

## Thioredoxin Reductase-1 Mediates Curcumin-Induced Radiosensitization of Squamous Carcinoma Cells

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### Abstract

Curcumin, a plant polyphenol, is a widely studied chemopreventive agent with demonstrated antitumor activities in preclinical studies and low toxicity profiles in multiple clinical trials against human malignancies. We previously showed that curcumin radiosensitizes cervical tumor cells without increasing the cytotoxic effects of radiation on normal human fibroblasts. Here we report that an inhibitory activity of curcumin on the antioxidant enzyme thioredoxin reductase-1 (TxnRd1) is required for curcumin-mediated radiosensitization of squamous carcinoma cells. Stable knockdown of TxnRd1 in both HeLa and FaDu cells nearly abolished curcumin-mediated radiosensitization. TxnRd1 knockdown cells showed decreased radiation-induced reactive oxygen species and sustained extracellular signal-regulated kinase 1/2 activation, which we previously showed was required for curcumin-mediated radiosensitization. Conversely, overexpressing catalytically active TxnRd1 in HEK293 cells, with low basal levels of TxnRd1, increased their sensitivity to curcumin alone and to the combination of curcumin and ionizing radiation. These results show the critical role of TxnRd1 in curcumin-mediated radiosensitization and suggest that TxnRd1 levels in tumors could have clinical value as a predictor of response to curcumin and radiotherapy. *Cancer Res*; 70(5); 1941–50. ©2010 AACR.

### Introduction

The success of radiotherapy depends on the ability to target malignant cells with radiation while simultaneously protecting surrounding normal tissues from its cytotoxic effects. One pharmacologic approach for increasing the clinical response to radiotherapy is the use of radiation response modifiers that either preferentially sensitize tumor cells or protect normal tissues from radiation-induced damage. Curcumin, a naturally occurring plant polyphenol, has an extensive history of use in diet and traditional medicine. Several preclinical studies carried out over the last 20 years have shown direct antitumor activity in mouse tumor models (1). Curcumin's antitumor properties occur through pleiotropic mechanisms, including inhibition of proliferation, induction of apoptosis, inhibition of angiogenesis, immune modulation, and suppression of inflammation (reviewed in ref. 2). Curcumin has a low toxicity profile and has been well tolerated in

multiple phase I and II clinical trials for malignant and non-malignant diseases (2).

Several groups, including our own, have shown that curcumin selectively radiosensitizes tumor cells (3–5) without enhancing the effects of radiation on nontransformed cells or normal tissue (6, 7). We previously showed that curcumin sensitized cervical tumor cells, but not normal fibroblasts to ionizing radiation (IR). Mechanistically, we found that pretreatment of tumor cells with curcumin and IR resulted in elevated levels of reactive oxygen species (ROS) leading to sustained activation extracellular signal-regulated kinase 1/2 (ERK1/2)–mitogen-activated protein kinase (MAPK) and that both were required for curcumin-mediated radiosensitization (3). However, the molecular mechanism accounting for the differential effects of curcumin in fully transformed and normal cells remained unclear.

Thioredoxin reductase 1 (TxnRd1) is one of several identified molecular targets of curcumin (8). TxnRds are essential mammalian selenocysteine containing flavoenzymes that act in homodimeric form to catalyze NADPH-dependent reduction of thioredoxin and small molecular weight oxidants including ROS (9, 10). TxnRd1 and TxnRd2 (the cytosolic/nuclear and mitochondrial form, respectively) are two ubiquitously expressed isoforms of this enzyme family. They play a key role in maintaining redox-regulated cellular functions, including transcription, DNA damage recognition and repair, proliferation, and apoptosis (9). In response to oxidative stress, TxnRds sustain signaling pathways that regulate transcription of genes to protect the cell from oxidative damage (11–13). Cytosolic TxnRd1 expression is often upregulated in human cancers, wherein it is associated with aggressive tumor

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growth and poor prognosis (14). TxnRd1 has been shown to confer protection against the lethal effects of IR in tumor cells (11). Moreover, agents that selectively target TxnRd1 have shown promising results as anticancer drugs in preclinical and clinical studies (15, 16) when used alone or when combined with IR (11). Fang and colleagues recently (8) showed that curcumin covalently binds to the selenocysteine residue of TxnRd1, thereby inhibiting its catalytic activity.

Based on these findings and our previous studies (3), we hypothesized that curcumin-mediated inhibition of TxnRd1 activity could enhance radiation sensitivity by decreasing the capacity of TxnRd1 to scavenge IR-induced ROS and/or decrease the activity of pro-survival signaling cascades activated by IR. We report that knockdown of TxnRd1 in squamous carcinoma cell lines significantly decreases curcumin-mediated radiosensitization. Conversely, overexpression in cells with low basal levels of TxnRd1 increased their sensitivity to curcumin alone and to the combination of curcumin and IR. These results show that TxnRd1 is required for curcumin-mediated radiosensitization.

## Materials and Methods

**Cell culture.** HeLa, SiHa, FaDu (American Type Culture Collection), SCC-1 (a gift from Dr. Bert O'Malley, Jr., University of Pennsylvania), *HEK293-pIRES*, and *HEK293-TxnRd1* (a gift from Dr. Giannis Spyrou, Academy of Athens, Greece) cell lines were grown in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum. MSK-Leuk1 (a gift from Dr. Peter Sacks and Memorial Sloane Kettering Hospital) and keratinocytes (Invitrogen) were maintained in defined keratinocyte serum-free media with growth factor supplements.

**Reagents.** All chemical reagents were obtained from Sigma-Aldrich. Curcumin and tetrahydrocurcumin were dissolved in DMSO (10 mmol/L stock solution). Puromycin and G418 were dissolved in water (1 and 200 mg/mL stock solutions, respectively). 2',7'-Dichlorofluorescein diacetate (DCF-DA) was dissolved in DMSO (20 mmol/L stock solution).

**Generation of stable TxnRd1 knockdown cells.** A set of nontargeting shRNA (shNT) and four shRNA plasmids targeting different coding regions of the *TxnRd1* gene (shTR1) were purchased (SA Biosciences, KH02104P). HeLa and FaDu cells were transfected using Lipofectamine 2000 reagent (Invitrogen). At 48 h posttransfection, equal numbers of cells were plated from mock and plasmid-transfected plates. At 24 h postplating, cells were treated with 2 µg/mL puromycin for 48 h, and clones were allowed to grow. Selected clones were maintained in 0.5 µg/mL puromycin.

**Clonogenic survival assays (low density).** HeLa cells were plated in 60-mm dishes at low densities and allowed to attach overnight. Cells were irradiated in the presence of curcumin or DMSO, using a <sup>137</sup>Cs source (dose rate of 1.3 Gy/min). Colony formation, fixation, and counting were performed as described (3). Survival curves were fitted using a second-order polynomial function. The average normalized surviving fraction from three independent experiments was reported (±SE). Dose enhancement ratio (DER) values were calculated at 0.37 survival fraction.

**Clonogenic survival assays (high density).** FaDu cells were plated in 60-mm dishes at high densities and allowed to attach overnight. Cells were pretreated for 8 h with curcumin and then irradiated using a <sup>137</sup>Cs source (dose rate of 1.3 Gy/min). At 1 h after irradiation, cells were trypsinized and subcultured at low densities. Colony formation, fixation, and counting were performed as described (3). Survival curves were fitted using a second-order polynomial function. The average normalized surviving fraction from three independent experiments is reported (±SE). DER values were calculated at 0.37 survival fraction.

**Cell growth assay (MTT assay).** MTT assays were performed using the cell proliferation kit (Roche Applied Sciences). Cells were plated in 24-well plates and treated with different curcumin doses for 8 h. Cell viability was assessed according to the manufacturer's protocol when the untreated controls reached confluence. Aliquots (200 µL) from each well were transferred into a 96-well plate, and absorbance was measured at 570 nm with an automated plate reader (Thermo Scientific).

**Analysis of ROS levels.** HeLa clones (shNT and shTR1) were treated with DMSO or 10 µmol/L curcumin for 8 h. Cells were incubated with 20 µmol/L DCF-DA for 30 min at 37°C. The probe was washed off with PBS, and the cells were either mock irradiated or exposed to a 4-Gy dose of IR. At 30 min later, the cells were trypsinized, resuspended in PBS, and analyzed for DCF fluorescence by flow cytometry (FACSCalibur, CellQuest Pro; BD Biosciences).

**Immunoblotting.** Whole-cell lysates were obtained as described previously (17). For detection of TxnRd1, TxnRd2, pERK1/2, ERK1/2, and β-actin, 20 to 50 µg of total protein was resolved on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membrane, blocked for 30 min in TBS-Tween solution containing 5% milk, and incubated for 1 to 2 h with the following antibodies in TBS-Tween solution containing 1% milk: anti-TxnRd1 antibody (Abcam), anti-TxnRd2 antibody (Abcam), anti-β-actin (Sigma), anti-pERK1/2, and anti-ERK1/2 (Cell Signaling). All membranes were incubated with horseradish peroxidase-conjugated antimouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology), and immunoreactive bands were detected using ECL Plus chemiluminescence (GE Healthcare).

**Real-time reverse transcription-PCR.** RNA was isolated from cells following TRI reagent protocol (Invitrogen). Reverse transcription was performed using AMV Reverse Transcriptase (Promega). Real-time PCR was done on Applied Biosystems 7300 Real-Time PCR System using Power SYBR Green PCR Master Mix. The primer sequences for detecting human *TxnRd1* cDNA were cactggtgaaagaccacgtt (forward) and aggagaaaagatcatcactgctgat (reverse). Primer sequences for 18S rRNA were aaacggctaccacatccaag (forward) and caatcagggcctcgaaag (reverse).

**TxnRd activity assay.** Cells were plated to 90% confluence, allowed to attach overnight, and treated with 10 µmol/L curcumin or tetrahydrocurcumin for 8 h. Following treatment, media were aspirated and replaced with 0.5 mL of media with Na-R-Lipoate (Na-RALA, GeroNova Research, Inc.) at a final concentration of 1 mmol/L. After a 2-h incubation, an aliquot was removed from the media overlying the cell monolayer and spun at 1,000 × g for 5 min to get rid of cellular debris. Fifty

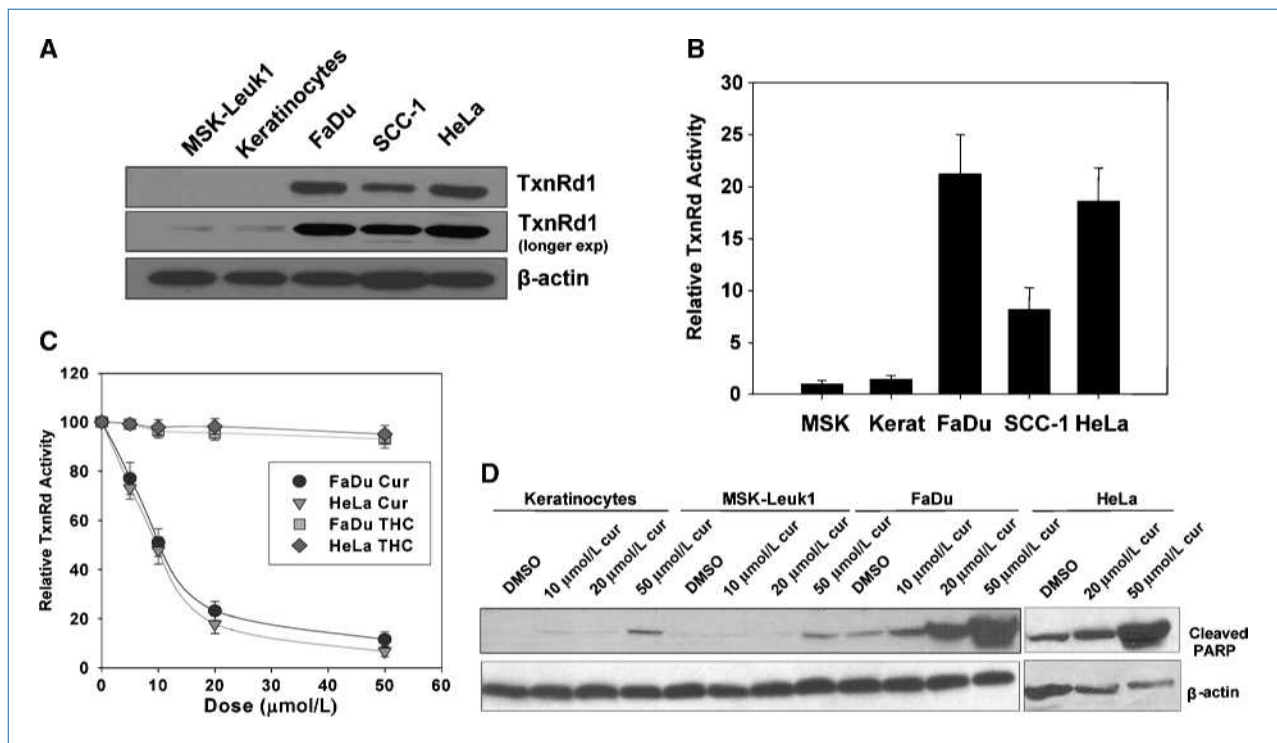
microliters of cleared media were then added to 100  $\mu\text{L}$  of 5,5'-dithiobis(2-nitrobenzoic acid) solution in triplicate wells of a 96-well plate. The plates were incubated for 10 min in the dark. Absorbance was measured at 450 nm using a plate reader (Thermo Scientific). TxnRd activity was defined by the concentration of sodium-lipoate (disulfide) reduced to dehydro-lipoate (dithiol) per milligram protein.

**Statistical analysis.** For all the clonogenic survival assays, mean and SEM of survival fraction from three independent experiments at 4 and 6 Gy for each treatment group were calculated. ANOVA was used to determine whether there were significant differences among three or more independent experimental conditions; specific pairwise comparisons were evaluated by planned contrasts. Student's *t* test was used to test for differences between two independent experimental conditions. Statistical significance was set at 0.05. All tests were two-sided. Statistical analyses were performed with SPSS Version 17 software (SPSS, Inc.).

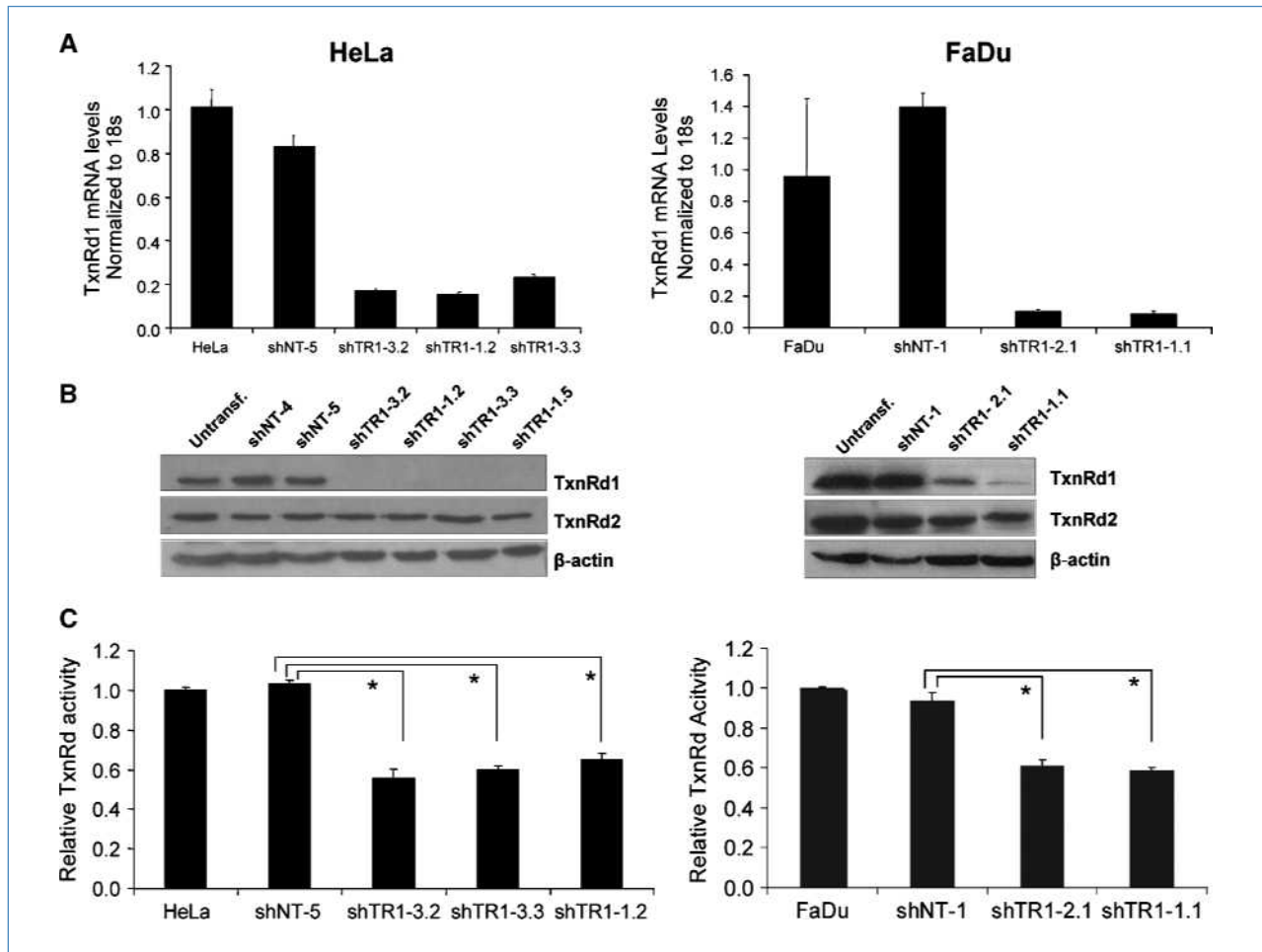
## Results

**Tumor cells express higher levels of TxnRd1 compared with normal or minimally transformed cells.** TxnRd1 ex-

pression is shown to be elevated in several primary human malignancies and in a number of human cancer cell lines (14, 18). A subset of aggressive head and neck cancers also exhibit elevated expression of *TxnRd1* mRNA and protein when compared with normal mucosa (14). We analyzed the expression of TxnRd1 protein levels and activity in various squamous carcinoma cell lines (FaDu, SCC-1, and HeLa) and compared it with that in primary keratinocytes and in MSK-Leuk1, a premalignant cell line derived from a human oral leukoplakia. Based on immunoblot analysis, TxnRd1 expression was elevated by >50-fold in FaDu and HeLa cells and by >20-fold in SCC-1 compared with either MSK-Leuk1 or keratinocytes (Fig. 1A). Total TxnRd activity was determined using an assay based on work by Arner and colleagues (19) and Biaglow and colleagues (20). These investigators showed that mammalian TxnRds reduce lipoate to dehydro-lipoate more efficiently than lipoate dehydrogenase (19) and that the reduction of lipoate in intact tumor cells was largely dependent on TxnRd activity (20). As shown in Fig. 1B, basal TxnRd activity correlated with levels of TxnRd1 protein; TxnRd activity was ~20-fold higher in FaDu and HeLa cells and ~7-fold higher in SCC-1 compared with that in keratinocytes or MSK-Leuk1 cells.



**Figure 1.** TxnRd1 protein and activity levels in cells with different transformation status correlate with response to curcumin. A, MSK-Leuk1, human keratinocytes, and three squamous carcinoma cell lines [HeLa (cervical), FaDu, and SCC-1 (head and neck)] were analyzed for TxnRd1 protein levels by immunoblot analysis.  $\beta$ -Actin was used as a loading control. B, basal levels of whole-cell TxnRd activity. Activity was measured as nanomoles dihydro-lipoate formed per milligram protein and was normalized to that in MSK-Leuk1 cells. Columns, average of three independent experiments; bars, SE. C, the effect of curcumin on TxnRd activity. FaDu and HeLa cells were treated with 0, 5, 10, 20, and 50  $\mu\text{mol/L}$  curcumin or tetrahydrocurcumin for 8 h and assayed for TxnRd activity. Activity was measured as nanomoles dihydro-lipoate formed per milligram protein and normalized to own cells untreated control. D, keratinocytes, MSK-Leuk1, FaDu, and HeLa cells were treated with DMSO at 10, 20, or 50  $\mu\text{mol/L}$  curcumin for 8 h. Whole-cell lysates were analyzed for curcumin-induced apoptosis by immunoblot assay using antibodies against cleaved PARP and  $\beta$ -actin.



**Figure 2.** Stable knockdown of TxnRd1 in HeLa and FaDu squamous carcinoma cell lines. Stable clones were generated from HeLa (left) and FaDu (right) cells by expressing a nontargeting shRNA (shNT) or an shRNA targeting human *TxnRd1* (shTR1). A, untransfected cells, shNT, and shTR1 clones were analyzed for *TxnRd1* mRNA levels by real-time reverse transcription–PCR analysis. *TxnRd1* mRNA levels were normalized to 18S rRNA control and normalized to mRNA levels in untransfected HeLa or FaDu cells. PCR reactions were done in triplicate ( $\pm$ SEM); \*,  $P < 0.05$ . B, TxnRd1 protein levels assayed by immunoblot analysis.  $\beta$ -Actin was used as a loading control. C, TxnRd enzymatic activity. Results represent the average of three independent experiments ( $\pm$ SEM); \*,  $P < 0.05$ .

**Curcumin inhibits TxnRd activity and enhances apoptosis in a manner-dependent on transformation status.** We subsequently examined the ability of curcumin to inhibit TxnRd activity in HeLa and FaDu cells. Curcumin treatment resulted in a dose-dependent decrease in TxnRd activity with an  $IC_{50}$  of  $\sim 10 \mu\text{mol/L}$  in both cell lines (Fig. 1C). These results are in agreement with those of Fang and colleagues, wherein TxnRd1 activity was measured by assaying the reduction of insulin in curcumin-treated and untreated HeLa cell lysates (8). In that study, the Michael acceptor function of curcumin was proposed to mediate the inhibition of TxnRd activity. To test this possibility more stringently, we used tetrahydrocurcumin, a curcumin metabolite lacking the  $\alpha,\beta$ -unsaturated carbon double bond that is presumably required for curcumin to covalently inhibit TxnRd1. As predicted, tetrahydrocurcumin had no significant effect on TxnRd activity at doses of up to  $50 \mu\text{mol/L}$  (Fig. 1C).

Curcumin has been shown to selectively induce apoptosis in transformed cells (21). Therefore, we determined the effects of curcumin on levels of cleaved poly(ADP-ribose) polymerase (PARP), a marker of apoptosis, in keratinocytes (normal untransformed cells), MSK-Leuk1 (minimally transformed), FaDu, and HeLa cells (fully transformed) at 24 hours after curcumin treatment. Levels of cleaved PARP did not appreciably increase in keratinocytes or MSK-Leuk1 cells treated with curcumin doses of up to  $20 \mu\text{mol/L}$ . However, there was a modest increase in cleaved PARP observed with  $50 \mu\text{mol/L}$  curcumin. In contrast, curcumin induced a robust increase in cleaved PARP in both FaDu and HeLa cells. This increase exhibited a dose dependence, which was evident at doses as low as  $10 \mu\text{mol/L}$  in FaDu (Fig. 1D). In accordance with reports that tetrahydrocurcumin retains the antioxidant effects, but not the proapoptotic activity, of the parental drug (22), we did not observe an increase in cleaved PARP in any of the cells

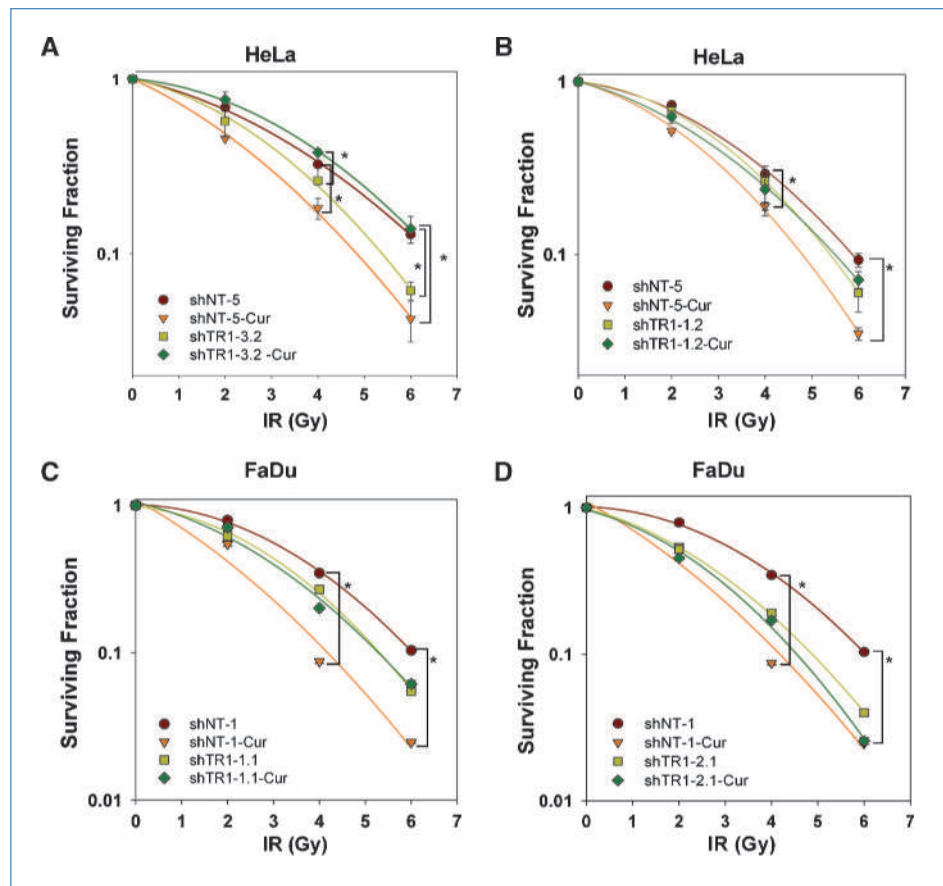
lines treated with tetrahydrocurcumin doses up to 50  $\mu\text{mol/L}$  (data not shown). These results show that curcumin enhances apoptosis preferentially in fully transformed squamous carcinoma cells and that this activity parallels its inhibitory activity on TxnRd1.

**Efficient knockdown of TxnRd1 in squamous carcinoma cell lines.** To test whether TxnRd1 is required for curcumin-mediated radiosensitization, we generated stable clones from both the HeLa and FaDu cell lines expressing an shRNA targeting a unique sequence in TxnRd1 gene (shTR1) or a non-targeting shRNA (shNT). For HeLa cells, two stable clones (shTR1-3.2 and shTR1-3.3) were selected using shRNA-3, whereas another two stable clones (shTR1-1.2 and shTR1-1.5) were selected using shRNA-1. For FaDu cells, one stable clone (shTR1-1.1) was selected using shRNA-1 and one stable clone using (shTR1-2.1) was selected using shRNA-2. All six knockdown clones exhibited >70% reduction in TxnRd1 mRNA levels compared with corresponding shNT clones transfected with the shNT plasmid (Fig. 2A). Consistent with mRNA levels, the HeLa-shTR1 clones showed over a 90% decrease (Fig. 2B, left) and FaDu-shTR1 clones showed an 80% decrease (Fig. 2B, right) in TxnRd1 protein levels as determined by immunoblot analysis. Moreover, there was a 50% reduction of TxnRd activity observed in HeLa-shTR1 clones (Fig. 2C, left) and 40% reduction in FaDu-shTR1 clones

(Fig. 2C, right) compared with the respective shNT clones. The residual activity most likely reflects activity due to the mitochondrial TxnRd2 isoform, whose levels are not substantially affected by these shRNA sequences (Fig. 2B).

**Stable TxnRd1 knockdown in squamous carcinoma cells inhibits curcumin-mediated radiosensitization.** To determine whether TxnRd1 is involved in the enhancement of the cytotoxic response of curcumin in combination with IR, we performed clonogenic survival assays on shNT and shTR1 clones from HeLa, SiHa (another cervical squamous carcinoma cell line), and FaDu cells. As predicted from our previous report (3), HeLa, SiHa, and FaDu cells stably expressing control shRNA (shNT clones) were significantly sensitized to IR by pretreatment with curcumin. The DERs at a surviving fraction of 0.37 ranged from 1.32 to 1.92 (Fig. 3A–D; Table 1). Knocking down TxnRd1 in HeLa and FaDu cells resulted in enhanced sensitivity to IR, in agreement with previous reports (ref. 12; Fig. 3A–D). Notably, curcumin failed to increase the radiosensitivity in the HeLa and FaDu shTR1 clones (Fig. 3A–D). Interestingly, in one of the HeLa clones (shTR1-3.2), curcumin induced a statistically significant radioprotective effect (Fig. 3A). Taken together, these results indicate that TxnRd1 is required for curcumin-mediated radiosensitization of squamous carcinoma cell lines.

**Figure 3.** Squamous carcinoma cells with TxnRd1 knockdown are less sensitive to curcumin-mediated radiosensitization. HeLa (A, B) and FaDu (C, D) shNT control and shTR1 clones were treated with DMSO or 10  $\mu\text{mol/L}$  curcumin for 8 h followed by 0, 2, 4, or 6 Gy doses of IR. Survival was assessed by clonogenic assays. Points, averages of three independent experiments; bars, SEM. Data were analyzed by pairwise comparisons. Treatment groups with statistically significant differences are indicated by brackets; \*,  $P < 0.05$ . Differences in survival in response to IR between untreated (no curcumin) shNT and shTR1 cells were analyzed by ANOVA and were found to be statistically significant at 6 Gy ( $P < 0.05$ ; see Results for details).



**Table 1.** Summary of DERs in TxnRd1 knockdown and overexpressing HEK293 cells

Cell line	Plasmid (clone)	n	DER (at 0.37 surviving fraction)
HeLa	shNT-5	3	1.32 ± 0.06
	shTR1-3.2	3	0.78 ± 0.18
	shTR1-3.3	2	0.97
	shTR1-1.2	3	1.04 ± 0.07
	shTR1-1.5	2	0.95
SiHa	shNT-2	2	1.92
	shTR1-1.1	2	1.50
FaDu	shNT-1	3	1.75 ± 0.28
	shTR1-1.1	3	1.10 ± 0.27
	shTR1-2.1	3	1.04 ± 0.14
HEK293	pIRES	3	1.06 ± 0.08
	TxnRd1	3	1.52 ± 0.19

NOTE: DERs (expressed as the ratio of radiation dose required to produce 0.37% survival with no curcumin present to the dose required to produce the same survival) in presence of 10 μmol/L curcumin. *n* represents the number of times the experiment was replicated.

Abbreviations: shNT, the control, nontargeting vector; shTR1, TxnRd1 knockdown clones; pIRES, empty expression vector; TxnRd1, TxnRd1 expressing vector.

**TxnRd1 knockdown leads to reduced ROS generation and ERK1/2 activation following curcumin and IR treatments.** In our previous study (3), we showed that pretreatment of tumor cells with curcumin led to enhanced production of IR-induced ROS resulting in sustained ERK1/2-MAPK activation. To test the effect of TxnRd1 knockdown on ROS generation following combined treatment with curcumin and IR, we used DCF-DA, a probe whose fluorescence is directly proportional to intracellular ROS levels (23). Treatment of HeLa shNT (control) and shTR1-3.2 (TxnRd1 knockdown) clones with curcumin did not significantly increase ROS levels (Fig. 4A). A single dose of 4 Gy induced an increase in ROS levels that was significantly higher in the shTR1 clones, which is consistent with the role of TxnRd1 as a ROS scavenger. Combined curcumin and IR treatment caused a substantial increase in ROS in the shNT clone, but not in the shTR1-3.2 clone. Quantitation of three independent experiments showed a similar trend (Fig. 4B). Similar results in terms of curcumin-mediated ROS changes were observed in a second HeLa clone, shTR1-1.2 (Supplementary Fig. S1). Interestingly, clone shTR1-3.2, which exhibited a larger increase in ROS induction upon IR treatment compared with shTR1-1.2, also showed a larger effect in terms of radiosensitization upon knockdown of TxnRd1. These results indicate that higher levels of TxnRd1 result in increased levels of ROS in response to the combined treatment of curcumin and IR.

Our previous study (3) indicated that increased ROS production following treatment with curcumin and IR led to sus-

tained ERK1/2 activation. To determine the effects of TxnRd1 knockdown on ERK1/2 activation, we performed immunoblot analysis on irradiated HeLa-shNT and HeLa-shTR1 cells that were pretreated with either curcumin or DMSO. As shown in Fig. 4C, irradiated cells showed a transient increase in phosphorylated ERK1/2 levels that decreased to baseline within an hour after irradiation. Pretreatment with curcumin prolonged IR-induced ERK1/2 activation in the HeLa-shNT cells for up to 6 hours after IR. This sustained ERK1/2 activation was not observed in the HeLa-shTR1 cells after curcumin treatment. These results show that the increased production of ROS and the sustained ERK1/2 activation (which we have previously shown is required for curcumin-induced radiosensitization) are dependent on the levels of TxnRd1 protein.

**Overexpression of TxnRd1 increases radiosensitivity to curcumin.** To determine if overexpression of TxnRd1 is sufficient to cause curcumin-mediated radiosensitization, we attempted to overexpress TxnRd1 in keratinocytes and the minimally transformed MSK-Leuk1 cells. However, the intricate mammalian selenoprotein synthesis machinery imposes a major obstacle in constructing stable selenoprotein-expressing mammalian cell lines. Specifically, previous studies showed that TxnRd1 expression is tightly regulated by multiple AU-rich sequences in the 3'-untranslated region (UTR; refs. 24, 25). Indeed, our attempts to overexpress TxnRd1 in either keratinocytes or MSK-Leuk1 cells were unsuccessful.

To overcome this problem, we searched for cell lines with low basal TxnRd1 levels, which could allow for TxnRd1 overexpression. One such line reported in the literature is the HEK293 cell line (24). We obtained the HEK293 cells stably expressing the full-length *TxnRd1* gene (HEK293-*TxnRd1*) and also HEK293 cells expressing the empty *pIRES* vector (HEK293-*pIRES*). Immunoblot analysis confirmed the overexpression of TxnRd1 protein in HEK293-*TxnRd1* cells (Fig. 5A). Moreover, TxnRd1 activity was 6-fold higher in HEK293-*TxnRd1* cells compared with HEK293-*pIRES* cells, indicating that the expressed protein was enzymatically active (Fig. 5B). Also as shown in Fig. 5C, HEK293-*TxnRd1* cells exhibited significantly reduced survival in response to curcumin compared with the HEK293-*pIRES* cells, whereas tetrahydrocurcumin did not result in significant cytotoxicity in either of the HEK293 cell lines, confirming the role of TxnRd1 in determining curcumin mediated cytotoxicity.

Using a dose of 10 μmol/L curcumin, we performed clonogenic survival assays on irradiated HEK293-*pIRES* and HEK293-*TxnRd1* cells. As predicted from the work of Smart and colleagues (12), HEK293-*TxnRd1* cells were more radioresistant than HEK 293-*pIRES* cells. Pretreating HEK293-*pIRES* cells with curcumin did not induce significant radiosensitization. In contrast, the HEK293-*TxnRd1* cells were significantly radiosensitized by 10 μmol/L curcumin, with a DER of 1.52 at 0.37 survival fraction (Fig. 5D). Taken together, these results indicate that TxnRd1 confers increased radioresistance and that TxnRd1-overexpressing cells exhibit enhanced sensitivity to combined treatment with radiation and curcumin, supporting the role for TxnRd1 as a crucial target mediating curcumin-induced radiosensitization.

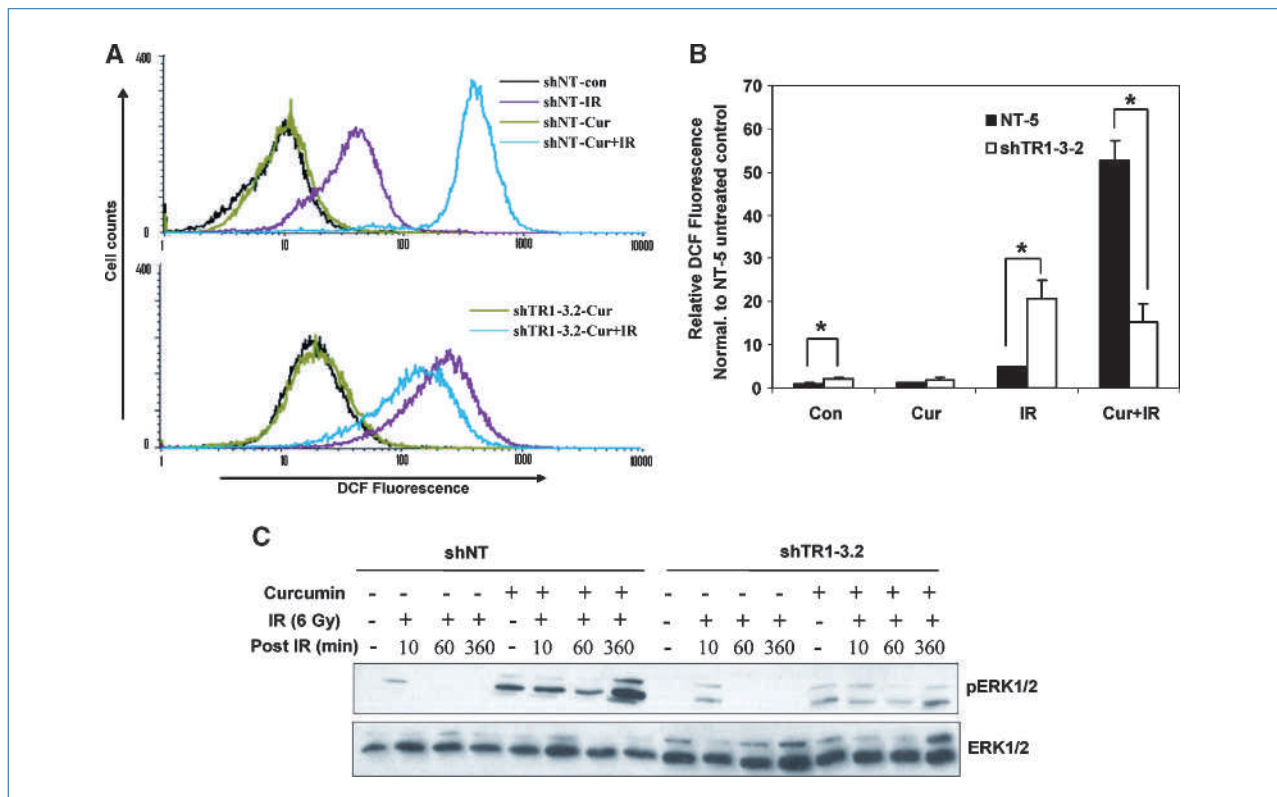
## Discussion

Several reports have identified curcumin as a potent protector of normal tissue against radiation-induced damage. Administration of curcumin significantly reduced various normal tissue toxicities in rodent models treated with whole-body radiation (6, 26, 27). Intriguingly, curcumin has also been shown to radiosensitize various tumor cell lines *in vitro* (3–5) and induce a pronounced tumor growth delay following irradiation in mouse tumor models (28, 29). These findings indicate that curcumin has the ability to preferentially radiosensitize tumor, but not normal tissue, a remarkable property for a radiation response modifier that could translate into substantial clinical benefit. However, the mechanism behind this selective property of curcumin has remained elusive, and potential targets, including NF- $\kappa$ B, Akt, etc. (4, 30, 31), have not been rigorously tested as causative factors in this effect.

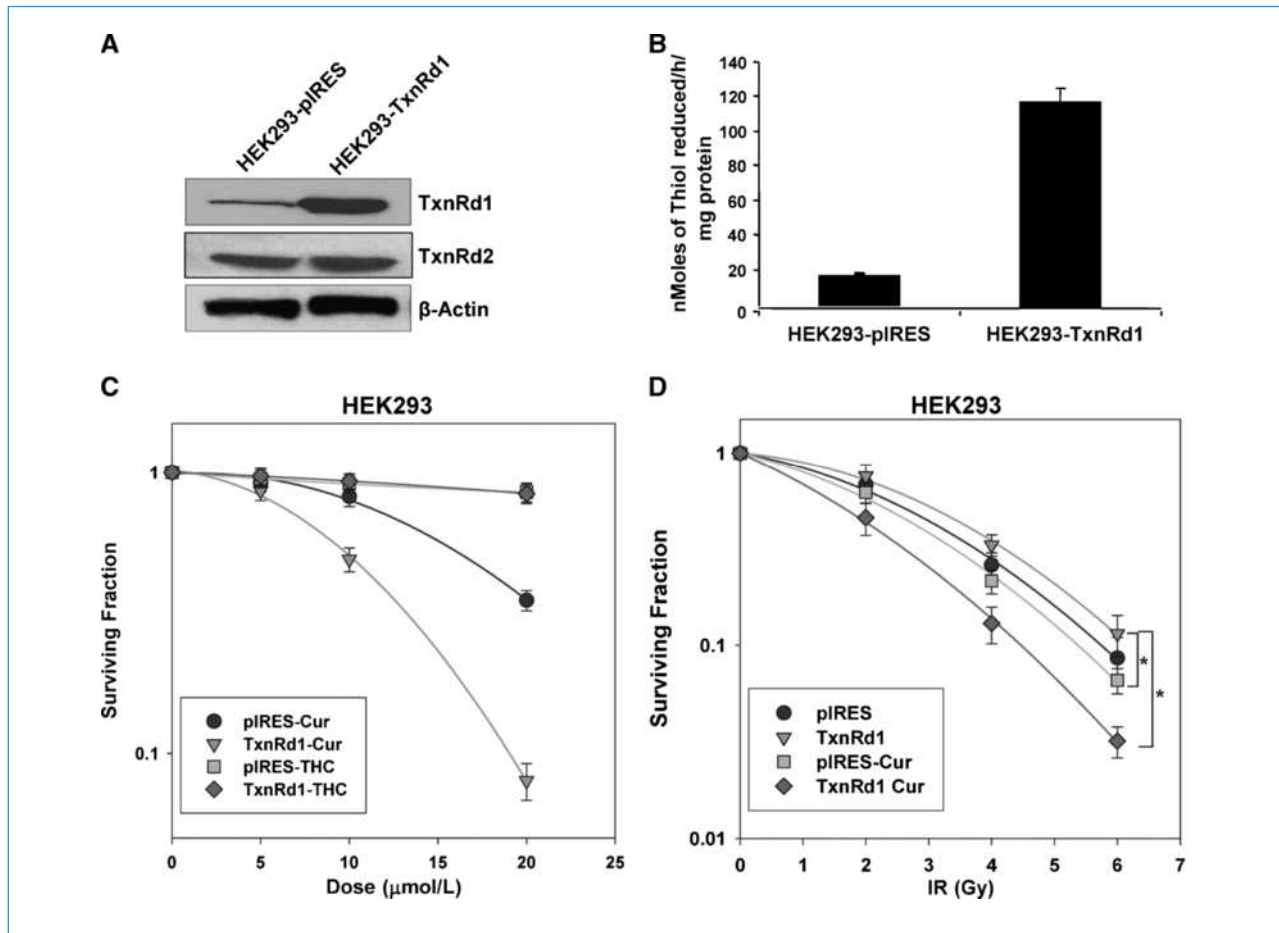
Growing evidence suggests that cancer cells produce higher basal levels of ROS (32) than normal cells. Under this persistent intrinsic oxidative stress, cancer cells develop an enhanced endogenous antioxidant capacity, which makes

them more resistant to exogenous oxidants (33, 34). The up-regulation of the antioxidant enzyme TxnRd1 is observed in multiple primary human malignancies, and its loss has been associated with a reversal of tumor phenotype and a decrease in tumorigenicity (35). These observations support the speculation that malignant cells could be sensitized to oxidants, including IR, by inhibition of this key antioxidant protein (12, 36).

It has been hypothesized that TxnRd1 may be necessary to counteract IR-induced changes in intracellular protein thiol oxidation/reduction status and to directly scavenge cytotoxic free radicals formed during exposure to IR. This hypothesis was tested by Smart and colleagues (12) using cell lines overexpressing wild-type or dominant-negative form of TxnRd1 (cysteine mutant). This study showed that HeLa cells overexpressing the wild-type but not the dominant-negative form of TxnRd1 were more resistant to the lethal effects of IR, suggesting that TxnRd1 is a clinically relevant target for novel radiosensitizing agents. Moreover, in a separate study, motexafin gadolinium, a potent inhibitor of TxnRd (37), has been shown to enhance tumor cell response to IR (38) and currently is in phase I clinical trials for patients with brain



**Figure 4.** Effect of TxnRd1 knockdown on ROS production and ERK1/2 activation. A, increase in ROS in HeLa-shNT or shTR1-3.2 clones was analyzed by flow cytometry using DCF-DA. At least 30,000 events were collected for each treatment condition. Results are representative of three independent experiments. B, results from A were quantified and represented as percentage increase in fluorescence normalized to shNT unirradiated control ( $\pm$ SEM); \*,  $P < 0.05$ . C, ERK phosphorylation in response to curcumin treatment. shNT and shTR1-3.2 cells were serum-starved for 24 h. The cells were pretreated with DMSO or 10  $\mu$ mol/L curcumin for 8 h, followed by mock irradiation or irradiation with 6 Gy. Whole-cell lysates were analyzed by immunoblot using antibodies against p-ERK1/2 and total ERK1/2. The results are representative of three independent experiments.



**Figure 5.** TxnRd1 overexpression enhances curcumin-induced toxicity and radiosensitivity in HEK293 cells. A, immunoblot analysis of HEK293 cells stably expressing an empty *pIRES-neo* vector (HEK293-*pIRES*) or the *pIRES* vector containing the full-length *TxnRd1* gene (HEK293-*TxnRd1*).  $\beta$ -Actin was used as a loading control. B, basal TxnRd activity in HEK293-*pIRES* and HEK293-*TxnRd1* cells. Columns, average of three independent experiments; bars, SEM. C, cytotoxic effect of curcumin and tetrahydrocurcumin on HEK293-*pIRES* and HEK293-*TxnRd1* cells. Cells were treated with increasing concentrations of curcumin or tetrahydrocurcumin for 8 h. When the untreated cells were confluent, MTT assays were performed. Points, averages of four independent experiments; bars, SEM. D, effect of curcumin on radiosensitivity of HEK293-*pIRES* and HEK293-*TxnRd1* cells. Cells were treated with DMSO or 10  $\mu$ mol/L curcumin for 8 h followed by 0, 2, 4, or 6 Gy doses of IR. Long-term survival was assessed by high-density clonogenic survival assay. Points, averages of three independent experiments; bars, SEM. Data were analyzed by pairwise comparisons. Treatment groups with statistically significant differences are indicated by brackets; \*,  $P < 0.05$ .

metastases from lung and breast cancers (15). Our results showing a significant increase in the radiosensitivity of HeLa and FaDu cells with knockdown TxnRd1 levels are in agreement with these data and further support the role of TxnRd1 as a major determinant of intrinsic tumor cell radiosensitivity (39).

Our attempts to overexpress TxnRd1 in nontransformed primary keratinocytes that normally express low levels of the protein were not successful, even when using a plasmid that had been used to express TxnRd1 in HEK293 cells (24). However, this was not unexpected, because previous reports showed that major obstacles exist to overexpressing TxnRd1 in mammalian cells (24, 25). TxnRd1 expression is regulated by sequences in the 3' UTR including AU-rich elements (ARE) that exert stringent regulatory control over translation of *TxnRd1* mRNA. ARE-binding proteins and microRNAs that

selectively recognize AU-rich sequences can affect the stability of *TxnRd1* mRNA levels and protein expression (40). One or more of these factors may contribute to the differential expression of TxnRd1 in tumor and normal cells. This is an area of ongoing investigation in our laboratory.

Based on the findings presented here, we propose that the preferential radiosensitization of tumor cells by curcumin is, in part, due to the elevated expression of the TxnRd1. The involvement of TxnRd1 in curcumin-mediated radiosensitization seems to be 2-fold. First, curcumin covalently binds to TxnRd1, irreversibly inhibiting its ability to reduce thioredoxin, which in turn is required for many of the pleiotropic antioxidant effects associated with TxnRd1 (9). Second, modification of the selenocysteine residue of TxnRd1 converts the protein from essential antioxidant enzyme to a protein with NADPH oxidase activity, thereby elevating oxidative stress. Both effects



would enhance the oxidative burden on the cell, leading to greater clonogenic cell death (a finding supported by our previous work showing ablation of curcumin-mediated radiosensitization by the antioxidant NAC; ref. 3). Normal cells that express very low levels of TxnRd1 are not radiosensitized by curcumin. Whereas this phenomenon could be explained by low levels of TxnRd1, we have not excluded other possible mechanisms of radioprotection. For example, at least *in vivo*, it has been postulated that the radioprotective effects of curcumin could be due to its inhibitory effects on the expression of inflammatory and fibrogenic cytokines (6).

Our findings further suggest that TxnRd1 may mediate radiosensitization by additional natural and chemical compounds. Curcumin reacts as a potent Michael acceptor due to the presence of  $\alpha,\beta$ -unsaturated ketone moieties coupled with electron withdrawing phenolic hydroxyl groups. Curcumin-induced inhibition of TxnRd1 is dependent on the Michael acceptor function by which curcumin binds covalently to the highly nucleophilic selenocysteine residue in the COOH terminus of TxnRd1 (8, 41). Tetrahydrocurcumin, which lacks the  $\alpha,\beta$ -unsaturated ketone moieties, does not inhibit TxnRd1 activity and does not radiosensitize tumor cells. These observations suggest that compounds with Michael acceptor functionality could represent a novel family of radiosensitizers that are selective for transformed cells. Moreover, direct inhibition of TxnRd1 activity could be a useful means for screening natural and synthetic compounds for Michael acceptor functionality. Notably, caffeic acid phenethyl ester and oleocanthal acid, two additional natural compounds with Michael accep-

tor functionality, are also inhibitors of TxnRd1 and potent tumor radiosensitizers.<sup>4</sup>

In conclusion, our results support a critical role of TxnRd1 in curcumin-mediated radiosensitization. Analysis of TxnRd1 levels in the tumors of patients enrolled in curcumin clinical trials could provide a valuable tool in identifying patients who would respond favorably to combined use of curcumin and radiotherapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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<sup>4</sup>S.W. Tuttle and colleagues, unpublished observations.

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