTTCAAA-3'
C
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RNAi1
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including the mitogen-activated protein kinase (MAPK) pathway (22, 41). Extracellular stimuli induce sequential activation of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK (29). Among the MAPK family, extracellular signal-regulated kinase (ERK) 1/2 has been elucidated extensively. The activation and nuclear localization ERK1/2 is regulated by MEK1/2 (16). The Ras-Raf-MEK-ERK cascade modulates cellular proliferation by regulating the expression of cell-cycle regulators such as cyclin D1 (3, 14).

We here investigated the physiologic role of TRX in cell survival and growth, showing that TRX knockdown barely affected cell death but instead caused cell-cycle arrest through downregulation of cyclin D1 transcription. These results suggest that TRX regulates cell growth by controlling the cyclin D1 transcription level as well as resistance to oxidative stress. We also showed that TRX regulates the ERK1/2–AP-1 pathway induced by epidermal growth factor (EGF), providing a novel clue for developing antitumor strategies based on the TRX system.

Materials and Methods

Plasmid constructs

We used a tRNA-shRNA expression vector (iGENE Therapeutics, Inc., Tokyo, Japan) to express an shRNA, which is composed of two complements of 68 nt sequences, and point mutations were introduced into the sequence for enhancement of the knockdown effect (Fig. 1B). Knockdown efficiencies were calculated by using Image J (http://rsb.info.nih.gov/ij/) software. The cyclin D1 promoter-luciferase reporter construct was kindly provided by Dr. H. Nakagawa (Pennsylvania University) (49). The cyclin D1 promoter mutant plasmid (pCyclinD1 AP-1mt-luc) was obtained by site-specific mutation by using polymerase chain reaction (PCR) primer sets: AP-1mt-luc (5′- AAAAATGAGTTGGAATGGA GATCACTGTTTCTCAGCTTTCC-3′, and 5′-GGAAAGCTG AGAAACAGTGATCTCCATTCCAACTCATTTTT-3′) with cyclin D1 by using a QuikChange II kit (Agilent Technologies, Santa Clara, CA). The expression vector for cyclin D1 was amplified by the standard PCR method. By using the vector (Human cDNA clone; Toyobo, Tokyo, Japan) as a template, the insert was obtained with PCR by using primers (forward 5′-GCGAATTCATATCCATATGACGTCCCAGACTATGCT AACACCAGCTCCTGTGCTG-3′ and reverse 5′-TCCTCTA GATCAGATGTCCACGTCCCGCA-3′). The forward primer contained the HA sequence (5′-TATCCATATGACGTCC CAGACTATGCT-3′). The products were then digested with *Eco*RI/*Xba*I and subcloned into the vector p3FLAG-CMV (Sigma, St. Louis, MO) to produce p3FLAG-HA-cyclin D1.

Cell culture

MCF7 and A549 cells were cultured in Dulbecco's minimum essential medium (DMEM) (Sigma) supplemented with penicillin and streptomycin (Sigma) and 10% fetal calf serum (FCS; Sigma) at 37°C in 5% CO_2.

Plasmids/siRNA transfections

Cells were transfected with empty vector (mock) or shRNA-TRX vector, respectively, by using TransIT LT1 (Mirus, Madison, WI) in accordance with the manufacturer's instructions. Transfected cells were cultured in medium containing 0.5 μg/ml puromycin (Invitrogen). Stable transfectants were obtained by selection for puromycin resistance and confirmed by Western blotting and reverse transcription (RT)-PCR analyses. We used only the cells from 14 to 28 days after transfection. Cells were transfected with 40 n*M* oligonucleotide duplex (QIAGEN) by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, and incubated for various time periods (from 24 to 96 h). siRNA duplexes targeting two different sites of human TRX, RNAi1 (#SI00753606) and RNAi2 (#SI00753613), and Control (Negative Control siRNA) (#1022076) were purchased from Qiagen.

Microarray analyses

Total RNA was prepared from cultured cells. Microarray analyses using Gene Chip Human Genome U133 Plus2.0 (Affymetrix, Santa Clara, CA) were performed according to the manufacturer's protocols. Data were analyzed by using GCOS (Affymetrix) and GeneSpring 7.3 software (Silicon Genetics, Redwood City, CA). In brief, per-chip normalization was done by using the median value, and then per-gene normalization was done by using the value $p < 0.05$.

Western blotting

Cells were lysed with RIPA buffer [150 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.0), 0.1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), protease inhibitor cocktail (Roche), PhoSTOP (Roche)] by sonication and subjected to SDS-PAGE. Western blotting was performed as described

FIG. 1. TRX knockdown mainly caused cell-growth arrest and induced cell death in only a small percentage of cells. (**A**) TRX knockdown by siRNA. A549 and MCF7 cells were transfected with oligonucleotides and analyzed with Western blotting. β-Actin was used as loading control (*left panel*). (*Right panel*) Densitometric analysis of the expression of TRX. Data are presented as mean ± SD and were analyzed with Student's *t* test compared with control. $^{**}p < 0.001$ ($n = 3$). Expression of TRX/β-actin levels in control oligonucleotide transfections was set at a relative value of 1. (**B**) The construct of TRX knockdown in the tRNA-shRNA expression vector. ▲ *Arrows* indicate the position of point mutations. Black characters indicate original sequences, and red characters indicate changed sequences. Cells were transfected with shRNA-expression vector and selected by using 0.5 μg/ml of puromycin. Fourteen days after transfection, the cells were collected, and TRX expression levels were tested with RT-PCR, as shown in Fig. 5A. (**C, D**) Cellular growth and survival. Cells were transfected with oligonucleotides (**C**), or sh-RNA expression vectors (**D**). The counting of cells was started at 24 h after transfection. Both trypan blue–stained and nonstained cells were counted and determined relative to the total cell number. Dead cells were determined as cells stained with trypan blue. Cell viability = 100×[(Total cell number) − (Dead cell number)]/(Total cell number). The results are shown as mean ± SD of triplicate cultures. Data were analyzed with Student's *t* test compared with control. $^{*}p < 0.05$; $^{**}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

previously (44). Antibodies against TRX (Redox Bioscience Inc., Kyoto, Japan), β-actin, Flag (Sigma), pJNK, JNK, ERK1/2, pERK1/2, cyclin D3 (Cell Signaling), cyclin D1, p21, p27 (Becton Dickinson), and rabbit and mouse IgG (Amersham) were used.

Reverse transcription PCR

Total RNA from each cell preparation was extracted by using TRIzol (Invitrogen). Reverse transcription was performed with a SuperScript III First-strand Synthesis System Kit (Invitrogen). PCRs were carried out by using the following oligonucleotide primers: human TRX sense (5′–CTGCTTT TCAGGAAGCCTTG-3′) and antisense (5′-TGTTGGCATGC ATTTGACTT-3′); and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense (5′-ATGGGGAAGGTGAA GGTCGGAGTC-3′) and antisense (5′-CCATGCCAGTGAGC TTCCCGTTC-3′); human cyclin D1 sense (5′-AGCTCCTGTG CTGCGAAGT-3′) and antisense (5′-GGGTGTGCAAGCCA GGTC-3′). PCR was performed under the following conditions: 96°C for 2 min; 21 cycles of 96°C for 30 s; 55°C for 30 s; 68°C for 30 s; and a final elongation at 68°C for 30 s to amplify the human TRX gene; 96°C for 2 min; 24 cycles of 96°C for 30 s; 60°C for 30 s; 68°C for 30 s; and a final elongation at 68°C for 30 s to amplify the human cyclin D1; 96°C for 2 min; 23 cycles of 96°C for 30 s; 55°C for 30 s; 68°C for 30 s; and a final elongation at 68°C for 30 s to amplify the human TRX and GAPDH genes. PCR products were then subjected to electrophoresis in a 1% agarose gel.

Quantitative real-time RT-PCR

Real-time RT-PCR was performed to measure the quantity of cyclin D1 and TRX mRNA, by using TaqMan Universal Master Mix (Applied Biosystems) with reverse-transcribed cDNA as a template. The amplification was performed by using an ABI Prism 7000 under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The oligonucleotides and TaqMan probes for cyclin D1 and TRX were purchased from Applied Biosystems. 18S ribosomal RNA was used as an internal control.

Dual luciferase assay of cyclin D1 promoter

Cells were plated in 24-well plates at a density of 1×10^5 cells per well. Plasmids (1 μg/well cyclin D1 promoter-luciferase: 1, pGL3-basic and pCyclin D1 AP-1mut-luc) and 40 n*M* oligonucleotides were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and after 24-h incubation, a luciferase assay was performed.

Cell-cycle analyses

Cells were cultured with serum-free medium for 24 h (G_0/G_1 synchronization) and maintained for 24 h after the addition of 10% serum. Cells were harvested, fixed with 70% ethanol, and incubated in phosphate-buffered saline (PBS) containing 50 ng/ml RNase (Qiagen) for 30 min at 37°C. Cells were then stained with 50 μg/ml propidium iodide (Calbiochem) for 1 h on ice in the dark. DNA content was analyzed with flow cytometry (Becton Dickinson FACSCaliber) by using Cellquest (Becton Dickinson) software.

Detection of cell death by using sub-G_1 analyses and staining with Annexin V-fluorescein isothiocyanate and propidium iodide

A sub-G_1 population was detected according to the cell-cycle protocol. Cells were resuspended in 500 μl of binding buffer (10 m*M* HEPES NaOH, pH 7.4, 150 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, and 1.8 m*M* $CaCl_2$), incubated for 5 min with 1 μl of Annexin V-FITC (MBL) at room temperature in the dark and subjected to flow cytometric analyses (Becton Dickinson FACSCalibur), using FlowJo (TreeStar, Inc.) software.

Estimation of intracellular ROS

Cells were incubated in medium with 5 μ*M* 2,7-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probe) for 30 min at 37°C and suspended in 1 ml of cold PBS. The intensity of fluorescence was analyzed by using flow cytometry.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Japan) and incubated with PBS containing 0.5% FCS for 12 h at 4°C. Cells were incubated with anti-ERK antibody (Cell Signaling) for 1 h at room temperature and incubated with Alexa-588 conjugated anti-rabbit antibody (Molecular Probe) for 1 h at 37°C. Cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) (Nacalai Tesque) for 5 min at room temperature. Images were acquired with Biozero (Keyence). The intensity of nuclear localization was analyzed by using Photoshop software.

Densitometric analysis

Density of bands in Western blotting or RT-PCR was analyzed by using Image J (http://rsb.info.nih.gov//ij/) software.

Results

The effects of TRX knockdown by RNA interference on cell growth

To examine whether TRX is necessary for cell survival, we analyzed the effect of TRX knockdown by RNA interference by using oligonucleotides and a shRNA-expression vector in A549 lung and MCF7 breast cancer cells. The knockdown with oligonucleotides caused significant reduction of TRX expression (Fig. 1A) but lasted for only a short period, whereas that of the shRNA expression vector provided a moderate but sustained knockdown effect. At the protein level, knockdown efficiencies by RNAi1 and RNAi2 were 0.25 ± 0.160 or 0.17 ± 0.193 in A549 cells, respectively, whereas those in MCF7 cells were 0.35 ± 0.20, or 0.29 ± 0.23 respectively (Fig. 1A). Expression levels in control oligonucleotide transfections were set at a relative value of 1. The appearance of TRX-knockdown cells did not show significant differences (data not shown). TRX knockdown by oligonucleotides or an sh-RNA expression vector caused cell-growth arrest in A549 and MCF7 cells, whereas the viability of the cells was reduced slightly (Fig. 1C and D) (Supplemental Fig. 1; see www.liebertonline.com/ars).

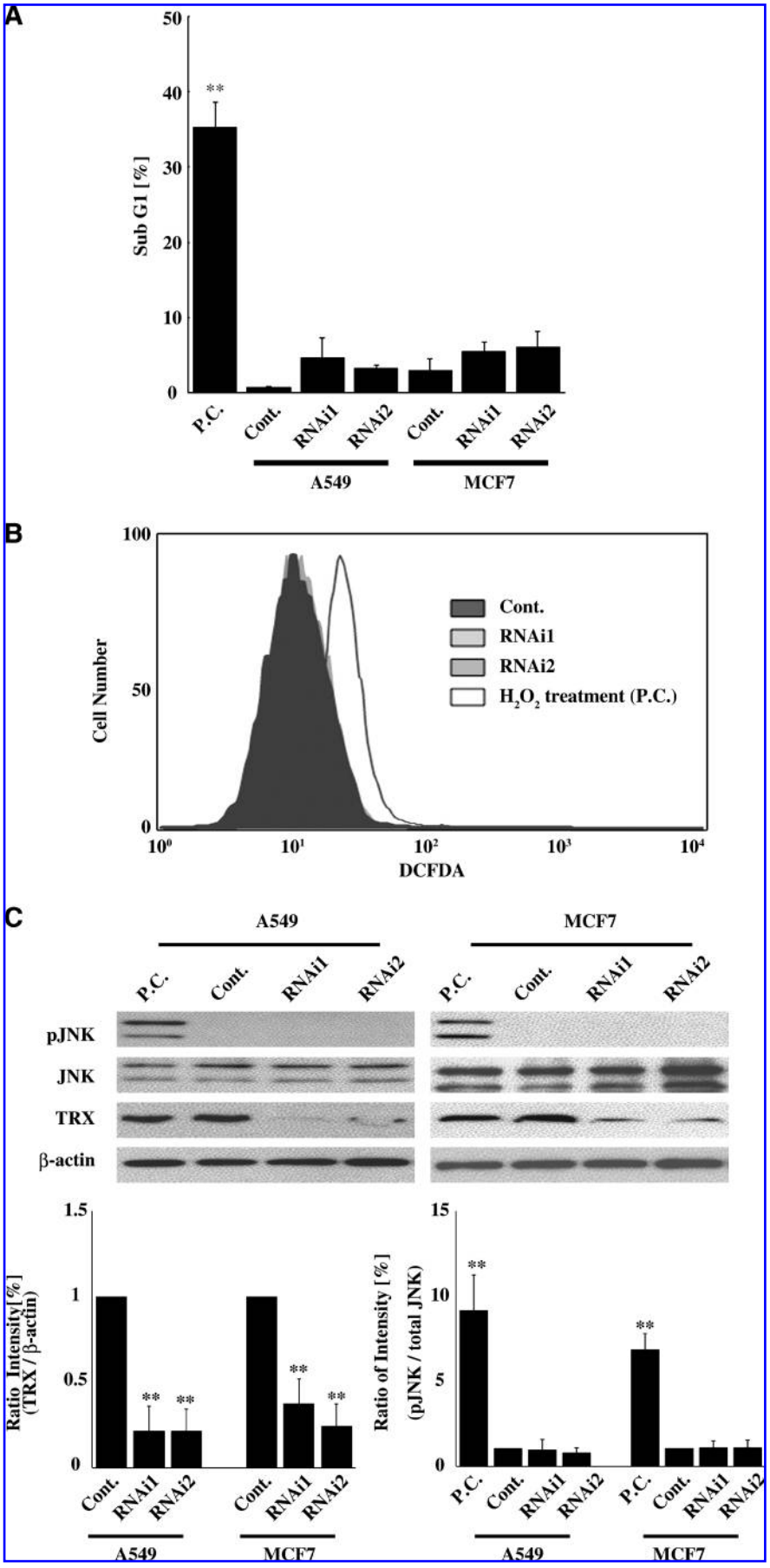

FIG. 2. TRX knockdown itself did not cause significant oxidative stress. (**A**) Effects of TRX knockdown on cell survival. Cells were transfected with oligonucleotides and cultured for 96 h, stained with PI, and analyzed with flow cytometry to quantify DNA content in cells. Positive control (*P.C.*): A549 cells were treated with 50 μM CDDP for 48 h. $^{**}p < 0.001$ (*t* test). (**B**) The effects of TRX knockdown on ROS production. A549 cells were transfected with oligonucleotides, cultured for 72 h, and analyzed with DCFDA by flow cytometry. Positive control (*P.C.*): Cells were treated with 1 mM H_2O_2 for 20 min. (**C**) Western blotting analyses of JNK activation. Cells were transfected with oligonucleotides and cultured for 48 h. Positive control (*P.C.*): Cells were treated with 10 μg/ml anisomycin for 1 h. JNK blot was used as loading control. (*Lower panel*) Densitometric analysis of pJNK. Data are presented as mean $\pm$ SD, and data were analyzed with Student's *t* test compared with control. $^{**}p < 0.001$ ($n = 3$). Expression of pJNK/JNK levels in control oligonucleotides transfections was set at a relative value of 1.

We then dissected the effect of TRX knockdown on cell death by using PI staining to quantify the numbers of cells with a subdiploid DNA content. As shown in Fig. 2A, only a few percent of TRX knockdown cells died, even though the expression of TRX protein was reduced to about 20% of control cells (Fig. 1A). TRX acts as a scavenger of reactive oxygen species (ROS), and ROS affects cellular redox balance and can cause activation of c-Jun N-terminal kinase (JNK) signaling. Thus, we examined the effects of TRX knockdown on ROS generation and found that it did not significantly enhance the production of ROS (Fig. 2B). TRX did not cause the activation of JNK at 48 h after transfection of oligonucleotides (Fig. 2C). We detected a slight augmentation of the level of phosphorylated JNK in RNAi2-treated cells at 72 h (data not shown), indicating slight augmentation of oxidative stress. These results suggest that reduction of TRX does not result in the production of massive ROS generation, and the low amount of TRX is sufficient for maintaining cell survival.

TRX knockdown enhanced CDDP-induced cell death

The cellular reducing system is composed mainly of the TRX and glutathione (GSH) systems. We tested whether an inhibitor of GSH synthesis, DL-buthionine-[*S*, *R*]-sulfoximine (BSO), augments the effect of TRX knockdown as determined by staining with Annexin V-FITC and PI. However, the treatment with BSO did not significantly enhance cell death (data not shown). The level of GSH was reduced to 20–40% compared with that in untreated MCF7 cells (data not shown). These results suggest that, without exposure to significant stress, cell survival can be maintained with reduced amounts of TRX and GSH.

Next, we examined the effect of the TRX knockdown on oxidative stress–induced cell death. TRX knockdown slightly augmented hydrogen peroxide–induced cell death analyzed by the sub-G_1 content (Supplemental Fig. 2; see www.liebertonline.com/ars). This confirms that decreased TRX expression enhances oxidative stress–induced cell death. Previous reports showed that the TRX expression level affects resistance to anticancer drugs such as *cis*-diaminedichloroplatinum (II) (CDDP) (34, 50). We analyzed the effect of TRX knockdown on CDDP-induced cell death and found that CDDP-induced cell death was enhanced (Fig. 3) (Supplemental Fig. 3; see www.liebertonline.com/ars). These results suggest that TRX plays a role in the protection against CDDP-induced cell death.

TRX knockdown induced G_1 cell-cycle arrest

As shown in Fig. 1C and D, TRX knockdown mainly induced retardation of cell growth. To examine the role of TRX in the cell cycle, we first analyzed which phase of the cell cycle is regulated by TRX. Flow-cytometric analyses revealed a significant accumulation of cells in G_1 phase in response to TRX knockdown (Fig. 4) (Supplemental Fig. 4; see www.liebertonline.com/ars). To identify genes regulated by TRX, we performed microarray analyses on MCF7 cells by using an

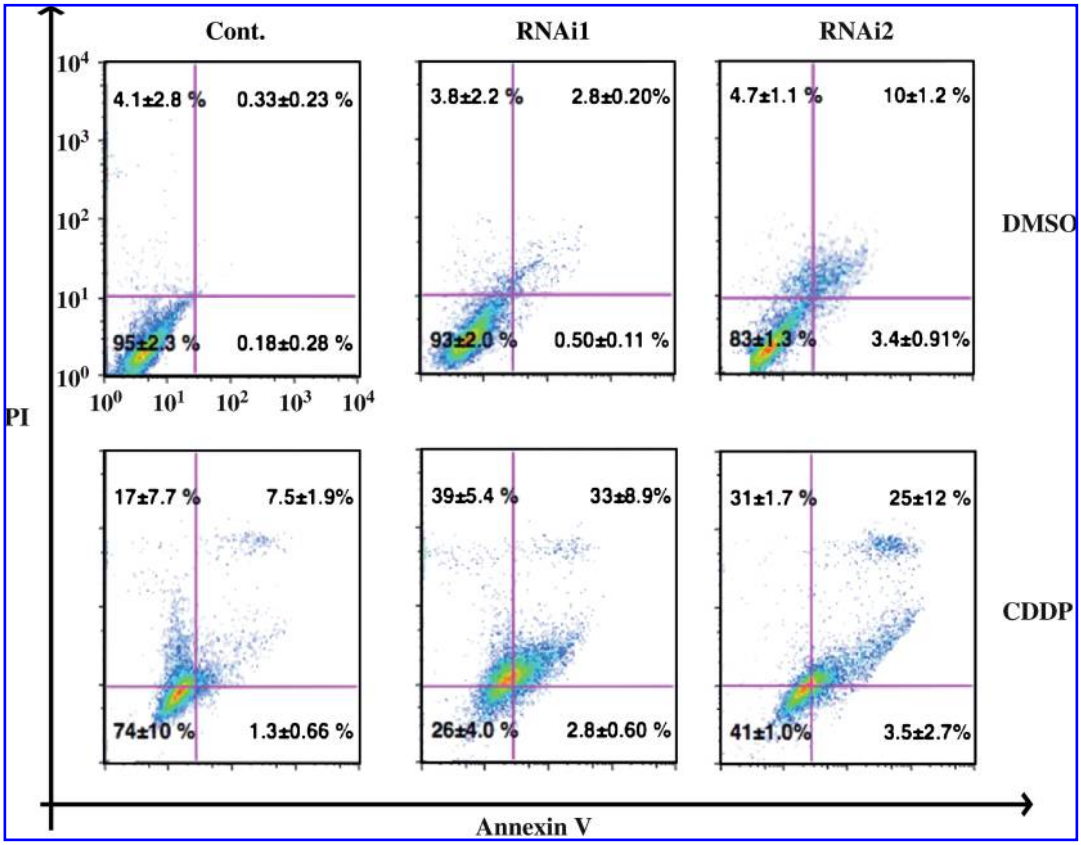

FIG. 3. TRX knockdown enhanced CDDP-induced cell death. A549 Cells were transfected with oligonucleotides for 24 h and treated with CDDP or dimethylsulfoxide (DMSO) and cultured for 48 h, and then stained with Annexin-V and PI and analyzed with flow cytometry, as described in Materials and Methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

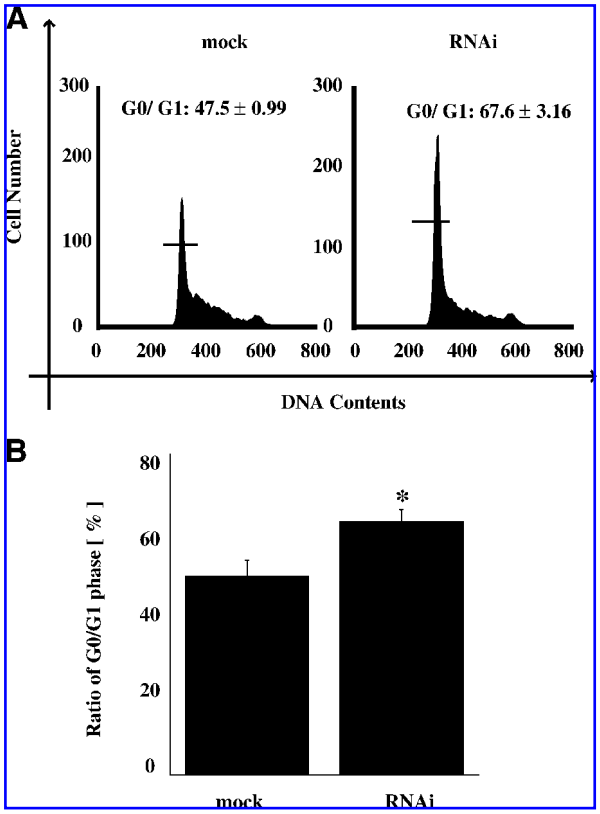

FIG. 4. TRX knockdown induced a significant accumulation of cells in G_1 phase. (**A**) Analyses of the cell cycle with flow cytometry. MCF7 cells were transfected with shRNA-expression vectors and analyzed with flow cytometry to quantify the DNA content of cells as described in Materials and Methods. (**B**) Ratio of G_0 to G_1 phase. Data are presented as mean ± SD, and data were analyzed with Student's *t* test compared with control. $^*p < 0.05$ ($n = 3$).

Affymetrix GeneChip (U133 Plus2.0), comparing control TRX knockdown cells from transfection of oligonucleotides or an shRNA expression vector. We detected 20 genes of which expression changed >1.3-fold in both the transfection of oligonucleotides and the transfection of the shRNA expression vectors. The genes were related to various cellular functions including several genes, such as cyclin D1, that are regulators of the cell cycle (Table 1). We next checked the mRNA expression levels of the cyclin D1 gene with RT-PCR and quantitative RT-PCR. The expression of cyclin D1 mRNA was downregulated by TRX knockdown in A549 and MCF7 cells (Fig. 5), as was the level of cyclin D1 protein (Fig. 6). We then analyzed the expression levels of G_1 phase–regulating proteins. TRX knockdown reduced the expression levels of cyclin D1, whereas the levels of cyclin D3, p21, and p27 were not significantly changed in both cells (Fig. 6A). If cyclin D1 plays a key role in the regulation of the cell cycle by TRX, cyclin D1 overexpression may rescue the cell-cycle arrest. Thus, we analyzed the effects of TRX knockdown by counting cell growth in cyclin D1 overexpression cells. Cyclin D1 overexpression restored the cyclin D1 expression in TRX knockdown cells (Fig. 7A). Cyclin D1 overexpression completely rescued cell growth in cells knocked down by RNAi2 and partially rescued cell growth in cells targeted by RNAi1 in MCF7 cells (Fig. 7B). These results indicate that TRX knockdown induced G_1 cell-cycle arrest mainly through the downregulation of the cyclin D1.

TRX knockdown reduced cyclin D1 transcription through the region including binding sites for AP-1 in the promoter

As shown in Fig. 5, TRX knockdown reduced cyclin D1 transcription. We analyzed the region of cyclin D1 promoter responsible for the effects of TRX knockdown. TRX knockdown reduced the luciferase activity from cyclin D1 promoter

Table 1. The Genes of Which Expression Changed by TRX Knockdown in Microassay Analyses

Vector/oligonucleotide	*tRNA-shRNA*		*Oligonucleotide*				
Name	*Mock*	*RNAi*	*NC*	*RNAi*	*UniGene*	*Affy_ID*	*Regulation*
AREG	0.037	0.10	0.79	1.2	Hs.270833	205239_at	UP
NPIP	0.043	0.057	1.2	1.6	Hs.498849	204538_x_at	UP
ASS	0.13	0.22	3.6	5.2	Hs.160786	207076_s_at	UP
HERPUD1	0.014	0.069	0.80	1.2	Hs.146393	217168_s_at	UP
SIAH2	0.091	0.13	2.8	4.1	Hs.20191	209339_at	UP
LOC283241	0.033	0.020	1.2	0.90	Hs.356719	224893_at	DOWN
GMNN	0.10	0.06	6.0	4.3	Hs.234896	218350_s_at	DOWN
DLG7	0.18	0.09	6.2	4.7	Hs.77695	203764_at	DOWN
RAB31	0.19	0.11	6.1	4.3	Hs.223025	217762_s_at	DOWN
HSPA1A	0.32	0.20	11	8.7	Hs.75452	200799_at	DOWN
MAD2L1	0.054	0.033	2.1	1.1	Hs.79078	203362_s_at	DOWN
RRM1	0.053	0.039	2.9	2.0	Hs.383396	201477_s_at	DOWN
MSH6	0.039	0.027	1.9	1.3	Hs.445052	202911_at	DOWN
CCND1	0.14	0.10	4.8	3.2	Hs.371468	208712_at	DOWN
TOP2A	0.052	0.030	1.8	1.2	Hs.156346	201292_at	DOWN
CDCA3	0.21	0.11	6.2	4.4	Hs.30114	223307_at	DOWN
ANLN	0.08	0.047	3.6	2.2	Hs.62180	222608_s_at	DOWN
MCM6	0.14	0.10	7.5	5.1	Hs.444118	201930_at	DOWN
CDC2	0.08	0.05	4.3	3.1	Hs.334562	203213_at	DOWN
TXNIP	0.42	0.19	16	11	Hs.179526	201008_s_at	DOWN

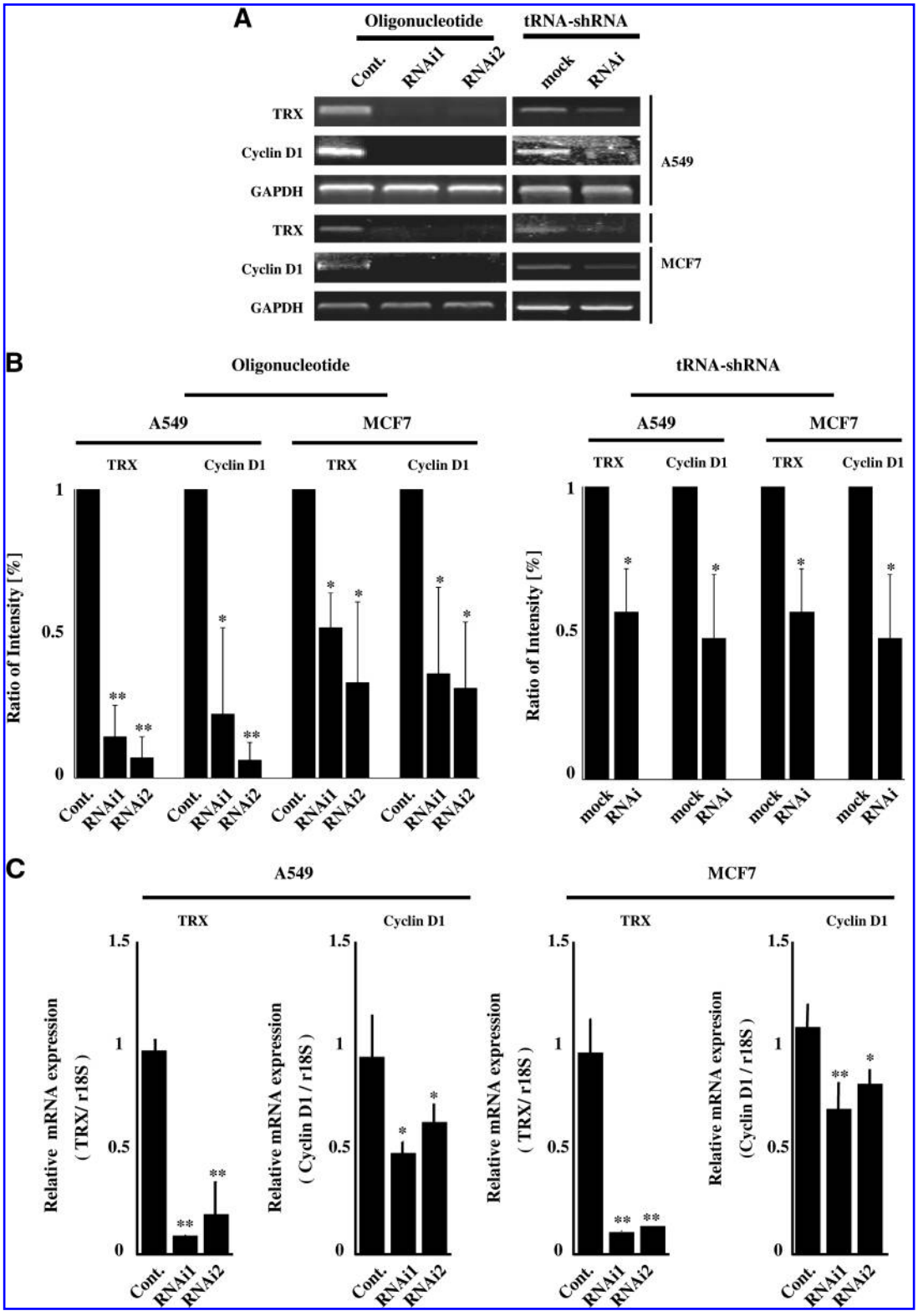

FIG. 5. TRX knockdown reduced cyclin D1 transcription. (**A**) Analyses of the expression of cyclin D1 mRNA in A549 and MCF7 cells with RT-PCR. Cells cultured for 24 h after transfection of oligonucleotides or shRNA-expression vector were used. GAPDH was used as loading controls. (**B**) Densitometric analysis of mRNA expression levels. Data are presented as mean ± SD, and data were analyzed with Student's *t* test compared with control. $*p < 0.05$; $**p < 0.001$ ($n = 3$). Expression of TRX/β-actin or cyclin D1/β-actin levels in control oligonucleotide transfections was set at a relative value of 1. (**C**) Quantitative real-time RT-PCR analysis of cyclin D1 mRNA expression. The results are shown as mean ± SD of triplicate cultures. Data were analyzed with Student's *t* test to compare with the control cells. $*p < 0.05$; $**p < 0.001$ ($n = 3$).

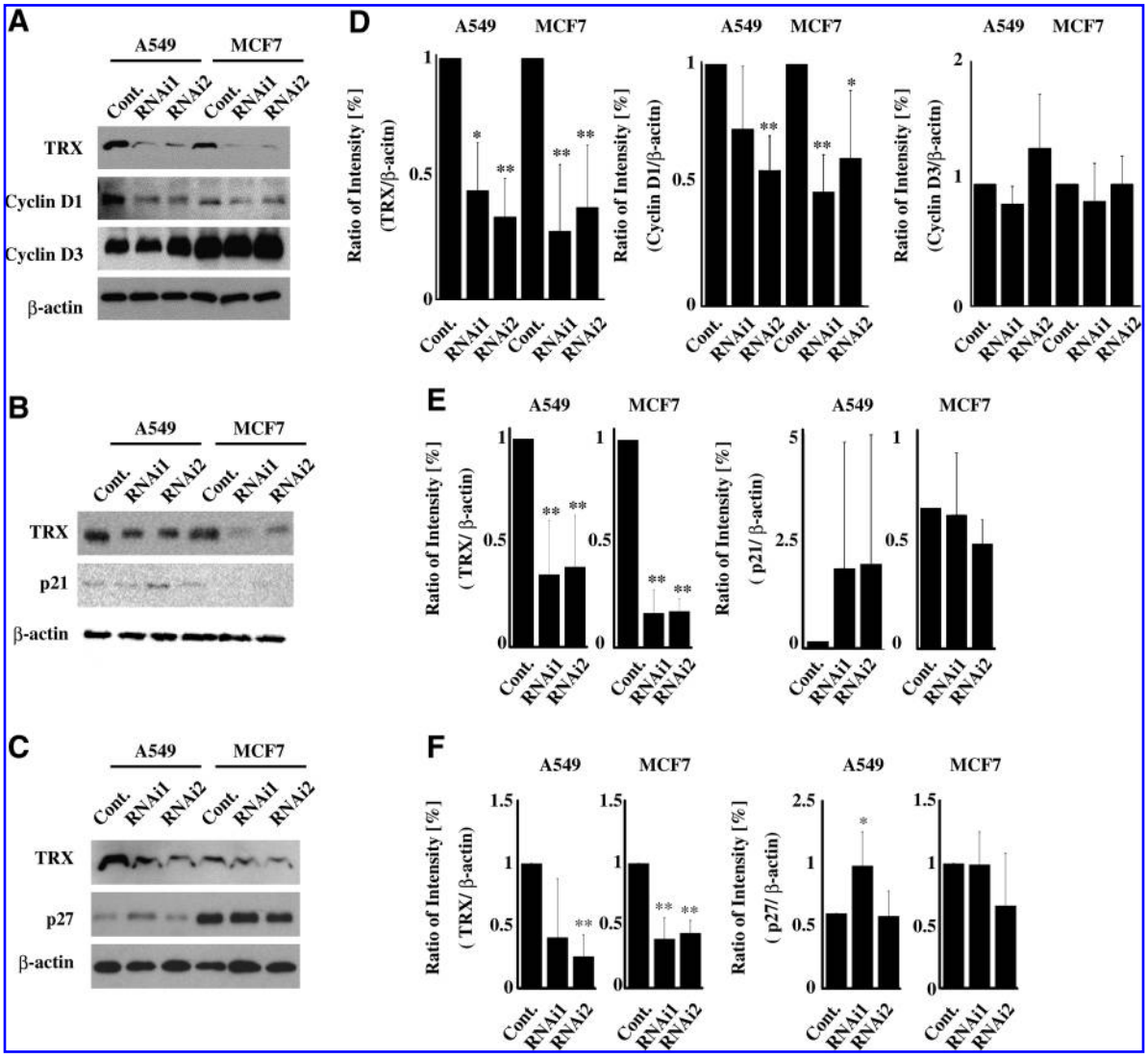

FIG. 6. TRX knockdown reduced the expression of cyclin D1 protein. (**A**) Western blotting of cyclin D1 and D3 in A549 and MCF7 cells. Cells were cultured for 48 h after transfection of oligonucleotides. β-Actin was used as a loading control. (**B**) Western blotting of p21 in A549 and MCF7 cells. (**C**) Western blotting of p27 in A549 and MCF7 cells. (**D–F**) Densitometric analysis of protein expression levels. Data are presented as mean $\pm$ SD, and data were analyzed with Student's t test compared with control. $^{*}p < 0.05$; $^{**}p < 0.001$ ($n = 3$). Intensity of the expression of TRX/β-actin, cyclin D1/β-actin, cyclin D3/β-actin, p21/β-actin, and p27/β-actin is shown. Levels in control oligonucleotides transfections were set at a relative value of 1.

vectors harboring regions from -1749 to $+35$ (21) (Fig. 8A). Cyclin D1 promoter contains binding sites for transcription factors including SP-1, AP-1, E2F, and T-cell factor (TCF). Because the role of AP-1 proteins in cyclin D1 expression and in the cell cycle is well characterized (35) and TRX is reported to reduce critical cysteine residues in the AP-1 protein to facilitate DNA interaction (6, 48), we analyzed the regulation through AP-1 sites. We prepared the mutant construct of the cyclin D1 promoter, which harbors mutations in the AP-1 binding site. Whereas TRX knockdown reduced the luciferase activity of a wild-type vector, the TRX-knockdown effect was cancelled in the vector with the mutations in the binding site for AP-1 (Fig. 8C and D). These results suggested that TRX regulates the expression of cyclin D1 through the AP-1 binding site in the cyclin D1 promoter.

TRX knockdown regulated AP-1 activity via the ERK1/2 pathway

The expression and activity of Jun and Fos family members is tightly regulated by the MAPK family (13). Activation of ERK leads to the coordinated stimulation of *c-fos* expression by acting on transcription factors bound to the *c-fos* promoter (13, 42). We thus analyzed the effect of TRX knockdown on the level of phosphorylated ERK1/2. TRX knockdown weakly reduced the levels of phosphorylated ERK1/2 at 24 to 72 h (data not shown). Then we analyzed the levels of protein and levels of phosphorylated ERK1/2 in EGF treatment. TRX knockdown also reduced the level of EGF-induced phosphorylation of ERK1/2 (Fig. 9). Because activated ERK1/2 translocates from the cytoplasm to the nucleus (20) and EGF

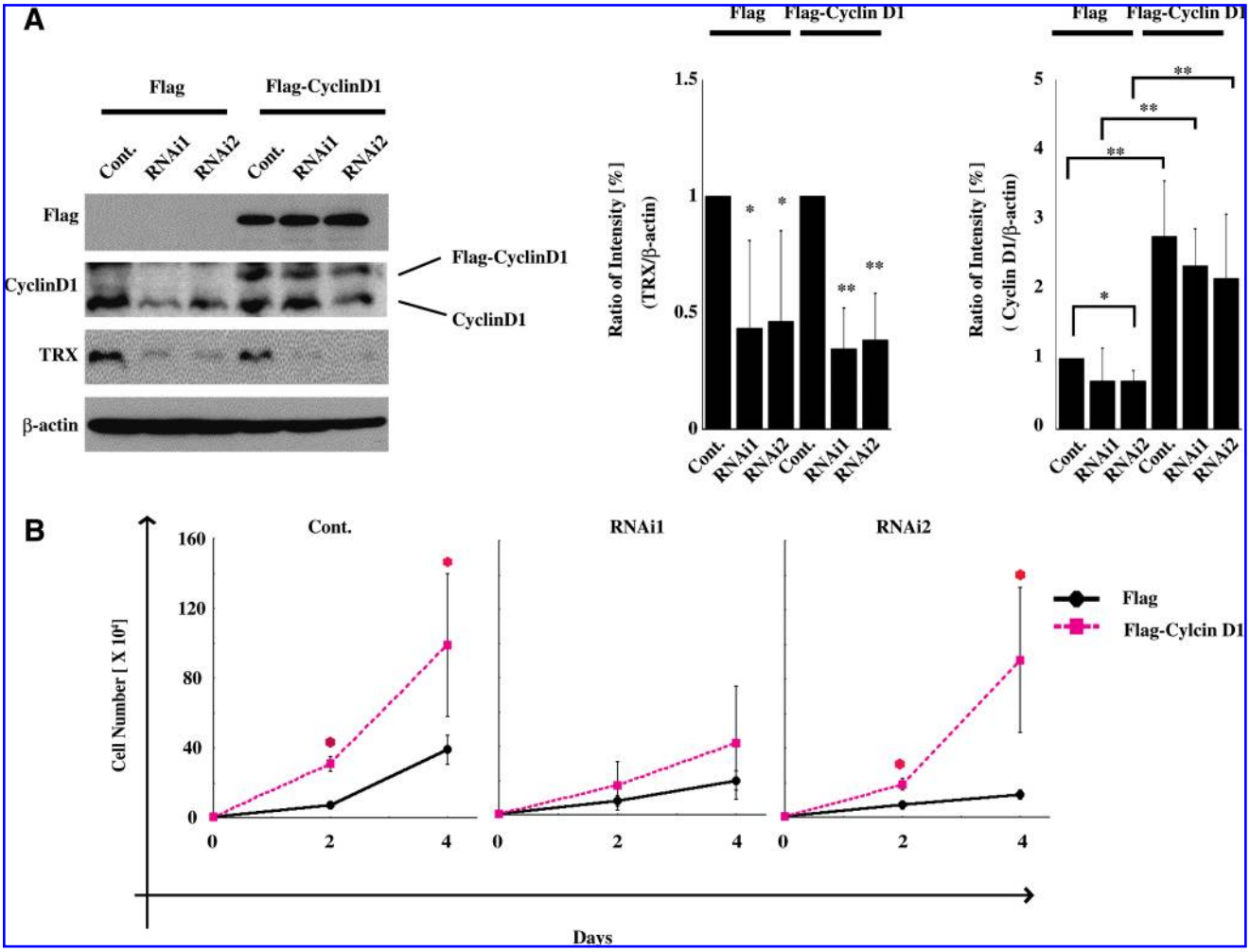

FIG. 7. Cyclin D1 overexpression rescued the TRX knockdown–induced cell growth arrest. (**A**) Western blotting analyses of TRX or cyclin D1. Cells were transfected with oligonucleotides in Flag or cyclin D1 overexpression cells for 24 h. Bar graph: densitometric analysis of TRX or cyclin D1 expression, normalized by the expression of β-actin. Data are presented as mean ± SD and were analyzed with Student's t test compared with control. *$p < 0.05$; **$p < 0.001$ ($n = 3$). Expression levels in control oligonucleotides transfections were set at a relative value of 1. (**B**) Cyclin D1 overexpression rescued cell growth arrest in TRX-knockdown cells. MCF7 cells were stably transfected with the p3FLAG-HA-cyclin D1 expression vector. Cell-growth analyses of cyclin D1 stable transfectants, transfected with oligonucleotides, were performed. The counting of cells was started at 24 h after oligonucleotide transfection. The results are shown as mean ± SD of triplicate cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

stimulation induces the strong nuclear translocation, we tested ERK1/2 translocation by immunofluorescence (Fig. 10 and Supplemental Fig. 5) and Western blotting (data not shown). In control cells, EGF stimulation induced nuclear translocation of ERK1/2 (Fig. 10) (Supplemental Fig. 5; see www.liebertonline.com/ars) and TRX (data not shown), whereas in TRX-knockdown cells, the translocation of ERK1/2 was suppressed. These results suggest that TRX regulates AP-1 activation *via* the ERK1/2 pathway.

Discussion

Based on previous reports (18, 21, 31, 33), we predicted that *in vivo* TRX knockdown by itself induces cell death. However, TRX knockdown barely affected the rate of cell death without exposure to stress (Fig. 2A). The ROS production was not significantly induced by TRX knockdown (Fig. 2B). These results indicated that in a resting state, TRX knockdown itself does not produce significant amounts of oxidative stress, and the low levels of TRX are sufficient for maintaining cell survival. Meanwhile, TRX and GSH are the main regulators of cellular redox homeostasis. Reduction of GSH did not significantly augment cell death in TRX-knockdown conditions (data not shown). These results also suggested that the low protein levels of TRX and GSH are sufficient for maintaining cell survival. However, the level of GSH in BSO-treated cells was reduced only to 20–40% compared with untreated MCF7 cells (data not shown). Accordingly, it is necessary to examine further the effect of TRX knockdown in cells with much lower levels of GSH. Previous reports showed that TRX is one of the key regulators of oxidative stress–induced cell death (1), and TRX expression levels affected the resistance to anticancer drugs (34, 50). Our results also showed that TRX knockdown enhanced hydrogen peroxide–induced (Supplemental Fig. 2) and CDDP-induced cell death (Fig. 3). It is reported that overexpressed TRX compensates for Fanconi anemia–related

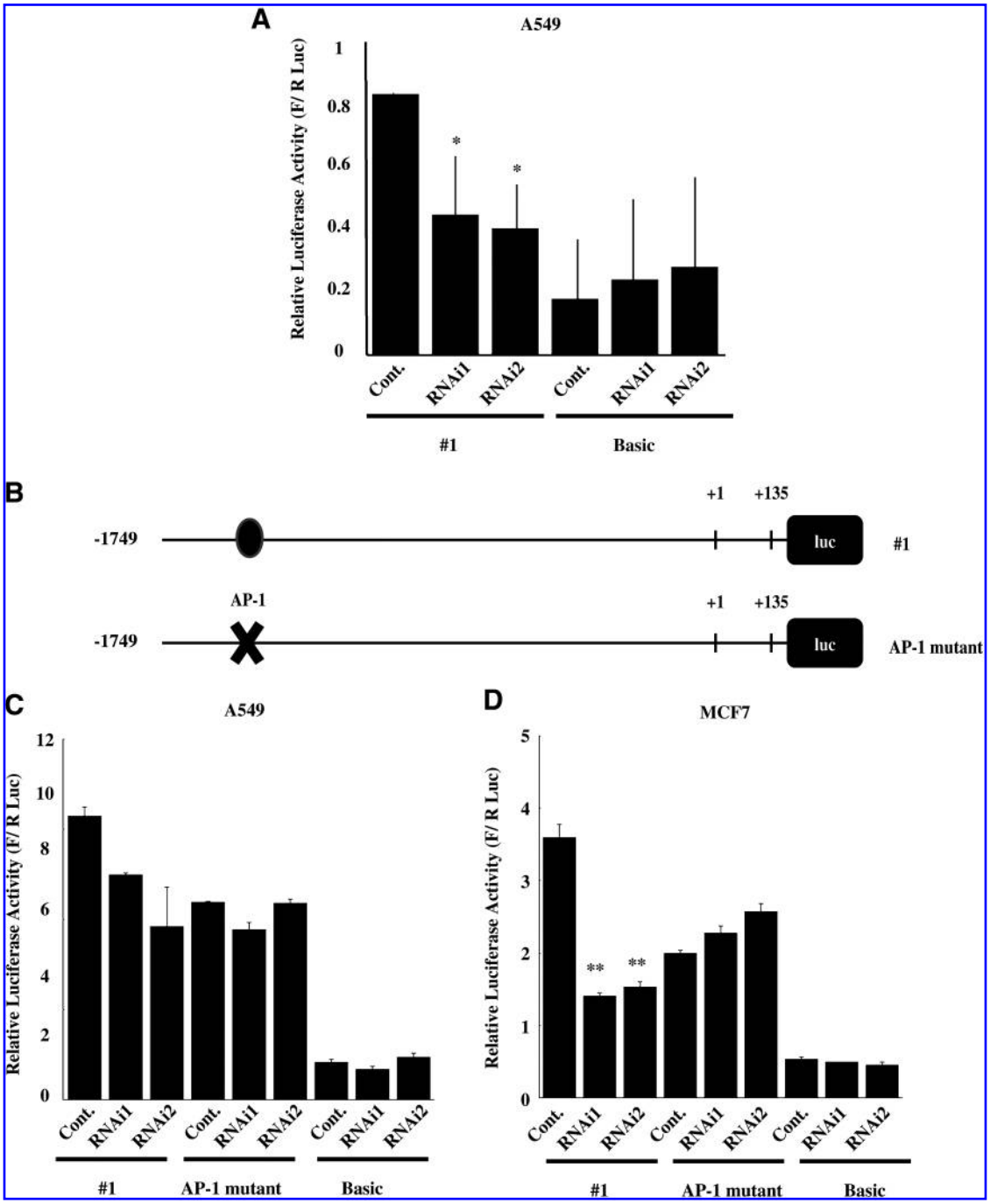

FIG. 8. Cyclin D1 promoter activity was reduced by TRX knockdown. (**A**) Effects of TRX knockdown on luciferase activity of the cyclin D1 promoter (#1; −1,749 to +135) in A549 cells. Cells were transfected with 1 μg/ml of the cyclin D1 promoter vector (#1) or pGL3-basic, 50 ng/ml of pRL-TK, and 40 n*M* oligonucleotides. Relative luciferase activities normalized by *Renilla* luciferase activities are shown as mean ± SD from three independent experiments. *$p < 0.05$ (*t* test). (**B**) The construct containing the cyclin D1 mutant promoter. (**C, D**) The effects of TRX knockdown on luciferase activity of AP-1 mutant vector of the cyclin D1 promoter in A549 (**C**) and MCF7 cells (**D**). Cells were transfected with 1 μg/ml of plasmid (#1, pCyclin D1 AP-1mut-luc, or pGL3 basic), 50 ng/ml of pRL-TK, and 40 n*M* oligonucleotides, and then cultured for 24 h. Luciferase activity normalized by pRL-TK expression is shown. The results are shown as mean ± SD of triplicate cultures samples. **$p < 0.001$ (*t* test).

chromosomal instability (17). TRX might play a physiological role in the protection against DNA damage. TRX may be indispensable at certain stages of development and in specific cell types, such as stem cells. Indeed, TRX was reported as one of the regulators of two-cell block (28). The role of TRX in cell survival and cell death in these conditions and cells should be further investigated.

A previous report showed that the cyclical changes of redox balance, which are controlled by TRX and other redox systems, regulate the cell cycle in early development (10). TRX

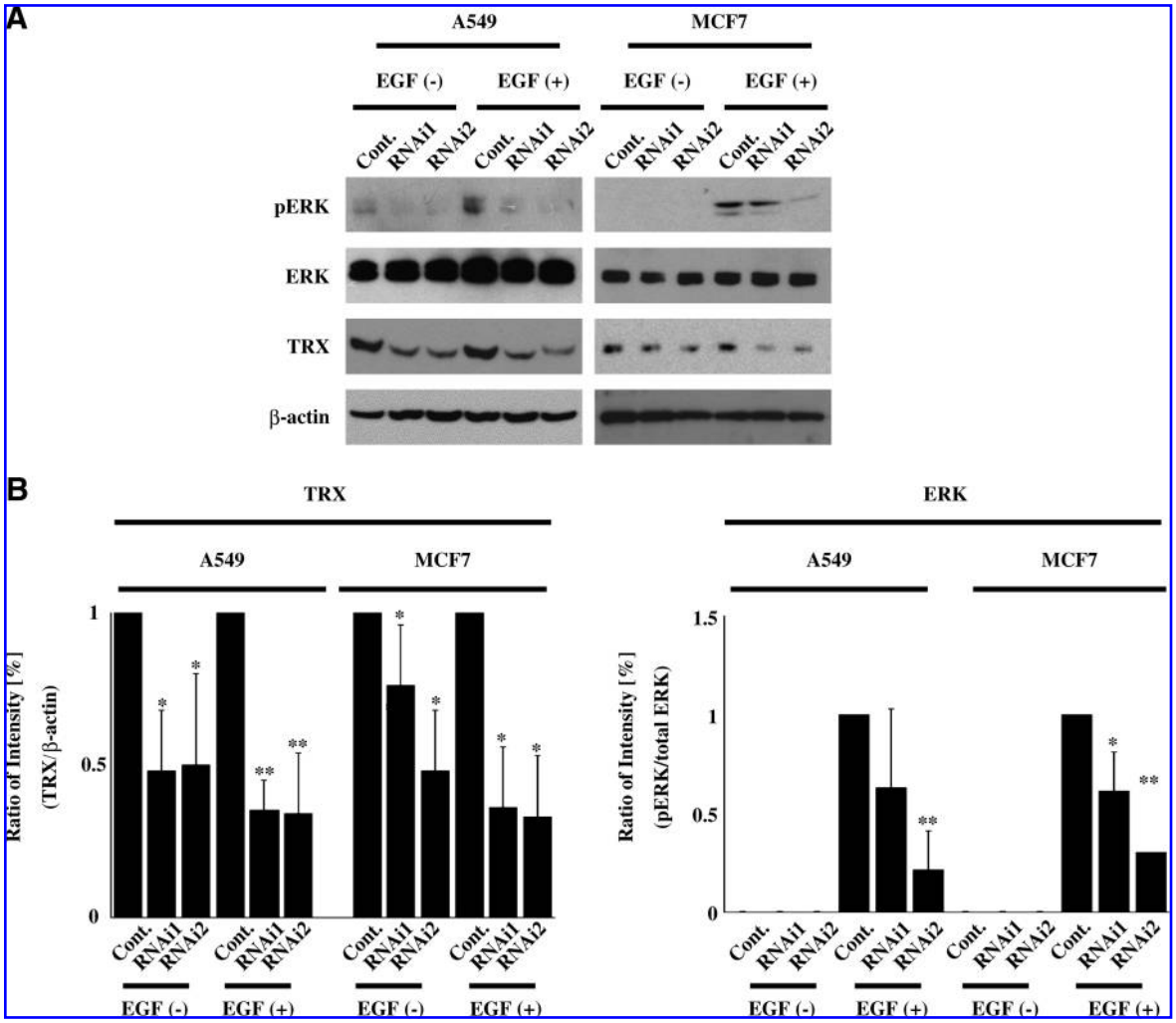

FIG. 9. TRX knockdown suppressed the phosphorylation of ERK1/2. (**A**) Western blotting analyses of ERK1/2 phosphorylation on EGF stimulation in A549 and MCF7 cells. Cells were cultured with DMEM containing 0.5% FCS for 12 h and transfected with 40n*M* oligonucleotides, cultured for 24 h, and then treated with 50 ng/ml EGF for 5 min and analyzed with Western blotting. (**B**) Densitometric analysis of the expression of TRX or cyclin D1 normalized by the expression of β-actin. Data are presented as mean ± SD and were analyzed with Student's *t* test compared with control. $^{*}p < 0.05$; $^{**}p < 0.001$ ($n = 3$). Expression levels in control oligonucleotides transfections were set at a relative value of 1.

also is reported to be a physiologically relevant electron donor for ribonucleotide reductase (RNR) during DNA-precursor synthesis (2, 15, 18, 26). We analyzed the effects of TRX knockdown on the level of dNDP to monitor RNR activity. A tendency for TRX knockdown to suppress the levels of dGDP, suggesting that TRX knockdown caused downregulation of RNR activity (data not shown). Thus, TRX seems to regulate the cell cycle in both the G_1 and S phases. In the current study, TRX knockdown caused G_1 cell-cycle arrest, which may lead to the blockage of entry into the S phase, in which DNA is synthesized (Fig. 4 and Supplemental Fig. 4).

Our data showed that TRX regulates the expression of cyclin D1 at transcriptional (Fig. 5) and protein levels (Fig. 6), whereas levels of other G_1 phase–regulating proteins such as cyclin D3, p21, and p27, did not change significantly (Fig. 6). The activation of the cyclin D1 gene is predominantly regulated at the level of transcription (37). Cyclin D1 transcription through the regions containing SP-1, AP-1, E2F, and TCF binding sites was suppressed in TRX knockdown analyzed with deletion mutants of cyclin D1 promoter (data not shown). The analyses using the construct harboring mutations in AP-1 indicated the involvement of this site in TRX-knockdown effects (Fig. 8C and D). We also showed that TRX knockdown suppressed binding of c-Jun and c-Fos in a chromatin immunoprecipitation assay (data not shown). The activation of AP-1 by TRX can be explained by a previously accepted view; TRX may reduce critical cysteine residues in the AP-1 proteins to facilitate DNA interaction (6, 48).

In addition to that view, we here presented a novel mechanism for the regulation of AP-1 activation by TRX. We showed that the basal levels of phosphorylated ERK1/2 were low in TRX-knockdown cells and that TRX knockdown suppressed the levels of phosphorylated ERK1/2 induced by EGF (Fig. 9). Extracellular stimuli, such as growth factors, induce sequential activation of Raf, MEK, and ERK to augment cyclin D1 transcription, inducing cells to enter into the S phase.

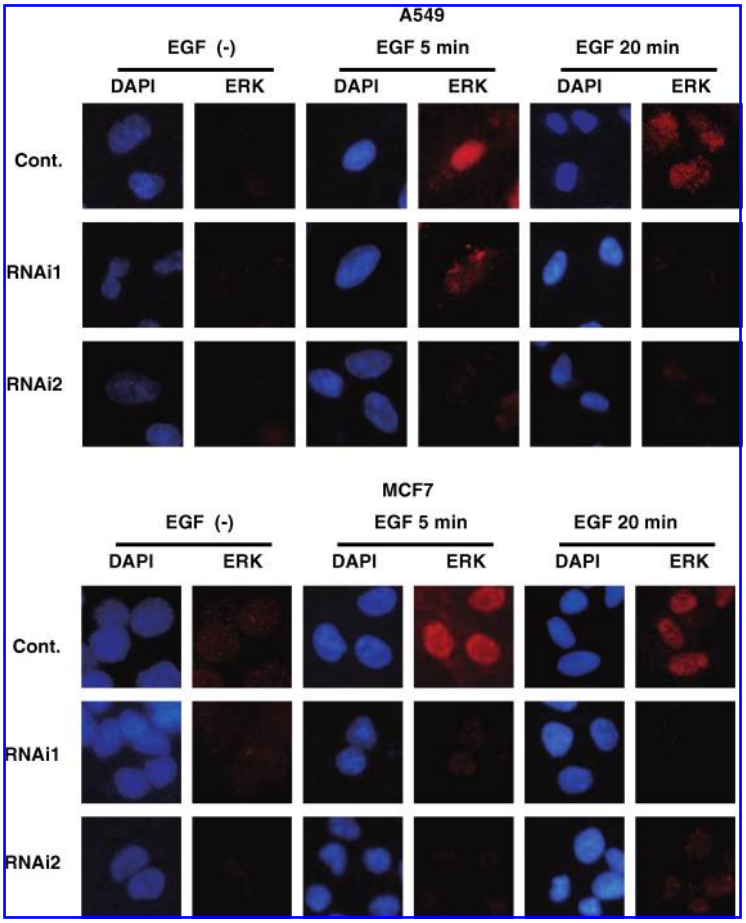

FIG. 10. TRX knockdown reduced EGF-induced ERK nuclear translocation. Cells were transfected with 40 n*M* oligonucleotides, cultured for 24 h, and then treated with 50 ng/ml EGF for 5 or 20 min. ERK1/2 localization was detected with anti-ERK1/2 antibody and DAPI in A549 and MCF7 cells.

Activated ERK translocates from the cytoplasm to the nucleus (20). In TRX-knockdown cells, EGF-induced nuclear translocation of ERK1/2 was suppressed (Fig. 10 and Supplemental Fig. 5). These results indicate that TRX regulates the levels of phosphorylated ERK1/2. TRX may regulate the oligomerization of receptor molecules or adaptor proteins, or augment the function of signaling molecules such as PKC, Ras, and Raf by reducing the cysteine residues of these molecules. For example, previous reports showed that TRX and TRX-related PKC-interacting protein (PICOT) play a critical role in the regulation of PKCs and AP-1 (12, 47). We also obtained results that the phosphorylation of Raf was reduced by TRX knockdown (data not shown). Because TRX was reported to interact with ASK1, one of the MAPKKKs, TRX may regulate ERK1/2 phosphorylation through the interaction with members of Raf family MAPKKKs. The underlying mechanism of how TRX regulates ERK1/2-cyclin D1 pathway is currently under investigation. Furthermore, it is still unclear how much of the effect of TRX knockdown on the cell cycle is mediated through its effect on the regulation of ERK1/2 phosphorylation. Further studies are needed to elucidate this finding.

Cyclin D1 expression is elevated in a wide variety of tumor cells, and we here demonstrated that TRX regulates the G_1 cell cycle through cyclin D1 transcription *via* the ERK1/2-AP-1 pathway. Thus, one might consider that enhanced levels of TRX result in the elevation of cyclin D1, leading to the facilitation of tumor formation. However, TRX overexpression itself does not seem to be oncogenic in a physiologic situation, because TRX transgenic (Tg)-mice have elongated life spans (24) and did not show an augmented ratio of tumorigenicity. In the context of tumor cell biology, nevertheless, elevated levels of TRX were shown in various cancer cell lines or tissues. Tumor cells may take advantage of TRX to sustain their growth by maintaining ERK/AP-1/cyclin D1 activation pathways. The elucidation of the mechanism with which TRX regulates EGF-mediated ERK activation might contribute to the development of new cancer-targeting approaches.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

AP-1 = activator protein-1
ASK1 = apoptosis signal-regulating kinase 1
BSO = DL-buthionine-[*S*, *R*]-sulfoximine
CDDP = *cis*-diaminedichloroplatinum (II)
DAPI = 4′,6-diamidino-2-phenylindole
DCFDA = 2,7-dichlorodihydrofluorescein diacetate
DMEM = Dulbecco's minimum essential medium
EGF = epidermal growth factor
ERK = extracellular signal-regulated kinase
FCS = fetal calf serum
FITC = fluorescein isothiocyanate
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GSH = glutathione
JNK = c-Jun N-terminal kinase
MAPK = mitogen-activated protein kinase
MAPKKK = MAP kinase kinase kinase
PCR = polymerase chain reaction
PI = propidium iodide
PKC = protein kinase C
RNAi = RNA interference
RNR = ribonucleotide reductase
ROS = reactive oxygen species
RT = reverse transcription
SD = standard deviation
SDS = sodium dodecylsulfate
TCF = T-cell factor
TRX = thioredoxin

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