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ACCELERATED PUBLICATION

Thioredoxin Reductase 1 Deficiency Reverses Tumor Phenotype and Tumorigenicity of Lung Carcinoma Cells*[S]

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Dietary selenium has potent cancer prevention activity. Both low molecular weight selenocompounds and selenoproteins are implicated in this effect. Thioredoxin reductase 1 (TR1) is one of the major antioxidant and redox regulators in mammals that supports p53 function and other tumor suppressor activities. However, this selenium-containing oxidoreductase is also overexpressed in many malignant cells and has been proposed as a target for cancer therapy. To further assess the role of TR1 in the malignancy process, we used RNA interference technology to decrease its expression in mouse lung carcinoma (LLC1) cells. Stable transfection of LLC1 cells with a small interfering RNA construct that specifically targets TR1 removal manifested a reversal in the morphology and anchorage-independent growth properties of these cancer cells that made them similar to those of normal cells. The expression of at least two cancer-related protein mRNAs, Hgf and Opn1, were reduced dramatically in the TR1 knockdown cells. Mice injected with the TR1 knockdown showed a dramatic reduction in tumor progression and metastasis compared with those mice injected with the corresponding control vector. In addition, tumors that arose from injected TR1 knockdown cells lost the targeting construct, suggesting that TR1 is essential for tumor growth in mice. These observations provide direct evidence that the reduction of TR1 levels in malignant cells is antitumorigenic and suggest that the enzyme is a prime target for cancer therapy.

There are 25 selenoproteins in humans and 24 in rodents (1), and of those with known functions, most serve as antioxidants (reviewed in Refs. 2 and 3). One of these selenoproteins, thioredoxin reductase 1 (TR1),[2] is one of the major antioxidant and redox regulators in mammalian cells. TR1 is an essential protein (4) that is expressed in all cell types and organs (2, 3), and the Sec moiety is essential for its activity (5, 6). Interestingly, it is overexpressed in many malignant cells (*e.g.* see Refs. 7–10). A variety of potent TR1 inhibitors have been shown to alter the cancer-related properties of tumors and numerous malignant cells (see Refs. 7–13 and references therein). For example, recently, a potent antitumor drug, 1,2-[bis (2-benzysoselenazolone-3(2H)-ketone)]ethane, was found to reverse the phenotype of five human carcinoma cell lines (13). Furthermore, reduction of TR1 activity in human hepatocellular carcinoma cells by transfection with TR1 antisense RNA inhibited cell growth (14). TR1 has therefore been implicated as a potential target for cancer therapy (*e.g.* see Refs. 7, 9, 10, and 15).

On the other hand, TR1 is a selenoprotein that activates tumor suppressor p53 (16) and is specifically targeted by carcinogenic electrophiles (17, 18). Dietary selenium also has potent cancer prevention activity (see Refs. 19 and 20 and references therein). These latter studies have implicated TR1 in tumor suppression, and thus, the overall role of TR1 in tumor progression remains unclear. To further assess the role of TR1 in tumor progression, we used RNA interference technology to knock down its expression in a mouse lung cancer cell line, mouse Lewis lung carcinoma (LLC1) cells, and have observed a reversal of the tumor phenotype. In addition, the tumorgenicity and metastatic properties of the TR1 knockdown cells were dramatically reduced further suggesting that TR1 is indeed a target for cancer therapy in malignant cell lines that overexpress this selenoprotein.

EXPERIMENTAL PROCEDURES

Materials—All materials were commercial products of the highest grade available and are described in the supplemental data or elsewhere (21, 22). Mouse Lewis lung carcinoma cells, designated LLC1, were obtained from the American Type Culture Collection (ATCC).

Knockdown of TR1—The pU6-m3 vector used for generating siRNA targets was constructed using the pSilencer 2.1-U6 Hygro vector as the backbone (details of this vector and its use as a knockdown construct are given in Ref. 22). Changes were inserted into the vector as follows: 1) GC bases at positions 468 and 469 were changed to an A making the U6 promoter more homologous to the corresponding wild type gene; 2) the EcoRI site was deleted at position 4110; and 3) a XhoI site was added at position 384. Changing these two cloning sites made it possible to insert multiple siRNA target sequences into the vector. The 3′-untranslated region of mouse TR1 mRNA (accession number: NM_015762) was surveyed using diDESIGN program (Dharmacon, Inc.) to select potential targeting sites for its knockdown. Sense-antisense oligonucleotides for TR1 knockdown and its control, which had mutations in the target sequences (*i.e.* the targeting construct encoded gtctcactttcaagctgtctaa and the corresponding siTR1 control encoded atcgctctgacaagctattag, where the underlined bases show mutations in the wild type targeting sequence), were annealed and inserted into the BamHI-HindIII cloning sites in pU6-m3 according to instructions detailed in the cloning manual of the pSilencer 2.1U6 Hygro vector. The sequences of the resulting constructs, designated siTR1 or pU6 control, were confirmed by sequencing.

Culture of Mammalian Cells and Transfection Studies—LLC1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution at 37 °C as described in the product description packet from ATCC. Cells were maintained by splitting every 3 days using trypsin-EDTA. Stably transfected siTR1 (knockdown) cells and stably transfected pU6 control cells were prepared by transfecting with the corresponding constructs using Lipofectamine 2000 and then selecting cells in the presence of 500 μg/ml of hygromycin B.

Northern Blot Analysis—Techniques for isolating RNA and carrying out Western blots have been detailed elsewhere (21, 22) and/or are given in the supplemental data. The full-length probe for TR1 mRNA was as described (22).

Western Blot Analysis—Techniques used for Western blot analysis have been described elsewhere (21, 22) and/or are given in the supplemental data.

[75]Se Labeling of Cells—Cells were seeded onto a 6 well plate (3×10^5 cells/well), incubated for 24 h, then labeled with 40 μCi of [75]Se (20 nM) for 24 h, harvested, and lysed as described above. 40 μg of each sample were applied to NuPAGE 10% BisTris gel, electrophoresed, proteins stained with Coomassie Blue staining solution, the gel dried, and exposed to a PhosphorImager (Amersham Biosciences) (see supplemental data). ^{75}Se-Labeled selenoproteins on exposed gels have been identified previously (see Refs. 21 and 22 and references therein).

Assay of TR Activity—TR enzyme activity was measured in control LLC1 cell lysates or LLC1 cell lysates from cells stably transfected with either pU6 control or siTR1 constructs as described (23).

Soft Agar Assay—A total of 1000 control or TR1 knockdown cells suspended in 3 ml of 0.35% noble agar (growth medium with 10% fetal bovine serum) were spread evenly onto 60-mm plates masked with a 4-ml basal layer of 0.7% noble agar in Dulbecco's modified Eagle's medium. The plates were then incubated in a humidified 37 °C incubator for 14 days, adding growth medium onto the
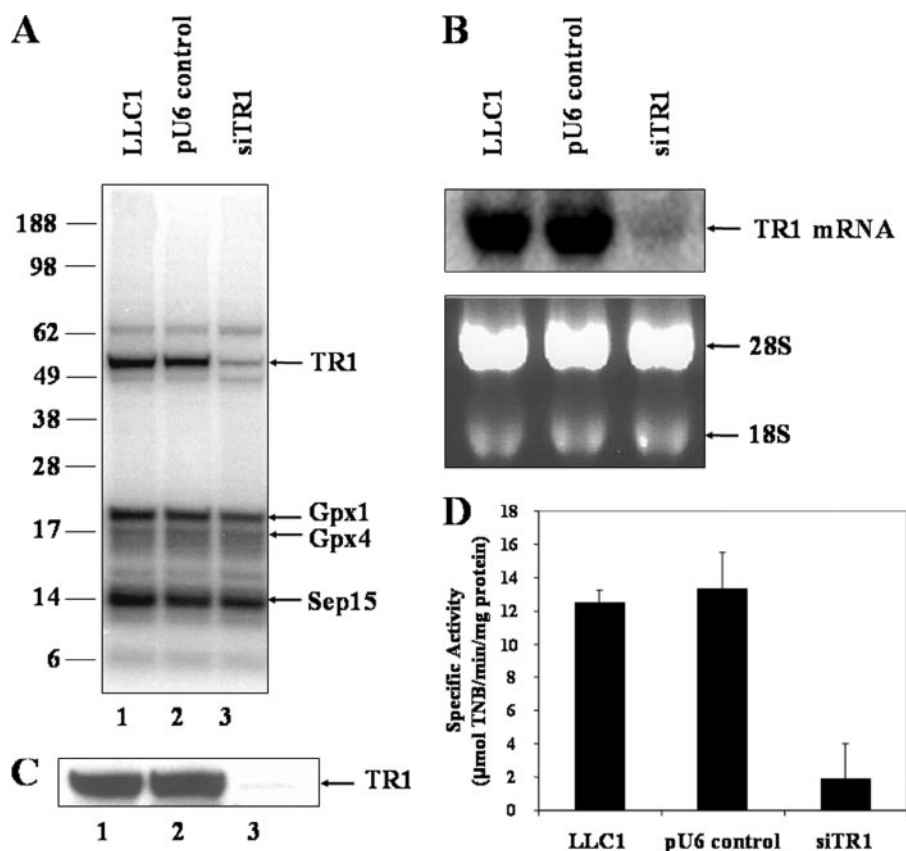

FIGURE 1. **Knockdown of TR1 in LLC1 cells.** LLC1 cells were either untransfected (designated LLC1) or stably transfected with the pU6 control or siTR1 construct (as indicated) and the expression of TR1 examined by labeling cells with [75]Se (*A*), Northern blotting (*B*), Western blotting (*C*), or TR enzyme assay (*D*). Lanes represent cell extracts of 1) untransfected LLC1 cells, 2) cells transfected with pU6 control, and 3) cells transfected with the siTR1 construct in *A–C*. Selenoproteins identified in previous studies are indicated on the *right side* and molecular weight markers on the *left side* of *A*; and 18 and 28 S ribosomal units are indicated on the *right side* of the *lower portion* of *C*.

agar plate every 5 days. The colonies that developed were visualized by staining with ρ-iodonitrotetrazolium violet overnight and counted.

Tumor Formation Assay—To check tumor formation capability, 2×10^5 of siRNA control cells (pU6 control) or TR1 knockdown cells (siTR1) that have been maintained in the growth phase were subcutaneously injected into the flanks of mice (female 5-week-old C57BL/6) and tumor formation monitored every 2 days. At 2 weeks following injection, the mice were euthanized and the tumors removed and analyzed. To assess the metastatic capability of these cell lines, 2×10^5 cells of each were injected into the tail veins of mice. After 4 weeks of intravenous injection, mice were euthanized and the lung tissues dissected and analyzed. Animal care was in accordance with the National Institutes of Health institutional guidelines under the expert direction of Dr. Kyle Stump (NCI, National Institutes of Health, Bethesda, MD).

RESULTS

Knockdown of TR1 mRNA and TR1—The siTR1 construct and the corresponding pU6 control construct were prepared as described under "Experimental Procedures" and used to transfect LLC1 cells. LLC1 cells were either stably transfected with the pU6 control construct or the siTR1 construct, or untransfected, and the levels of TR1, TR1 mRNA, and TR activity determined (Fig. 1). Each cell line was initially labeled with ^{75}Se to examine the levels of TR1 and other selenoproteins and the ability of the siTR1 construct to knockdown TR1 expression. The relative intensities of the various ^{75}Se-labeled bands were determined using a PhosphorImager that demonstrated that TR1 levels were most severely affected (see Fig. 1*A* and supplemental Fig. 1). Northern blot analysis (Fig. 1*B*), Western blot analysis (Fig. 1*C*), and direct assay (Fig. 1*D*) also demonstrated that the expression of TR1 mRNA, TR1, and TR1 activity, respectively, was efficiently knocked down by the siRNA vector.

Phenotypic Changes in TR1 Knockdown Cells—Since the pU6 control construct did not appear to have any effect on TR1 expression in LLC1 cells (see Fig. 1), further studies were carried out only with the LLC1 cells transfected with pU6 and siTR1 constructs. As expected, cells transfected with the pU6 control construct grew in multilayer and loosely attached to the culture dish (Fig. 2*A*), which are characteristics of malignant cells. However, cells transfected with the siTR1 construct grew in monolayer and tightly attached to the culture dish, which are growth properties characteristic of normal cells. The

growth rate of the siTR1-transfected cells was slightly retarded compared with pU6-transfected cells; that is, it was reduced by ~25% at 72 h (Fig. 2*B*). While the inhibition of growth rates in the TR1 knockdown was noticeable, this phenotype was mild, considering that this enzyme is essential during embryonic development (4).

Another characteristic of many cancer cells is that they can grow unanchored in soft agar, while many normal cells do not grow under such conditions. The colonies present after 2 weeks growth in soft agar of the two cell lines transfected with either the pU6 control or siTR1 construct were photographed and the data quantitated as shown in Fig. 2, *C* and *D*, respectively. Clearly, growth of the TR1 knockdown cells in soft agar was inhibited as evidenced by colony numbers and colony size compared with the pU6-transfected cells.

Changes in Cancer-related Components in TR1 Knockdown Cells—RNA extracts from LLC1 cells transfected with either the pU6 control or siTR1 construct were examined for differences in the expression of 96 cancer-related marker mRNAs (see supplemental Fig. 2). Two of the boxes, designated H for hepatocyte growth factor (Hgf) mRNA and O for osteopontin (Opn1) mRNA, were reduced in RNA extracts from siTR1-transfected compared with pU6-transfected cells. Boxes corresponding to several other factors and to controls are also identified in the supplemental data. Both Hgf (24) and Opn1 (25) play important roles in metastasis and tumor growth. The mRNA levels of both cancer promoting factors were quantitated as described under "Experimental Procedures" and found to be reduced more than 2.5-fold in siTR1-transfected cells as compared with pU6-transfected cells (data not shown). The mRNA levels of both components were further examined by Northern blot analysis and also found to be substantially reduced in siTR1-transfected cells as compared with control cells (Fig. 2*E*).

Tumorigenesis and Metastasis of siTR1 Knockdown Cells—To assess the tumorigenicity and metastasis of the pU6- and siTR1-transfected cells, mice were injected in the flank with either of the cell lines. Tumor progression was analyzed after 2 weeks by euthanizing the animals and examining the tumors. Tumors were much larger in mice injected with the pU6 control-transfected cells with an average weight of 0.341 g compared with an average tumor weight of 0.063 g in mice injected with the siTR1-transfected cells (see Fig. 3*A* and legend). This dramatic difference in tumor growth in mice contrasted with slight changes in growth rates due to TR1 knockdown in cell culture (compare
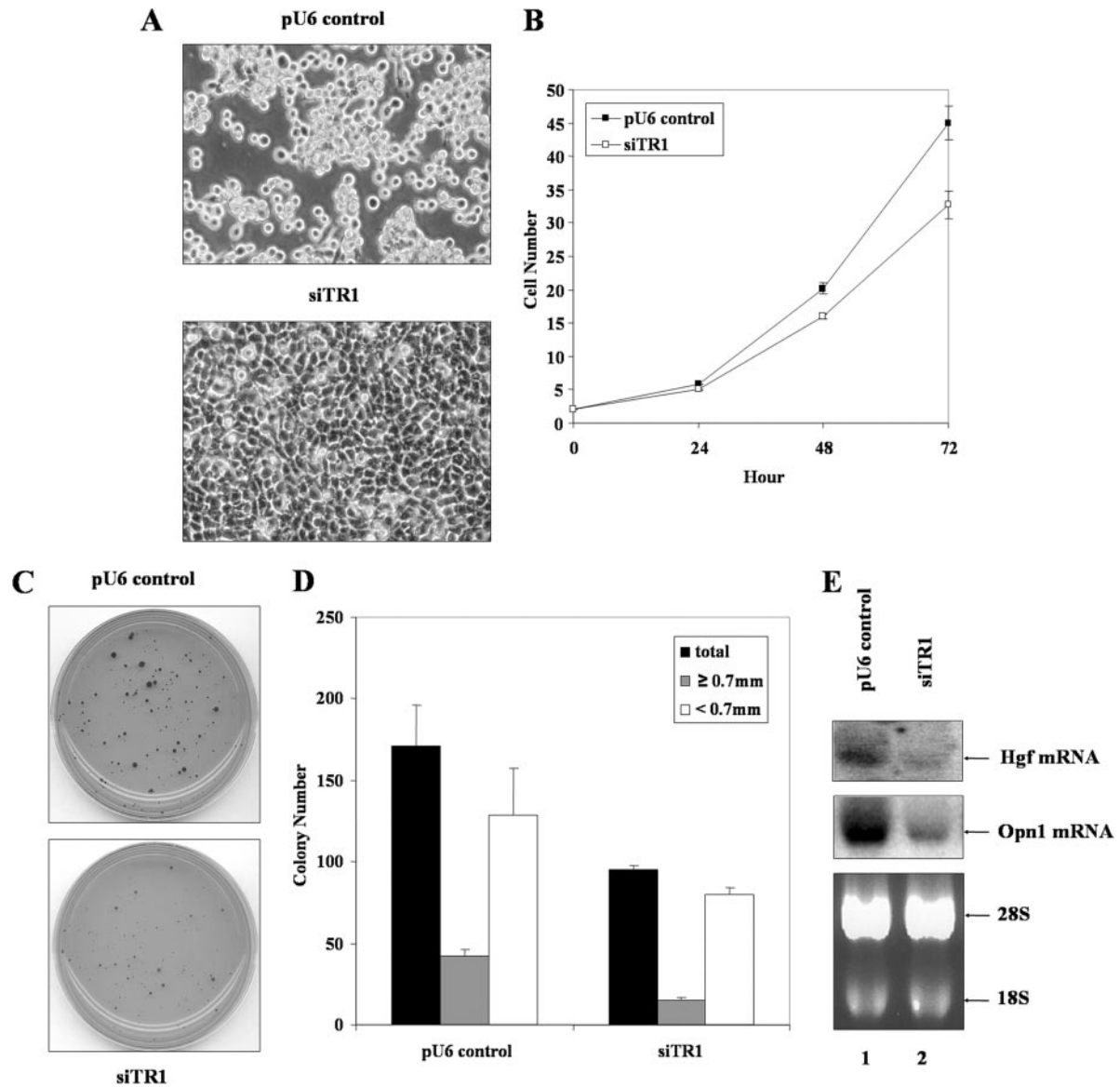
FIGURE 2. **Phenotypic changes in TR1 knockdown cells and analysis of gene products associated with malignancy.** LLC1 cells were transfected with the pU6 control or siTR1 construct and their morphology examined during exponential growth (*A*) or their growth rates quantitated (*B*). Cells were photographed with an inverted phase contrast microscope. Cells were seeded at a density of 2×10^5 cells/60-mm culture dish and growth rates determined by counting cell numbers at 24, 48, and 72 h. *C*, colonies were stained with ρ-iodonitrotetrazolium violet overnight at 37 °C and the stained colonies counted and recorded as the total number of colonies (*D*) (*solid dark rectangles*), colonies greater than 0.7 mm in size (*gray rectangles*), and colonies 0.7 mm or less in size (*white rectangles*). *E*, Northern blots of RNA extracts from pU6 control (*lane 1*)- and siTR1 (*lane 2*)-transfected cells. Relative intensities of 18 and 28 S ribosomal subunits are shown in *lower panel* of *E*.

FIGURE 3. **Tumorigenicity and metastasis of LLC1-transfected cells.** Mice were injected in the flank with cells expressing pU6 or TR1 knockdown constructs. *A*, tumor formation was monitored every 2 days; after 2 weeks, mice were euthanized, tumors removed from the flanks, weighed (weights were averaged from three separate mice), and photographed. *A'*, TR1 Western blot analysis in tumor extracts; and *A''*, PCR analysis of genomic DNA from tumor masses for the presence of the pU6 and TR1 siRNA construct. *B*, metastasis was assessed after 4 weeks whereby mice were euthanized and the lungs removed and photographed; and *B'*, lung tissue slices examined for pathological changes. *Arrows* indicate tumor regions resulting from injected pU6 control cells.
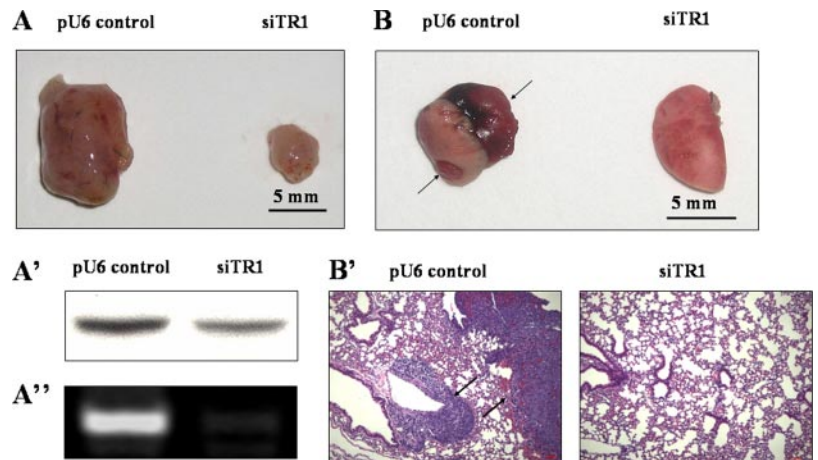
Figs. 3*A* and 2*B*). In addition, since the constructs were retained in stably transfected cells with the counter-selecting antibiotic, hygromycin B, and the injected mice could not be treated with this drug, it was possible that tumor growth in mice injected with the siTR1-transfected cells might be due to the loss of the siTR1 targeting vector. Indeed, Western blot analysis showed that tumors developed from both siTR1- and pU6-transfected cells expressed high levels of TR1, further suggesting that low levels of TR1 did not support tumor growth and that tumors reappeared due to reversal of the TR1 knockdown (Fig. 3*A'*). Consistent with this observation, PCR analysis of genomic DNA from both siTR1 and pU6 tumors showed that the TR1 siRNA construct was lost from tumor cells that were injected with the knockdown vector (Fig. 3*A''*).

Tumor metastasis was analyzed after 4 weeks by euthanizing the mice and examining the lungs. Tumors were readily apparent in the lungs of mice injected in a tail vein with the pU6 vector-expressing cells, while no tumors were visible in mice injected with cells containing the siTR1 construct (Fig. 3*B*). Histological analysis of lung tissue showed extensive malignancy in mice injected with the pU6 construct, but only normal tissue was found in mice injected with the siTR1 construct (Fig. 3*B'*).

DISCUSSION

Redox-sensitive signaling factors govern multiple cellular processes, such as proliferation, cell cycle events, and numerous signaling cascades relating to proper cell function (reviewed in Refs. 26 and 27). TR1 is an essential protein (4) and a central component in several redox-regulated pathways. Its main function is to keep thioredoxin in the reduced state (28). In turn, thioredoxin donates electrons to disulfides in cytosolic and nuclear proteins, thus maintaining cysteine residues in these proteins in the reduced state.

Among other pathways dependent on thioredoxin, TR1 is critical for the proper function of tumor suppressor p53, and inhibition of TR1 by carcinogenetic, electrophilic compounds implicated this protein in cancer prevention (17). In addition, TR1 contains an essential selenocysteine residue. Selenium is known as a trace element with potent cancer prevention function (19, 20). On the other hand, since overexpression of TR1 is consistently observed in many tumors and several antitumor drugs are known inhibitors of TR1 (29–31), this selenoenzyme was proposed as a target for tumor therapy (7, 9, 10, 15, 32). In addition, selenium deficiency has been reported to decrease tumor incidence in some cancer models in animals (33). Thus, the contrasting functions of TR1 in regard to tumor development and growth complicate the assessment of its role in cancer.

In this study, we inhibited TR1 expression using RNA interference technology without affecting the expression of other major selenoproteins. TR1 deficiency was not lethal in cell culture and in fact only slightly reduced growth characteristics of the knockdown cells compared with the cells transfected with the control vector. In contrast, we observed dramatic changes in tumor growth and metastasis, when these cells were injected into mice. Further studies on the knockdown cells revealed that the TR1 deficiency reversed the phenotype of malignant cells rather than simply reducing cell growth. Importantly, the tumors observed in mice injected with the knockdown cells lost the targeting construct, suggesting that TR1 is essential for tumor growth but not for growth of cells in culture. It is clear that some cell types, *e.g.* T cells,[3] and even organs, *e.g.* liver (34), can function without TR1, or for that matter, all selenoproteins, as the targeted removal of the Sec tRNA[Ser][Sec] gene in this cell type or organ, is not lethal. Thus, TR1 expression is not essential to all cell types as seems to also be true of LLC1 cells. Overall, our data show that down-regulating TR1 expression alters the redox homeostasis and in turn reverts the phenotype of malignant cells more in line with that of normal cells. Thus, TR1 provides an excellent molecular target for cancer therapeutic intervention.

How can this apparently essential function of TR1 in cancer development be reconciled with the role of TR1 in tumor suppression and the known anticancer role of selenium? We speculate that TR1 and selenium act as cancer prevention factors by regulating cellular redox homeostasis (a balance between reductive and oxidative processes in the cell) and protecting against mutations and oxidative damage to DNA and proteins. However, in newly emerged tumors, TR1 is required to sustain tumor growth, probably because of the increased dependence on the reducing equivalents provided by thioredoxin for various biosynthetic processes. Apparently, this TR1 dichotomy requires the thioredoxin pathway, due to the many targets of this protein. This hypothesis could explain both the potent cancer prevention activity of dietary selenium (19, 20) and the potent antitumor activity of drugs that target TR1 (7–13). Most importantly, this study provides a foundation to explain literature data on the role of TR1 in cancer and establishes this enzyme as a prime target for cancer therapy.

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[3] M.-H. Yoo, X.-M. Xu, B. A. Carlson, V. N. Gladyshev, and D. L. Hatfield, unpublished data.

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Materials - ^{75}Se (specific activity 1000 Ci/mmol) was obtained from the Research Reactor Facility, University of Missouri, Columbia, MO, [α-^{32}P]CTP (specific activity 6000 Ci/mmol) from Perkin Elmer and Hybond-N$^+$ nylon membrane, Ready-To-Go DNA labeled Bead and probeQuant G-50 MicroColumns from Amersham Biosciences and PVDF membranes, NuPage 10% Bis-Tris gels, Trizol reagent, hygromycin B and lipofectamine 2000 from Invitrogen Life Technologies. siRNA vector pSilencer 2.1-U6 Hygro was purchased from Ambion, Inc., Mouse Cancer Pathway Finder Gene Array from SuperArray Bioscience Company, BCA protein assay reagent from Pierce ρ-iodonitrotetrazolium violet (INT) from Sigma and Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution and 10% fetal bovine serum from Life Technologies, Inc.

Northern blot analysis - Total RNA was isolated from cultured cells with Trizol reagent according to the manufacturer's protocol. 12 μg of total RNA were electrophoresed on a 1.2% formaldehyde-agarose gel, transferred to a Hybond-N$^+$ nylon membrane and cross-linked using a UV-Stratalinker. Probes were prepared by random labeling with [α-^{32}P]CTP using the Ready-To-Go DNA Labeled Bead kit and non-incorporated nucleotides removed using probe Quant G-50 MicroColumn following the manufacturer's procedure. Membranes were hybridized with labeled probes, washed and exposed to a PhosphorImager (Molecular Dynamics). TR1 mRNA expression ratios were obtained using ImageQuant program (Molecular Dynamics).

Western blot analysis - Cells were washed in cold PBS and whole cell lysates prepared using lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM NaF, 5 mM EGTA, 0.5 mM PMSF and proteinase inhibitor cocktail) as described (S1). Protein concentrations were measured using BCA protein assay reagent and 30 μg of protein samples were electrophoresed on NuPAGE 10% Bis-Tris gels. The separated proteins were transferred to PVDF membrane, then incubated initially with primary antibody (polyclonal anti-TR1), and finally with HRP-conjugated secondary antibody. Membrane was reacted with ECL reagent and exposed to X-ray film.

Supplementary reference

S1. Yoo, M. H., Song, H., Woo, C. H., Kim, H., and Kim, J. H. (2004) *Oncogene*, 23, 9259-9268

<u>Supplementary Figure 1.</u> **Analysis of ^{75}Se-labeled selenoproteins.** The gel shown in Figure 1 in the manuscript text was subjected to PhosphorImager analysis and the relative intensities of Thioredoxin reductase 1, glutathione peroxidase 1 and selenoprotein 15 bands were determined using a ImageQuant 5.2 (Molecular Dynamics) program. It should also be noted that SPS2 and TR3, which are expressed in all cell types, albeit in lower levels than TR1, migrate at the similar position as TR1 on gels, contribute to the intensity of the band shown at ~55 kDa and most certainly are responsible for most of the remaining low ^{75}Se-labeling at ~55 kDa shown in Fig. 1A, lane 3, of the text.
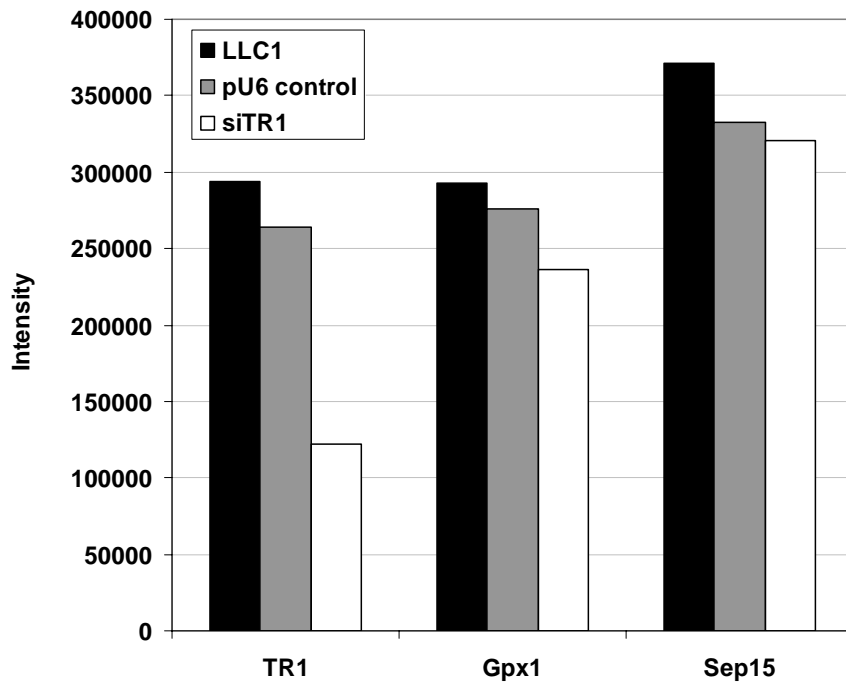
Supplementary Figure 2. **Gene products associated with malignancy.** RNA extracts from LLC1 cells stably transfected with either pU6 control or siTR1 constructs were examined for expression profiles of 96 candidate cancer molecules using a Mouse Cancer Pathway Finder Gene Array as described in Materials and Methods of the manuscript. Boxes designated H and O, which are reduced approximately 2.5 fold in siTR1 transfected cells, correspond to hepatocyte growth factor (Hgf) mRNA and osteopontin (Opn1) mRNA, respectively. These factors have important roles in metastasis and tumor growth (see text of manuscript). Several additional boxes in the figure are identified as further discussed in the text.