The cyclin-dependent kinase inhibitor, p21WAF1, promotes angiogenesis by repressing gene transcription of thioredoxin-binding protein 2 in cancer cells

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The cyclin-dependent kinase inhibitor, p21WAF1, induces cell-cycle arrest and can act as a tumor suppressor. However, increasing evidence indicates that p21WAF1 can also increase resistance to some anticancer therapies and thus promote tumor growth. The mechanisms explaining this paradox have not been explained. We found that conditioned media from MCF-7 breast cancer cells transfected with a p21WAF1-specific small interfering RNA (siRNA) significantly reduced endothelial cell migration, invasion and vascular sprouting. Liquid chromatography/mass spectrometry analysis of the conditioned media revealed that p21WAF1 knockdown significantly reduced secretion of thioredoxin (Trx), a redox protein known to promote tumor angiogenesis. p21WAF1 knockdown decreased Trx enzymatic activity in cancer cells, by effects on the expression levels of intracellular thioredoxin-binding protein 2 (TBP2), known to bind and inactivate Trx. Consistent with these findings, media from cancer cells transfected with TBP2 siRNA promoted endothelial cell invasion and blocked the anti-angiogenic effect of p21WAF1 siRNA. Addition of Trx siRNA blocked the pro-angiogenic effects of TBP2 siRNA. Chromatim immunoprecipitation assays showed p21WAF1 bound TBP2 gene promoter. Taken together, our data suggests that p21WAF1 can induce Trx secretion and angiogenesis in cancer cells, by direct transcriptional repression of the TBP2 promoter.

Introduction

The cyclin-dependent kinase inhibitor p21WAF1 (CDKN1A) is well known to function as a tumor suppressor by inducing cell-cycle arrest and tumor growth inhibition. However, p21WAF1 gene expression is often activated by various chemotherapeutic drugs and radiotherapy, and the upregulation of p21WAF1 not only renders cancer cells resistant to the treatments in vitro (1,2) but also correlates with worse clinical outcome in cancer patients on clinical trials (3,4). Moreover, we have found that p21WAF1-knockout mice develop significantly less tumors than their wild-type counterparts in various organs after ionizing radiation (5), and additional deficiency of p21WAF1 paradoxically inhibits the development of thymic lymphoma in p53-knockout mice (6), further supporting the hypothesis that p21WAF1 can also act as an oncogene in some settings (6,7).

Angiogenesis is vital for tumor progression; tumor growth is arrested at a size of 1–2 mm³ in the avascular stage (8). When pro-angiogenic factors outbalance the action of anti-angiogenic molecules, the tumor acquires an angiogenic switch, that leads to endothelial cell migration, invasion, vascular sprouting, the formation of new blood vessels, tumor growth and metastasis (9). One of the key factors that promote tumor-driven angiogenesis is thioredoxin (Trx) (10).

Trx is a hydrogen donor and a scavenger of reactive oxygen species. Trx increases the transcriptional activity of the pro-angiogenic protein, hypoxia-inducible factor 1α, to enhance gene transcription of hypoxia-inducible factor 1α target genes, such as vascular endothelial growth factor in the nucleus of cancer cells (10). Moreover, when secreted into the extracellular matrix, Trx inhibits the enzymatic activity of tissue inhibitor of metalloproteinases 1 and 2, and therefore increases the enzymatic activity of metalloproteinases 2 and 9, which stimulates tumor cell invasion and tumor-driven angiogenesis (11). Importantly, the enzymatic activity of intracellular Trx is mainly inactivated by direct binding to thioredoxin-binding protein 2 (TBP2) (12), which is also known as Trx-interacting protein. However, TBP2 does not inactivate extracellular Trx, as TBP2 cannot be secreted into the extracellular matrix (13).

In this study, we show that p21WAF1 can promote tumor growth by inducing angiogenesis in cancer cells. We demonstrate that TBP2 transcription is directly repressed by p21WAF1, which leads to increased secretion of Trx and angiogenesis. These data provide a novel mechanism whereby p21WAF1 may act to promote tumor growth.

Materials and methods

Cancer cell line culture

Breast (MCF-7), lung (H460) and prostate (LNCaP) cancer cell lines were purchased from American Type Culture Collection (Manassas, VA); MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, whereas H460 and LNCaP cell lines were cultured in Roswell Park Memorial Institute Medium (Invitrogen) supplemented with 10% fetal calf serum. All cell lines were maintained in a humidified incubator at 37°C and 5% CO2 in air. The cancer cells were placed in hypoxic chamber for angiogenesis studies. Hyposoxic conditions were maintained in a chamber filled with 1% oxygen and 99% nitrogen gas mixture.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were a gift from Dr K MacKenzie (Children’s Cancer Institute Australia, Sydney, Australia). HUVECs were maintained in 0.1% gelatin-coated tissue culture flasks in Medium 199 (Invitrogen) supplemented with 20% fetal bovine serum, 5% human serum (Sigma, St Louis, MO), 10 μM heparin (Pharmacia & Upjohn, Peapack, NJ), 5 ng/ml fibroblast growth factor-basic (Sigma) and 20 μg/ml endothelial cell growth factor (Roche, Mannheim, Germany). Only passages five and six were used in the experiments.

Small interfering RNA transfection

Cancer cells were transfected with a validated scrambled control small interfering RNA (siRNA) or siRNA specifically targeting genes of interest, p21WAF1 (SMARTpool siRNA, Dharmacon Research, Lafayette, CO); Trx (SMARTpool siRNA, Dharmacon) or TBP2 (SMARTpool siRNA, Dharmacon) with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendation. Eight hours after siRNA transfection, cells were removed from the medium containing the siRNA and lipofectamine and replenished with fresh cell culture medium.

Semiquantitative-competitive reverse transcription–polymerase chain reaction

Semiquantitative-competitive reverse transcription–polymerase chain reaction was carried out to analyze target gene expression in cancer cells as described previously (14). As a control, β2-microglobulin was amplified from the same complementary DNA samples and used to determine relative gene expression by dividing the densitometric volume of the band of the polymerase chain reaction (PCR) product from the target gene by that from the control β2-microglobulin.

Whole cell protein extraction

Cells were lysed using cold radio-immunoprecipitation assay buffer [150 mM NaCl (Ajax Finechem, Sydney, Australia); 1% Nonidet P-40 (Fluka, St Louis, MO); 0.5% sodium deoxycholate (Sigma); 0.1% sodium dodecyl sulfate (MP Biomedicals LLC, Aurora, OH)] and 50 mM Tris, pH 7.5 (Sigma) containing...
protease inhibitor cocktail (Sigma). Protein quantification using BCA Protein Assay (Pierce, Rockford, IL) was done according to manufacturer’s guidelines.

Western blotting

Twenty micrograms of protein per sample were mixed with loading buffer containing 20% β-mercaptoethanol (Sigma) and boiled for 5 min at 95°C. After electrophoresis, the proteins were transferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). Following blocking, the membranes were incubated with primary antibodies against p21WAF1 (Santa Cruz Biotechnology, Santa Cruz, CA), Trx (FL-105, Santa Cruz Biotechnology), or TBP2 (MBL International, Woburn, MA) in TBST (1× Tris-buffered saline and 0.1% Tween-20) for 2 h at room temperature. Following washing, secondary antibodies conjugated with horseradish peroxidase were incubated with the membranes for 1 h at room temperature in Tris-buffered saline with 0.5% skim milk. Chemiluminescent detection was performed using SuperSignal reagents (Pierce). Membranes were then stripped with 0.2 M glycine pH 2.8 and 0.5 M NaCl and reprobed with an anti-β-actin antibody (Pierce), as a loading control.

End point insulin assay for measurement of Trx activity

Intracellular protein in cancer cells was extracted using a radio-immunoprecipitation assay buffer containing N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (Sigma) and protease inhibitors were diluted to an equal concentration. Two reaction mixes were prepared for each sample. Reaction mix A contains 33 μl master mix [85 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer solution (Sigma), 0.3 mM insulin from bovine pancreas (Sigma), 660 μM β-nicotinamide adenine dinucleotide 2- phosphate reduced salt (Sigma) and 3 mM ethylenediaminetetraacetic acid and 10 μl 250 mM Trx reductase from rat liver (Sigma)] and 7 μl protein sample, and a reaction mix B contains 33 μl master mix, 10 μl 200 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer solution (Sigma) and 7 μl protein sample. Following incubation for 20 min at 37°C, 500 μl of 5.5'-dithiobis-(2-nitrobenzoic acid)/guanidine solution [1 mM 10 mM 5.5’-dithiobis (2-nitrobenzoic acid) (Sigma) and 9 mM 6 guanidine hydrochloride (Sigma)] was added to each sample. Absorbances at 412 nm of reaction mix A and B for each sample were determined and the difference (A–B) correlated with the relative Trx activity in the protein sample.

Preparation of protein samples for liquid chromatography/mass spectrometry

Dubelco’s modified Eagle’s medium containing no phenol red (Invitrogen) was conditioned by MCF-7 cancer cells for 3 days in hypoxic conditions and then concentrated 15× using Amicon Ultra-15 Centrifugal Filter Devices (Milipore, Billerica, MA). Samples were analyzed by liquid chromatography/mass spectrometry at University of New South Wales Bioanalytical Mass Spectrometry Facility.

Endothelial cell migration assay

HUVEC migration toward medium conditioned by cancer cells was tested using a BD BioCoat Angiogenesis System (Endothelial Cell Migration System, Becton Dickinson), according to manufacturer’s instructions. Total DNA was purified and concentrated using phenol–chloroform extraction and ethanol precipitation method and then subjected to PCR analysis. Specific primers used for PCR analyses were as follows: 5’-tttaggtggtaagaggagcat-3’ and 5’-cactctgttggttcoccatatta-3’ for the E2F-binding site of TP2B promoter and 5’-cattgtaagatctgggct-3’ for the region ~1500 bp upstream of the E2F-binding site.

Statistical analyses

All experiments were repeated for at least three times. All data for statistical analyses were presented as mean ± SE. Differences were analyzed for significance using t-test among groups. A probability value of ≤0.05 was considered significant.

Results

Repression of p21WAF1 expression in cancer cells decreases in vitro angiogenesis

To determine whether p21WAF1 plays a role in tumor-driven angiogenesis, we examined whether p21WAF1 expression in cancer cells promoted endothelial cell invasion, migration and vascular sprouting. MCF-7 breast cancer cells were transfected with scrambled control or p21WAF1 siRNA and left in a hypoxic chamber (1% O2). Conditioned cell culture medium from the cells 72 h after siRNA transfection was assessed in HUVEC invasion, migration and vascular sprouting assays for pro-angiogenic properties. As shown in Figure 1A, reverse transcription–polymerase chain reaction and immunoblot analysis showed that p21WAF1 siRNA repressed p21WAF1 messenger RNA (mRNA) expression by 90% (P < 0.001) and protein expression by 70% (P < 0.001). The Alamar blue assay confirmed that transfection of p21WAF1 siRNA had no effect on MCF-7 cell proliferation (supplementary Figure 1 is available at Carcinogenesis Online). HUVEC invasion, through Matrigel toward the culture medium from MCF-7 cells transfected with p21WAF1 siRNA, decreased by 40%, compared with those transfected with scrambled control siRNA (Figure 1B). In endothelial cell migration assays, significantly smaller numbers of HUVECs migrated toward the culture medium conditioned by MCF-7 cells transfected with p21WAF1 siRNA, in comparison with the medium conditioned by cells transfected with scramble control siRNA (Figure 1C). Consistently, stimulation of HUVECs with conditioned medium collected from MCF-7 cells transfected with p21WAF1 siRNA produced a small but significant decrease in the number of complete branches at each branching point in the vascular sprouting assay, compared with control (Figure 1D).

To test whether a secreted product regulated by p21WAF1 promoted invasion by other cancer types, we performed the HUVEC invasion assay with conditioned cell culture medium from lung cancer (H460) and prostate cancer (LNCaP) cells 72 h after scrambled control or p21WAF1 siRNA transfection under hypoxic conditions. reverse transcription–polymerase chain reaction confirmed that p21WAF1 siRNA dramatically reduced p21WAF1 gene expression (Figure 1E and F), and an Alamar blue assay revealed that p21WAF1 siRNA had no effect on cell proliferation in H460 and LNCaP cells (supplementary Figure 1 is available at Carcinogenesis Online). As shown in Figure 1E and F, 25% less HUVECs invaded toward conditioned cell culture medium from H460 and LNCaP cells transfected with p21WAF1 siRNA than control. Thus, p21WAF1 expression promoted HUVEC invasion, and potentially angiogenesis, as a general feature of cancer cells.

Repression of p21WAF1 expression in MCF-7 breast cancer cells increases the secretion of Trx

To identify the factor responsible for p21WAF1-induced angiogenesis, we performed liquid chromatography/mass spectrometry analysis of proteins secreted into the cell culture medium from MCF-7 cells. As
shown in supplementary Table 1 (available at Carcinogenesis Online), transfection with p21WAF1 siRNA, compared with scrambled control siRNA, increased the secretion of aldolase A, protein kinase C inhibitor protein-1, thioredoxin peroxidase, glucocorticoid receptor AF-1-specific elongation factor and lactate dehydrogenase A but decreased the secretion of nucleolar phosphoprotein product B23.1, nuclear autoantigen RA33/A2 heterogeneous nuclear ribonucleoproteins homolog and Trx.

Because previous literature has supported a role for Trx in tumor invasion and angiogenesis (10,11), we sought to analyze the role of...
p21\textsuperscript{WAF1} in regulating the secretion of Trx by cancer cells and performed immunoprecipitation and immunoblot analyses of the conditioned culture medium with an antibody against Trx. As shown in Figure 2A, repression of p21\textsuperscript{WAF1} expression for 3 days in MCF-7 cells significantly repressed Trx secretion under hypoxic condition.

\begin{figure}[h]
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\caption{Repression of p21\textsuperscript{WAF1} decreases Trx secretion by increasing TBP2 expression. MCF-7 cells were transfected with scrambled control or p21\textsuperscript{WAF1}-specific siRNA and placed in a hypoxic chamber for 48 h. The effect of p21\textsuperscript{WAF1} siRNA on p21WAF1 and TBP2 mRNA expression in MCF-7 cells was analyzed by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR), with the housekeeping gene \(\beta_2\text{-microglobulin} (\beta2\text{M})\) as a loading control, or by immunoblot with an anti-actin antibody as a loading control, respectively. (\(\text{Fig. 2A}\)) Conditioned cell culture medium was immunoprecipitated with an anti-Trx antibody and then immunoblotted with an anti-TBP2 antibody. (\(\text{Fig. 2B}\)) Expression of Trx mRNA and intracellular Trx protein in the MCF-7 cells was analyzed by RT–PCR and immunoblot, respectively. (\(\text{Fig. 2C}\)) Intracellular Trx enzymatic activity in MCF-7 cells was analyzed by an end point insulin assay. The Trx activity was measured as the presence of active Trx in the experimental samples relative to standards; \(P < 0.05\). Either (\(\text{Fig. 2D}\)) MCF-7, (\(\text{Fig. 2E}\)) H460 or (\(\text{Fig. 2F}\)) LNCaP cells were transfected with scrambled control or p21\textsuperscript{WAF1} siRNA and placed in a hypoxic chamber for 48 h. The effect of p21\textsuperscript{WAF1} siRNA on p21\textsuperscript{WAF1} and TBP2 mRNA expression in the cancer cells was analyzed by semiquantitative RT–PCR, whereas intracellular TBP2 protein expression in MCF-7 cells was analyzed using an anti-TBP2 antibody in an immunoblot.

\(p21\textsuperscript{WAF1}\) upregulates Trx secretion by decreasing TBP2 expression

The expression level of extracellular Trx protein is determined by the expression level of intracellular Trx and its secretion, the latter of which is modulated by the intracellular ratio of Trx to TBP2 protein (12), since Trx protein bound to TBP2 is inactive and cannot be secreted into the cell culture medium or extracellular matrix (13). We, therefore, tested the effect of \(p21\textsuperscript{WAF1}\) siRNA on the expression level of intracellular Trx and TBP2 and the intracellular Trx enzymatic activity measured by the end point insulin assay, in MCF-7 cells. As shown in Figure 2B, \(p21\textsuperscript{WAF1}\) siRNA had no effect on the expression of Trx. Importantly, in contrast to the total protein level, the activity of intracellular Trx was decreased by 45\% by \(p21\textsuperscript{WAF1}\) siRNA (\(P = 0.03\)) (Figure 2C). We next examined the effect of \(p21\textsuperscript{WAF1}\) siRNA on TBP2 expression, in MCF-7 cells. We found that a 75\% decrease in \(p21\textsuperscript{WAF1}\) mRNA increased TBP2 mRNA and protein expression (Figure 2D) by 40\% (\(P < 0.0001\)) and 170\% (\(P = 0.01\)), respectively, in the breast cancer cells. Thus, basal \(p21\textsuperscript{WAF1}\) expression in these cancer cells repressed TBP2 expression, leading to increased intracellular Trx activity and secretion.

To test whether the modulation of TBP2 gene transcription by \(p21\textsuperscript{WAF1}\) is a general phenomenon among cancer cells arising in different organs, we analyzed TBP2 mRNA expression in lung cancer (H460) and prostate cancer (LNCaP) cells. As shown in Figure 2E and F, \(p21\textsuperscript{WAF1}\) siRNA reduced \(p21\textsuperscript{WAF1}\) mRNA expression by 40\% (\(P < 0.0001\)) in lung cancer H460 cells and by 50\% (\(P < 0.0001\)) in prostate LNCaP cells. Reduced \(p21\textsuperscript{WAF1}\) expression correlated with an increase in TBP2 mRNA expression by 40\% (\(P < 0.01\)) in H460 cells and 30\% (\(P < 0.01\)) in LNCaP cells.

\(\text{Trx and TBP2 have opposing effects on endothelial cell invasion}\)

Next, we investigated the role of Trx and TBP2 on tumor-driven endothelial cell invasion. As shown in Figure 3A, transient transfection of MCF-7 cells with Trx siRNA decreased Trx gene expression by 65\% (\(P < 0.0001\)) and the total intracellular Trx protein by 95\% (\(P < 0.0001\)). Although TBP2 mRNA expression was not affected, protein expression of TBP2 was decreased by 85\% (\(P = 0.002\)) upon knockdown of Trx, suggesting that the regulation of intracellular TBP2 levels is directly linked to the availability of its substrate, Trx. The HUVEC invasion assay showed that the number of HUVECs that invaded through the Matrigel toward the culture medium from MCF-7 cells transfected with Trx siRNA, was reduced by 35\% (\(P = 0.0004\)), compared with MCF-7 transfected with scrambled control siRNA (Figure 3B).

Since TBP2 is a negative regulator of Trx, we next examined the hypothesis that repression of TBP2 would generate results opposite to the repression of Trx. As shown in Figure 3C, transient transfection of MCF-7 cells with TBP2 siRNA resulted in a 40\% (\(P = 0.0002\)) reduction in Trx mRNA expression and a 90\% (\(P < 0.0001\)) reduction in TBP2 protein expression. However, total Trx protein expression was unchanged (Figure 3C). In the HUVEC invasion assay, 70\% more HUVECs invaded toward the conditioned culture medium from MCF-7 cells transfected with TBP2 siRNA than those transfected with control siRNA (\(P < 0.0001\)) (Figure 3D). These data suggested that cancer cell-derived Trx promoted, whereas TBP2 repressed, invasion.

\(\text{TBP2 and p21\textsuperscript{WAF1} have opposing effects on Trx activity and endothelial cell invasion}\)

Next, we transiently transfected MCF-7 cells with scrambled control siRNA or a combination of \(p21\textsuperscript{WAF1}\) siRNA and TBP2 siRNA. Consistent with Figure 1A and Figure 3C, simultaneous repression of \(p21\textsuperscript{WAF1}\) and TBP2 with siRNAs in MCF-7 cells decreased the expression of \(p21\textsuperscript{WAF1}\) and blocked transcriptional activation of TBP2 induced by p21 siRNA (Figure 4A). Similarly, we observed no significant change in the expression level of Trx mRNA and protein (Figure 4A) or in intracellular Trx enzymatic activity (Figure 4B). Importantly, simultaneous repression of both \(p21\textsuperscript{WAF1}\) and TBP2 in MCF-7 cells resulted in no change in endothelial cell invasion.
compared with significant changes induced by either p21WAF1 siRNA or TBP2 siRNA alone (Figure 4C). These results demonstrate that p21WAF1-induced HUVEC invasion is dependent on repression of TBP2 and the subsequent increase in Trx activity.

**p21WAF1 represses TBP2 transcription by binding to an E2F-binding site in the TBP2 gene promoter**

p21WAF1 is known to repress gene transcription by direct binding to DNA-binding sites of target gene promoters recognized by the E2F family of transcription factors (15,16). Consistent with literature (17), our promoter analysis confirmed an E2F-binding site in the proximal TBP2 gene promoter. To test whether p21WAF1 directly regulated TBP2 gene transcription through binding to the E2F-binding site, we performed a chromatin immunoprecipitation assay with an anti-p21WAF1 antibody. PCR analysis with primers targeting the E2F-binding site of the TBP2 promoter revealed that p21WAF1 bound directly to the E2F-binding site in MCF-7 cells transfected with scrambled control siRNA but not in those cells transfected with p21WAF1 siRNA (Figure 5).

**Discussion**

Recent *in vitro* and *in vivo* data from other laboratories and ourselves suggest that p21WAF1 can promote tumor growth (6) (reviewed in ref. 7). Upregulation of p21WAF1 protects cancer cells from DNA damage-induced apoptosis by suppression of cyclin-dependent kinase activity that is required for activation of the caspase cascade (18). Moreover, upregulation of p21WAF1 has been shown to block therapy-induced tumor regression in mouse models of cancer (19) and correlates with poor prognosis in cancer patients in some clinical trials (3,4). These observations have lead to the hypothesis that the induction of p21WAF1 expression in cancer cells responding to some cellular and genotoxic stress may promote tumor growth by mechanisms that are still poorly understood. Here, we show that p21WAF1 may contribute to tumor progression by promoting tumor-driven angiogenesis. Moreover, we have found, for the first time, that p21WAF1 promoted angiogenesis by directly repressing TBP2 transcription, leading to increased Trx activity and secretion. These results demonstrate a context-dependent role for p21WAF1 in tumor tissue and have important implications for drug resistance in cancer patients and the choice of combination anticancer therapies.

Beyond associating with cyclins/cyclin-dependent kinases, p21WAF1 participates in many protein–protein interactions and has the potential of physically associating with transcription factors and co-activators, thereby modulating gene transcription (16,20). p21WAF1 binds to the transcription factor E2F1 and the histone acetyl-transferase, p300, at E2F-binding sites of target gene promoters (15,16). Binding of p21WAF1 to p300 reduces recruitment of p300, causing histone hypoacetylation at target gene promoters, leading to inhibition of E2F1-mediated target gene expression (15,16). Our results point to a new role for p21WAF1 as a repressor of TBP2 transcription in breast, prostate and lung cancer cells. A consequence of repressed TBP2 transcription was increased secretion of Trx into the extracellular environment, with the potential to enhance tumor-related angiogenesis. It is unclear which *in vivo* context favors this mechanism of tumor angiogenesis, since in preliminary experiments in tumor tissues from p21WAF1 knockoout mice, we did not show enhanced TBP2 expression.
for TBP2 in cell invasion and angiogenesis has not been reported. In this study, we have confirmed that repression of TBP2 with siRNA in cancer cells lead to increased intracellular Trx protein levels and activity in cancer cells and promoted cancer cell-driven angiogenic activity of endothelial cells. We have further confirmed that repression of TBP2 in cancer cells blocked the p21WAF1 siRNA-induced reduction in Trx activity and angiogenesis. Taken together, our results indicated that the tumor suppressor TBP2 repressed tumor-driven angiogenesis through binding and inactivating Trx, whereas p21WAF1 promoted tumor-driven angiogenesis through repression of TBP2 transcription and subsequent increases in extracellular Trx.

In addition to stimulating tumor-driven angiogenesis, Trx enhances tumor cell invasion and possibly also metastasis through inhibiting the enzymatic activity of tissue inhibitor of metalloproteinases 1 and 2 and subsequently increasing the enzymatic activity of matrix metalloproteinases 2 and 9 (11). Consistently, overexpression of Trx in tumor tissues from cancer patients correlates with advanced tumor stage, invasive phenotype, metastasis and poor prognosis (reviewed in ref. 24). Since our data demonstrate that p21WAF1 increases intracellular Trx activity and secretion through repression of TBP2 gene transcription, p21WAF1 may promote tumor invasion and metastasis through increasing Trx secretion into extracellular matrix. Our findings, therefore, highlight the importance of targeting p21WAF1 or Trx in cancer therapy. The small molecule inhibitors of p21WAF1, such as Flavopiridol (25), and small molecule inhibitors of Trx, such as PX-12 (26), are already in clinical trials for the treatment of cancer. Our data suggest the addition of Flavopiridol or PX-12 in future clinical trials for patients with high level of p21WAF1 expression in tumor tissues either before or after anticancer therapy.

In summary, we have shown that repression of TBP2 decreased the activity of intracellular Trx and that TBP2 repressed, whereas Trx promoted, tumor-driven angiogenesis. We have also shown that p21WAF1 repressed TBP2 gene transcription through direct binding to an E2F-binding site of its gene promoter, increased intracellular Trx activity and secretion and thus promoted angiogenesis in vitro. The effects of p21WAF1 on angiogenesis were counteracted by TBP2. We conclude that p21WAF1 may operate either as a tumor suppressor or a tumor promoter, dependent on the cellular and, perhaps, treatment-related context. Moreover, our findings suggest a role for inhibitors of p21WAF1 or Trx in some cancer patients with high basal or therapy-induced p21WAF1 expression in tumor tissues.

We hypothesize that the effect of p21WAF1 on TBP2 expression may depend on the form of cellular stress, its site and timing within the tumor. Further experiments on tumor tissues from human clinical trials or experimental models, following different anticancer therapies, will determine the circumstances under which p21WAF1 can promote tumor growth.

While overexpression of TBP2 is known to induce cell-cycle arrest, growth inhibition and apoptosis (21,22) (reviewed in ref. 23), the role of TBP2 in cancer cells is not well understood. In this study, we have shown that repression of TBP2 decreased the activity of intracellular Trx and that TBP2 repressed, whereas Trx promoted, tumor-driven angiogenesis. We have also shown that p21WAF1 repressed TBP2 gene transcription through direct binding to an E2F-binding site of its gene promoter, increased intracellular Trx activity and secretion and thus promoted angiogenesis in vitro. The effects of p21WAF1 on angiogenesis were counteracted by TBP2. We conclude that p21WAF1 may operate either as a tumor suppressor or a tumor promoter, dependent on the cellular and, perhaps, treatment-related context. Moreover, our findings suggest a role for inhibitors of p21WAF1 or Trx in some cancer patients with high basal or therapy-induced p21WAF1 expression in tumor tissues.
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Conflict of Interest Statement: None declared.

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