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Mouse Embryonic Fibroblast Cells From Transgenic Mice Overexpressing tNOX Exhibit an Altered Growth and Drug Response Phenotype

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Abstract Mouse embryonic fibroblast (MEF) cells prepared from transgenic mice overexpressing a cancer-specific and growth-related cell surface NADH oxidase with protein disulfide-thiol interchange activity grew at rates approximately twice those of wild-type embryonic fibroblast cells. Growth of transgenic MEF cells overexpressing tNOX was inhibited by low concentrations of the green tea catechin (−)-epigallocatechin-3-gallate (EGCg) or the synthetic isoflavene phenoxodiol. Both are putative tNOX-targeted inhibitors with anti-cancer activity. With both EGCg and phenoxodiol, growth inhibition was followed after about 48 h by apoptosis. Growth of wild-type mouse fibroblast cells from the same strain was unaffected by EGCg and phenoxodiol and neither compound induced apoptosis even at concentrations 100–1,000-fold higher than those that resulted in apoptotic death in the transgenic MEF cells. The findings validate earlier reports of evidence for tNOX presence as contributing to unregulated growth of cancer cells as well as the previous identification of the tNOX protein as the molecular target for the anti-cancer activities attributed to both EGCg and phenoxodiol. The expression of tNOX emerges as both necessary and sufficient to account for the cancer cell-specific growth inhibitions by both EGCg and phenoxodiol. J. Cell. Biochem. 101: 295–306, 2007.

Key words: ECTO-NOX; tNOX; NADH oxidase; transgenic mouse; phenoxodiol; (−)-epigallocatechin-3-gallate (EGCg); growth

Tumor-associated NADH oxidase (tNOX) is a novel cancer-specific member of a family of growth-related cell-surface ECTO-NOX proteins with protein disulfide-thiol interchange (ECTO-NOX) and hydroquinone (NADH) oxidase activities [Chueh et al., 2002]. A defining characteristic of tNOX is that its activity is inhibited by a series of quinone site or putative quinone site inhibitors including capsaicin [Morré et al., 1995a], anti-tumor sulfonylureas [Morré et al., 1995b], adriamycin [Morré et al., 1997], the green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCg) [Morré et al., 2000], and phenoxodiol [Morré et al., 2007] all with anti-cancer activity. These agents inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting the constitutive ECTO-NOX (CNOX) or growth of non-cancer cells [Morré et al., 1995a,b, 2000]. ECTO-NOX activities correlate with the enlargement phase of cell growth [Pogue et al., 2000; Morré et al., 2001; Morré and Morré, 2003]. Cancer cells expressing tNOX not only grow in an unregulated manner but the unregulated growth is inhibited by the quinone site inhibitors that also inhibit tNOX [Morré et al., 1995a,b, 1997, 2000; Morré, 1998; Morré and Morré, 2003]. The cells, once having divided, fail to enlarge [Morré and Morré, 2003]. Such cells, unable to enlarge, fail to undergo further divisions even though DNA and protein synthesis are not inhibited and, after a few days, undergo apoptosis [Morré et al., 2000; Morré and Morré, 2003].

Results with tNOX overexpression in non-cancer cell lines and results with antisense in cancer cell lines have been consistent with the

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hypothesis that functional cell surface expression of tNOX is both necessary and sufficient for the cancer-specific cell growth inhibitions attributed to EGCg in cancer cells propagated in culture [Chueh et al., 2004]. As an extension of these observations, transgenic mice overexpressing tNOX previously were shown to exhibit the same level of unregulated cell enlargement and sensitivity to EGCg as observed with cancer cells [Yagiz et al., 2006]. Wild-type mice were resistant to EGCg. Administration of EGCg in the drinking water (500 mg/kg body weight) reduced the growth rate of the transgenic mice to that of the wild-type mice. Also potentially targeted specifically to tNOX [Morré et al., 2007] is a synthetic anti-cancer isoflavene, phenoxodiol [2H-1-benzopyran-7-0, 1,3-(4-hydroxyphenyl)] [Kelly and Husband, 2003]. Phenoxodiol inhibits both the NADH and hydroquinone oxidase activities of tNOX, as well as protein disulfide-thiol interchange activity and growth, without corresponding effects on normal cells.

To examine the effects of EGCg and phenoxodiol at the cellular level, mouse embryonic fibroblast (MEF) cells were isolated and analyzed from both tNOX transgenic embryos and, as controls, from FVB wild-type embryos. The findings show that the transgenic MEF cells exhibit tNOX expression, growth and drug-response characteristics consistent with their transgenic origins that recapitulate similar characteristics observed with tNOX transfected human cell lines in vitro [Chueh et al., 2004].

MATERIALS AND METHODS

Mice and Cell Lines

The tNOX transgenic mice were generated as described [Yagiz et al., 2006]. Four transgenic female mice were bred with one transgenic male littermate and checked for vaginal plugs daily. Control FVB/N mice were bred in parallel. Primary MEF cells were isolated from transgenic and wild-type mouse embryos by the Purdue University Transgenic Mouse Core Facility. Cells were plated into DMEM-Knockout media with 2 mM L-glutamine, 100 μM non-essential amino acids, 50 U Penicillin/Streptomycin (Gibco, Carlsbad, CA), 10% fetal bovine serum, and β-mercaptoethanol (Sigma, St. Louis, MO). To obtain confluent cells, $1 \times 10^6/175\ cm^2$ flasks were seeded and cultured with a change of medium every second day.

Genotyping Mouse Embryonic Fibroblast (MEF) Cells

Genomic DNA and total RNA was isolated from transgenic and wild-type MEF cells by using DNAzol and TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) reagents according to the manufacturer's instructions. Genotyping of MEF cells was performed by PCR analysis using tNOX transgene-specific primers: 5′-TACCCATACGATGTTCCGGAT-3′ (forward) and 5′-CAGAGGTTCTGCCTGTGATAC-3′ (reverse). PCR amplifications were in a total volume of 25 μl. Amplified products were analyzed using 1% agarose gels. A β-globin primer pair (Genbank, J00413) was used as an internal positive control.

RT-PCR Analysis With MEF Cells

Total RNA was isolated from transgenic and wild-type MEF cells by using TRI- REAGENT (Molecular Research Center, Inc.) reagents according to the manufacturer's instructions. The first strand cDNA was used as the template in the PCR reactions. The integrity of each tissue RNA sample was checked by RT-PCR with primers (5′-ACCCATACGATGTTCCGGATT-3′ (forward), and 5′-GGTCAGCTTCAAGCCTCGAAGC-3′ (reverse)). Mouse GAPDH primers (5′-TCACCATCTTCCAGGAGC-3′ (forward), 5′-CTGCTTCACCACCTTCTTGA-3′ (reverse)) were used as internal standards. The negative control reactions included reagents without reverse transcriptase to ensure that the RT-PCR was RNA-dependent.

Western Blot Analysis of tNOX Expression

The plasma membranes of transgenic and wild-type MEFs were prepared as described [Morré and Morré, 1989]. The membranes were stored in 50 mM Tris-MES (pH 7.5). Proteins were separated on 10% SDS–PAGE and then transferred by electroblotting onto nitrocellulose membranes [Towbin et al., 1979] and detected as described [Chueh et al., 2002]. The blots were incubated in primary (anti-HA-tag or 2281.1) antibody solution. The antibody 2281.1 (MorphoSys, Martinsreid, Germany) is a tNOX-specific recombinant antibody generated by phage display to the N-terminal peptide of 50 amino acids of recombinant tNOX that contains the cancer-specific, potentially drug reactive quinone binding sequence and lacks the conserved potentially drug reactive adenine

nucleotide binding region. The secondary antibody was either goat anti-mouse linked to the alkaline phosphates for anti-HA tag or monoclonal anti-polyhistidine clone His-1 (Sigma) for anti-tNOX 2281.1

Spectrophotometric Assay of NADH Oxidase Activity

NADH oxidase activity of MEF cells was determined from the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-MES buffer (pH 7.5), 1 mM KCN to inhibit mitochondrial oxidase activity, and 150 μM NADH at 37°C by using a Hitachi U-3210 spectrophotometer (Hitachi, Japan) with continuous recording over 1.0 min at intervals of 1.5 min each. EGCg (Sigma) (1 μM) was added as a tNOX inhibitor. Proteins were estimated by the bicinchoninic acid method with bovine serum albumin as standard [Smith et al., 1985].

Growth Measurements

Transgenic and wild-type MEF cells were grown in small Petri dishes (10 × 35 mm) with media for 24 h. Direct measurements were of attached cells with an Olympus Vanox-S Microscope, Model AHBS, using bright field and/or differential interference contrast optics. The microscope was attached to a Hamamatsu CE400-07 video camera system with a Hamamatsu camera head for viewing and data recording. Hamamatsu Argus-10 and Argus-20 real time digital contrast and low light enhancement image processing were used. Increase in area was monitored for 60 min. Temperature control was provided by a temperature heating system connected to the microscope. Using freeze frame technology, the tape was stopped at 30 s intervals. The cell perimeter was then digitally traced. Cell areas were calculated using the Hamamatsu Argus-10, equipped with the appropriate software (ImageJ). Each frame was measured three times. Cells for measurement were selected on the basis of their morphological characteristics of having typical appearance. Excessively rounded or enlarged cells were avoided. The three measurements were averaged and standard deviations were determined [Pogue et al., 2000].

In parallel, cells were counted daily for 5 days with the aid of a hemocytometer. Media were replaced with fresh MEF media every 2 days. Counts were repeated on three parallel determinations from three different cell culture preparations. Doubling time was determined from the slope of a logarithmic fit of numbers of cells with time plotted against the cell seeding density according to the following log base 2 formula: $\log_2 (x) = \log_{10} (x)/\log_{10} (2) = \ln (x)/\ln (2)$.

For flow cytometry, cells were cultured (1×10^5 cells/ml in triplicates) for 48 and 72 h by replacing the medium every other day. The cells were gently washed in PBS, pelleted and resuspended thoroughly to get single-cell suspensions in fresh PBS as 1×10^6 cells/ml. Following, propodium iodide (50 μg/ml stock) labeling, samples were analyzed using a FC 500-2 Cytometer at the same flow rate and for the same time period for each sample to obtain relative cell numbers. As an internal control, chicken red blood cells were both analyzed separately and analyzed mixed with the MEF cells.

For absorbance assays, cells were seeded in 96-well microplates (2×10^3 cells/well in triplicate) and incubated at 37°C with 5% CO_2 for 24 h. EGCg or phenoxodiol was added at the concentrations indicated and the cells were incubated for an additional 24 or 48 h. The absorbency of cells fixed and stained was determined at 580 nM in an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Cell number was determined microscopically in parallel by counting the number of cells over defined areas consisting of a grid of 5 mm squares. The data were represented as percent survival rate.

Apoptosis Assays

Transgenic and wild-type MEF cells (1×10^5) were seeded on small cover slips with MEF media in 10 × 35 mm Petri dishes and incubated at 37°C with 5% CO_2 for 24 h. The next day, the medium was replaced with fresh MEF medium. Then, EGCg or phenoxodiol was added at 1, 5, 25, and 50 μM and the cells were incubated for an additional 48 h. After incubation, the cells on the cover slips were washed in PBS solution and fixed in 1% paraformaldehyde for 15 min. The fixed cells were labeled for 2 h at 37°C with green fluorescence Chroma Tide® BODIPY® FL-14-dUTP reaction solution (Molecular Probes, Inc., Eugene, OR), which end-labels the fragmented DNA of apoptotic cells. The labeling mixture consisted of 0.5 mM $CoCl_2$, 66.7 U of

TdT (terminal deoxynucleotidyl transferase), and 167 nM BODIPY reagent. The control labeling solution excluded BODIPY reagent. The cells were washed twice in PBS, twice with 15 mM EDTA (pH 8.0), and once in PBS with 0.1% Triton X-100 buffer. The appearances of labeled nuclei in the cells were recorded using fluorescence microscopy at the Purdue University Cytometry Laboratory. The excitation/emission maxima for the labeled nucleotide were 505/515 nm.

Statistical Analyses

Statistical analyses were based primarily on paired *t*-tests. Treatments that differed from controls by more than two standard deviations were considered as significant. Individual *P*-values are reported.

RESULTS

tNOX Expression in Mouse Embryonic Fibroblast Cells

Transgenic MEF cells were genotyped by PCR analysis and compared to wild-type MEF cells (Fig. 1A). The tNOX transgene-specific primers verified the presence of the tNOX transgene in transgenic MEF cells as confirmed by the presence of tNOX mRNA in the transgenic MEF cells and its absence from wild-type MEF cells (Fig. 1B). The ratio of tNOX mRNA (Fig. 1B) to GAPDH mRNA (Fig. 1C) suggested that tNOX expression in transgenic MEF cells was low. However, Western blot analysis with tNOX-specific 2281.1 antibody revealed 36, 34, and 32 kDa processed forms of the tNOX protein in transgenic MEF cells (Fig. 2). These processed tNOX forms were absent from wild-type MEF cells. Processed tNOX proteins of similar molecular weights were observed previously in tissues of transgenic mice and were shown to be absent from tissues of wild-type mice (Yagiz et al., 2006).

Inhibition of NADH Oxidation by EGCg and tNOX Antibody

NADH oxidase activities of both wild-type and transgenic MEF cells were analyzed from the decrease in absorbance at 340 nm as one of the characteristics of ECTO-NOX proteins (Table I). Wild-type MEF cells contained only EGCg-resistant CNOX activity. Transgenic MEF cells contained both EGCg-resistant CNOX activity and EGCg-inhibited tNOX activity. The latter was absent from wild-type cells. The tNOX activity was inhibited as well by the specific antibody 2281.1 in transgenic MEF cells, but CNOX activities of both wild-type and

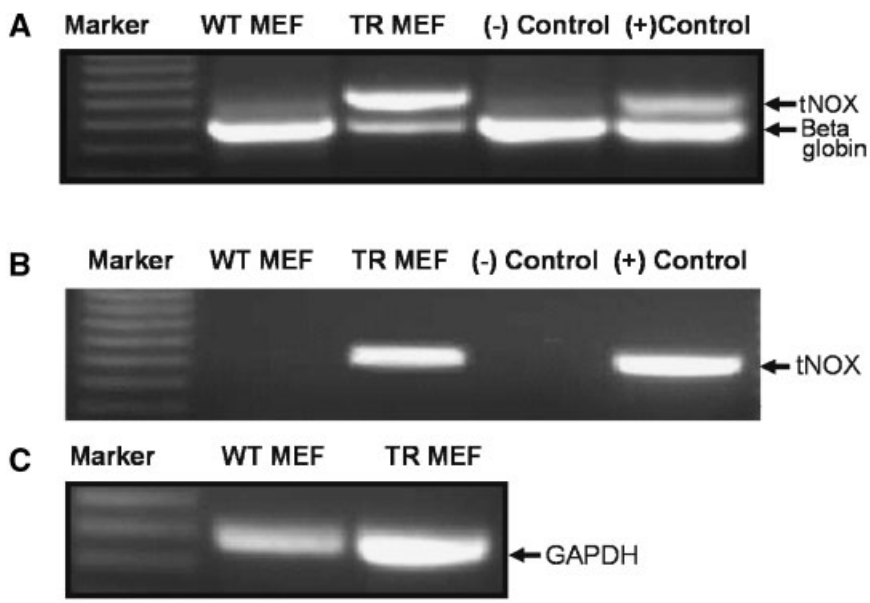

Fig. 1. Genotyping and RT-PCR analyses of mouse embryo fibroblast (MEF) cells. **A**: Genotyping. **Lane 1** wild-type (WT) MEF cells, **lane 2** transgenic (TR) MEF cells, **lane 3** (-) Control = wild-type mouse DNA, **lane 4** (—) Control = transgenic founder mouse DNA. **B**, **C**: RT-PCR analysis (B) tNOX mRNA was present in only transgenic MEF cells. **C**: Glyceraldehyde phosphate dehydrogenase (GAPDH) primers used to show integrity of the extracted RNAs.

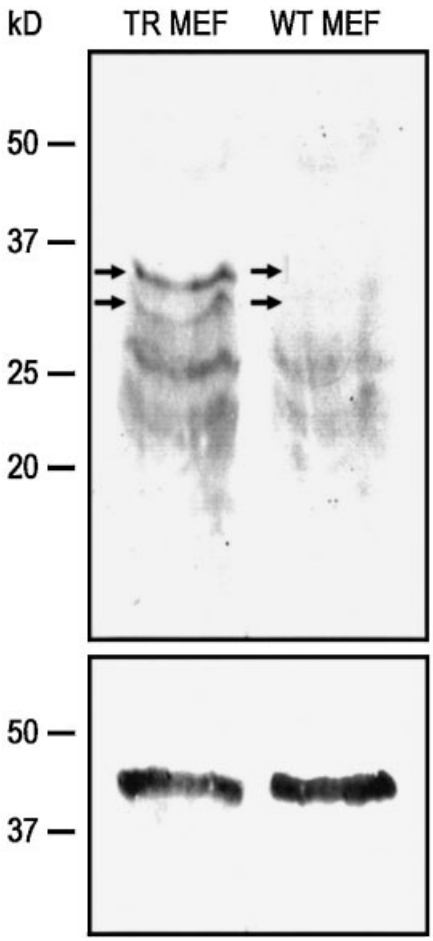

Fig. 2. Western blot analysis with MEF cells. **Top**: tNOX antibody 2281.l. **Lane 1** microsomes from transgenic (TR) MEF cells, **lane 2** microsomes from wild-type (WT) MEF cells. Thirty micrograms of microsomal proteins from MEF cells were loaded. Arrows indicate 36 (upper arrow) and 32 (lower arrow) kDa processed forms of the tNOX protein absent from a corresponding amount of wild-type microsomes. **Bottom**: Anti β-actin antibody from parallel lanes of same Western blot to demonstrate uniformity of loading.

transgenic MEF cells were refractory to inhibition by antibody 2281.1.

Growth of Transgenic Mouse Embryonic Fibroblast Cells

Based on the 96-well plate analysis, transgenic MEF cells showed higher absorbance levels compared to wild-type MEF cells under conditions of no treatment (Fig. 3A). When cell numbers were determined, the transgenic MEF cells exhibited approximately 50% more cells. However, with 96-well plate assays based on total absorbance of cellular material present, the increase was approximately twofold between 24 and 48 h. The fold increase decreased progressively as confluency was reached and/or the culture was depleted of media so that by 84 h, the total cell mass of transgenic MEF cells was only 30% greater than that of wild-type MEF cells (Fig. 3B). Based on cell counts the fold increase was less being about 50% greater for transgenic MEF cells compared to wild-type MEF cells after 48 and 72 h of growth and became more nearly equal at 96 and 120 h (Fig. 3B). The calculated doubling times were 28 h for wild-type and 24.4 h for transgenic MEF cells. Analysis by flow cytometry revealed no differences in cell-cycle phase comparing transgenic and wild-type MEF cells (not shown).

Cell Enlargement

MEF cells when examined microscopically enlarged rapidly for about 30 min after which their rate of enlargement slowed perceptibly due to the less favorable environment afforded by the microscope chambers (Fig. 4). With transgenic MEF cells (Fig. 4B), the initial rapid rate of cell enlargement was approximately twice that of wild-type MEF cells (Fig. 4A). After addition of 0.1 μM EGCg to wild-type MEF cells they continued to elongate slowly (Fig. 4A) whereas when 0.1 μM EGCg was added to the transgenic MEF cells net cell elongation ceased (Fig. 4B).

With EGCg or phenoxodiol added during the initial rapid enlargement phase, the rate of enlargement of transgenic MEF cells was reduced by 70% following treatment with 0.1 μM EGCg or 0.1 μM phenoxodiol while wild-type cells were insensitive to this concentration of EGCg or phenoxodiol (Table II).

The enlargement rate was not linear and exhibited a periodic behavior as described previously [Pogue et al., 2000] with maxima at intervals of 24 min (single arrows) for both the wild-type MEF cells (Fig. 4A) and the transgenic cells following EGCg treatment (Fig. 4B). The 24 min interval corresponds to the period length of CNOX [Morré and Morré, 2003]. After 24 h of growth, well-separated wild-type MEF cells based on measurements from six wells, exhibited an area post-fixation of 2160 ± 940 μm^2 whereas transgenic MEF cells had an area of 3120 ± 690 μm^2. Assuming a proportionate increase in cell height corresponding to ca. 10% of the leading edge, the transgenic MEF cells would have a volume approximately twice that of the wild-type MEF cells.

TABLE I. Total NADH Oxidase Activities of Embryo Fibroblast Cells From Wild-Type and Transgenic Mice and Inhibition by (—)-Epigallocatechin-3-Gallate (EGCg) and to 2281.1 tNOX-Specific Antibody

		nmoles/min/10^6 cells	
		Constitutive	Tumor-specific
Cell Line	Addition	ECTO-NOX (CNOX)	ECTO-NOX (tNOX)
Wild-type	None	2.3 ± 0.4	—
	1 μM EGCg	2.4 ± 0.4	—
Transgenic	None	2.0 ± 0.2	1.8 ± 0.3
	1 μM EGCg	1.9 ± 0.3	0.35 ± 0.2*
Wild-type	None	2.0 ± 2.5	—
	Ab 2281.1	1.9 ± 0.1	—
Transgenic	None	2.1 ± 0.1	1.6 ± 0.2
	Ab 2281.1	2.2 ± 0.2	0.3 ± 0.1*

Average ± standard deviation.
*Highly significant $P < 0.001$.

Dose Response of Growth of Mouse Embryonic Fibroblast Cells to EGCg and Phenoxodiol and Induction of Apoptosis

The inhibition of growth of transgenic MEF cells by EGCg or phenoxodiol was dose-dependent with an EC50 of about 0.5 μM (Fig. 5A). After 48 h of treatment with 10 μM EGCg or phenoxodiol, >90% of the transgenic MEF cells failed to survive (Fig. 5F,G) and had undergone apoptosis (Fig. 6F). Wild-type MEF cells, even after 48 h of treatment with 10 μM EGCg or phenoxodiol, survived (Fig. 5C,D) and showed no evidence of apoptosis (Fig. 6E). Results with EGCg and phenoxodiol were similar.

Induction of apoptosis was evidenced by the presence of BIODIPY-stained nuclei (Fig. 6). No fluorescence (autofluorescence) was observed in either unlabeled wild-type (Fig. 6A) or transgenic (Fig. 6B) MEF cells or in unlabeled wild-type (Fig. 6C) or transgenic (Fig. 6D) MEF cells grown in the absence of EGCg. After 48 h of

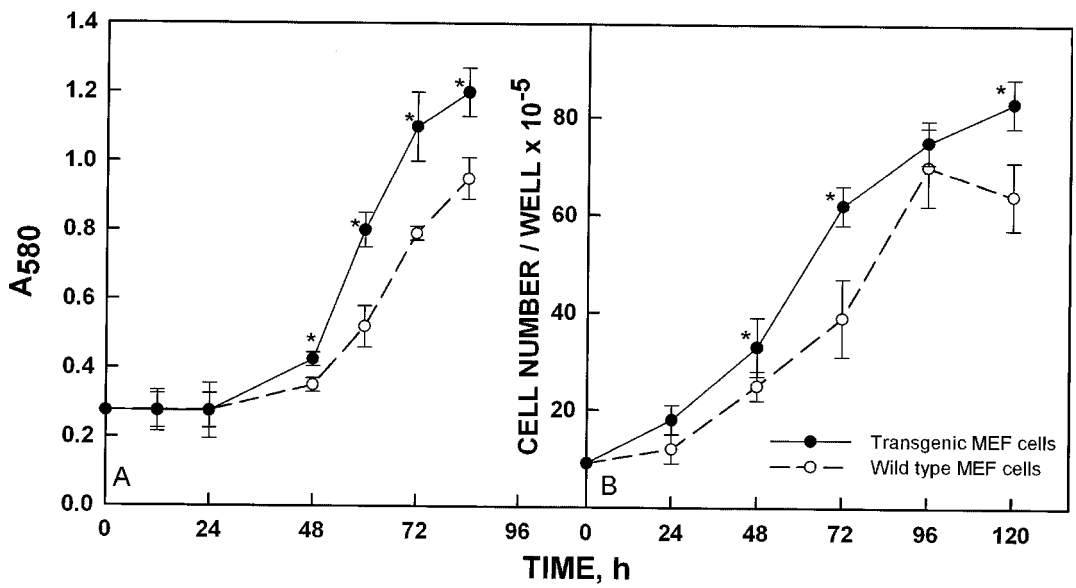

Fig. 3. Growth comparing transgenic and wild-type MEFs cells in culture. **A**: Change in A^0_{58} based on growth analysis using 96-well plates. Using this criterion, growth of transgenic cells in culture was nearly twice that of wild-type cells between 24 and 60 h of culture. **B**: Cell numbers of transgenic and wild-type MEF cells. Data are means of three experiments ± standard deviations. $P < 0.001$.

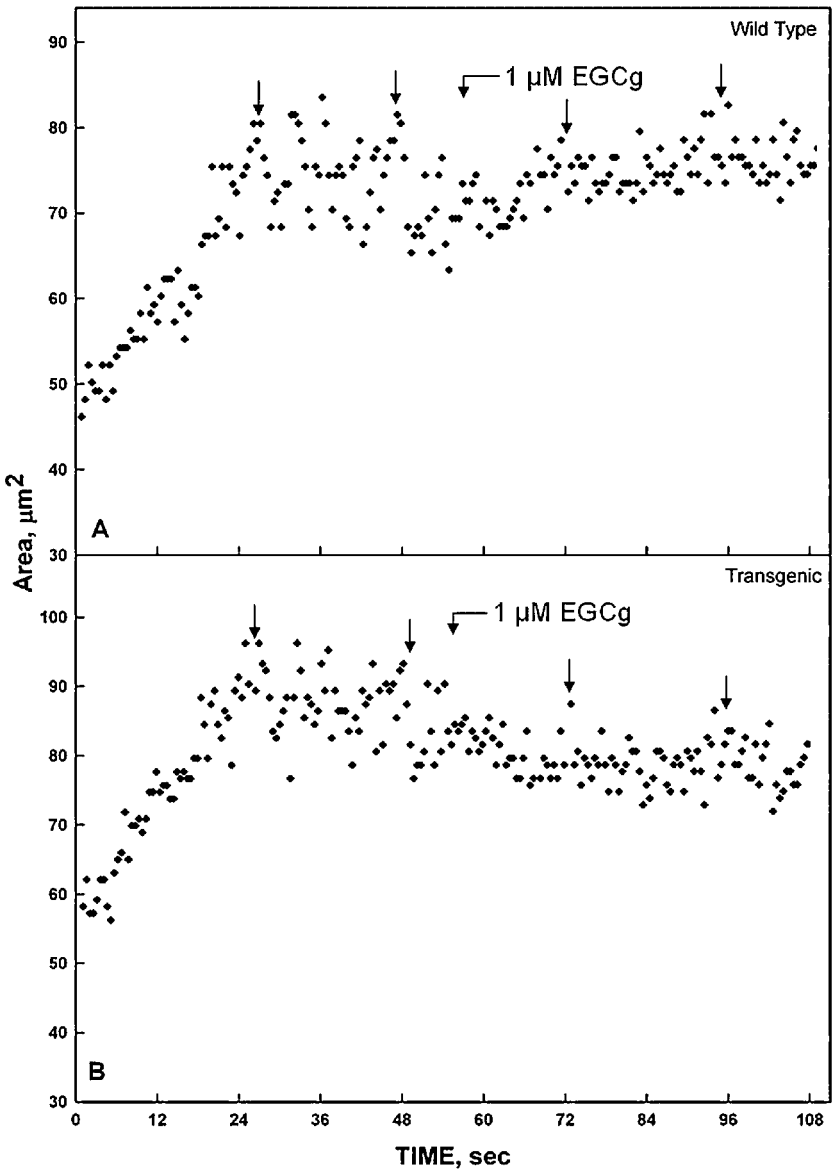

Fig. 4. Increase in area (enlargement growth) of small rounded MEF cells post-cytokinesis comparing wild-type (**A**) and transgenic (**B**) cells as determined by image-enhanced light microscopy. The growth rates fluctuated with a complex pattern of periodicity but were approximately twofold greater with the transgenic MEF cells. The single arrows separated by intervals of 24-min indicate periods of rapid enlargement alternating with resting periods. (−)-Epigallocatechin-3-gallate (EGCg) (0.1 μM) was added after 60 min (angled arrow).

treatment with 25 μM EGCg, wild-type MEF cells still showed no evidence of apoptosis (Fig. 6E). However, with transgenic cells (Fig. 6F), extensive apoptosis was revealed by BIODIPY-stained nuclei.

DISCUSSION

Our laboratories have identified a novel cancer-specific and growth-related cell-surface protein with protein disulfide-thiol interchange and hydroquinone (NADH) oxidase activities designated as tNOX (GenBank Accession No. AF207881) [Chueh et al., 2002]. tNOX responds to several known or putative quinone-site inhibitors all of which have anti-cancer activity [Morré, 1998] including capsaicin [Morré et al., 1995a; Chueh et al., 1997], adriamycin [Morré et al., 1997], EGCg [Morré et al., 2000], and the anti-tumor sulfonylureas [Morré et al., 1995b].

TABLE II. Initial Rates of Cell Enlargement Averaged Over 20 min of Wild-Type and Transgenic MEF Cells as Determined by Video-Enhanced Light Microscopy

	Wild-type MEF cells	Transgenic MEF cells
Treatment	Cell area, μm^2/min	
No addition	0.73 ± 0.0	1.4 ± 0.3
0.1 μM EGCg	0.63 ± 0.09	0.41 ± 0.08*
0.1 μM Phenoxodiol	0.80 ± 0.2	0.43 ± 0.03*

Results are averages of three determination ± standard deviation.
*Significant $P < 0.05$.

For EGCg, as one example, previous results have suggested that tNOX is both necessary and sufficient for the cancer-specific cell growth inhibitions attributed to EGCg in cancer cell lines [Chueh et al., 2004]. While transgenic mice overexpressing tNOX grew at an accelerated rate compared to wild-type mice [Yagiz et al., 2006], consumption of EGCg (500 mg/kg body weight), a tNOX inhibitor, prevented the accelerated growth resulting from tNOX overexpression. EGCg was without effect on either

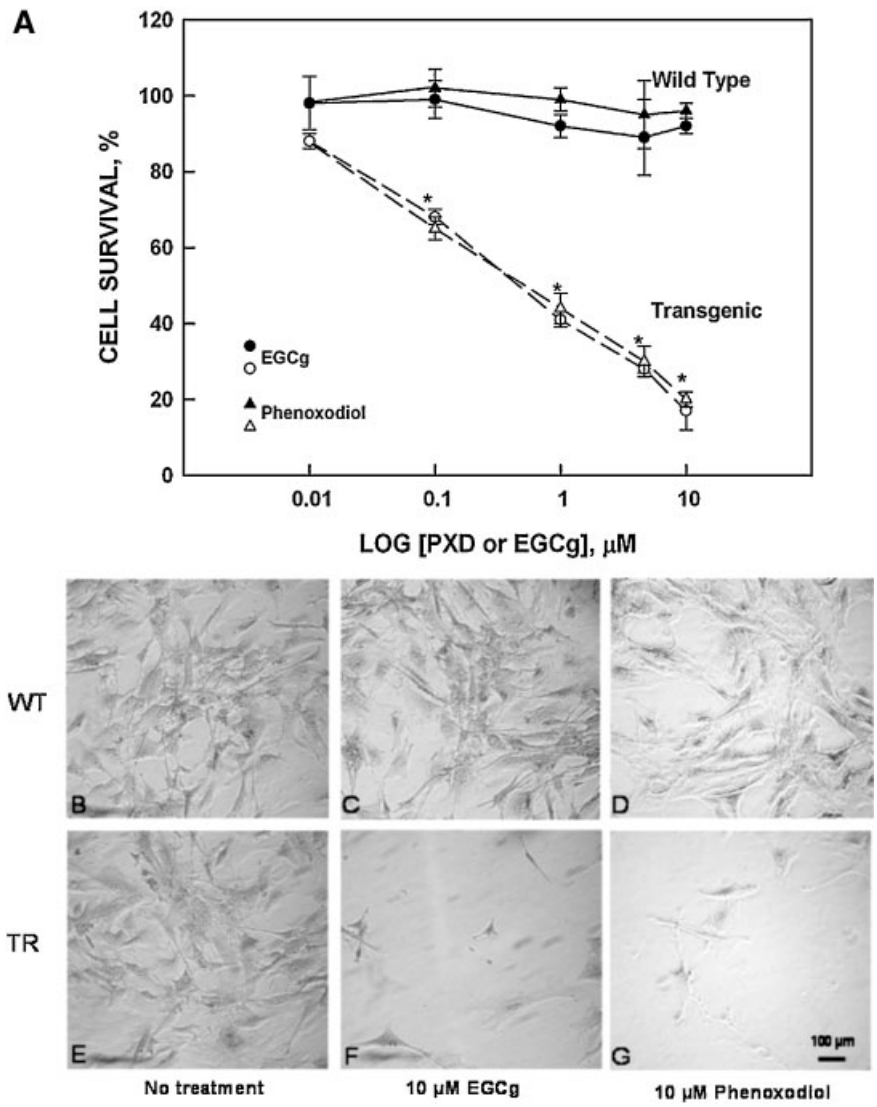

Fig. 5. Growth (**A**) and morphology (**B–G**) of wild-type and transgenic MEF cells in response to (−)-epigallocatechin-3-gallate (EGCg) and phenoxodiol (PXD). In A, numbers of viable cells were determined. Data are mean ± standard deviations of three experiments. Growth was inhibited during 48 h incubation with 10 μM phenoxodiol or with 10 μM EGCg in transgenic MEF cells. Wild-type MEF cells exhibited normal growth morphology with both phenoxodiol and EGCg even at the highest concentration tested of 10 μM. $P < 0.05$ versus wild-type control. Bar = 100 μm.

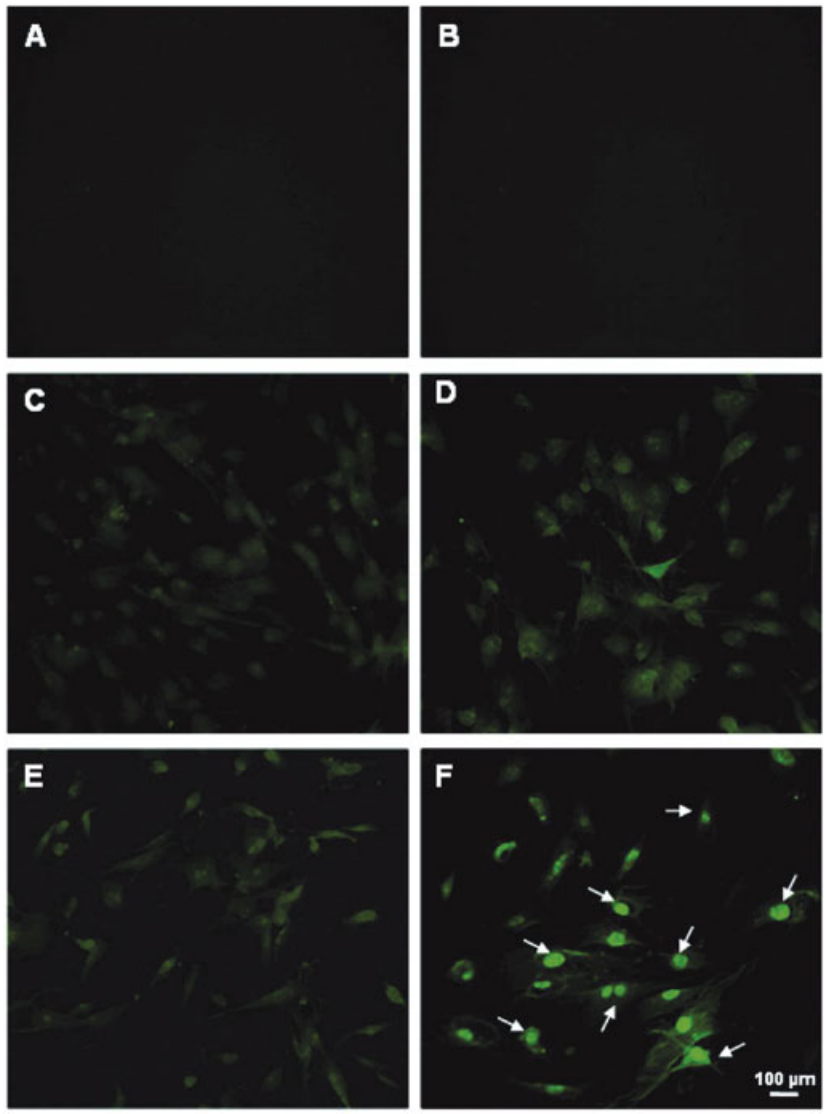

Fig. 6. Apoptosis induction by 48 h of treatment with (−)-epigallocatechin-3-gallate (EGCg) in MEF cells. **A**, **C** and **E**: Wild-type MEF cells. **B**, **D** and **F**: Transgenic MEF cells. A and B, unlabeled. C and D, labeled in the absence of EGCg. E and F, labeled after teatment with 25 µM EGCg. In F, nuclei were fluorescent (arrows) indicative of DNA fragmentation. Wild-type MEF cells showed normal morphology after 25 µM EGCg treatment and nuclei were unlabeled indicative of the absence of apoptosis. Results with 25 µM phenoxodiol were similar. Bar = 100 µm.

the growth or the NADH oxidase activity of wild-type mice.

To test further the implication that tNOX was both necessary and sufficient for the cell growth inhibitions attributed to EGCg in the transgenic mice, the reported experiments with fibroblasts from transgenic and wild-type mouse embryos were carried out. As with other systems [Morré, 1998], ECTO-NOX activity and growth of the mouse embryo fibroblasts were highly correlated [Morré, 1998]. Measurements of initial rates of cell enlargement revealed that the rate of enlargement of transgenic MEF cells was approximately twice that of wild-type cells. Both transgenic and wild-type MEF cells had constitutive CNOX activity. In addition, transgenic MEF cells exhibited tNOX activity. tNOX activity was absent in wild-type MEF cells. The tNOX activity present in the transgenic MEF cells was strongly inhibited by EGCg and by a tNOX-specific antibody. CNOX activity was refractory to both EGCg and phenoxodiol and

to the antibody in both cell lines. Drug inhibition is one of the defining tNOX characteristics. The implication is that tNOX activity is somehow directly linked to the actual process by which cells enlarge following cell division as neither EGCg nor phenoxodiol interfere with protein synthetic activity. In this context, inhibition of growth by tNOX inhibitors can be understood on the basis of their direct inhibitory effects on tNOX activity rather than in indirect effects following established downstream pathways [Yang et al., 2000; Vayalil et al., 2003; Chueh et al., 2004].

When increases in cell area were measured, the rates of increase fluctuated corresponding to the period length of the ECTO-NOX proteins present [Pogue et al., 2000; Morré and Morré, 2003]. Overexpression of tNOX cDNA in COS cells led to a more rapid rate of cell enlargement and several fold increase in cell volume compared to non-transfected COS cells [Chueh et al., 2004]. In all respects, our findings with enlargement of transgenic MEF cells recapitulated those of previous studies with HeLa cells [Pogue et al., 2000; Chueh et al., 2004].

The differences in cell number and cell size or cell mass comparing wild-type and transgenic MEF cells is likely due to the response to tNOX presence of an enhanced rate of cell enlargement. If MEF cells from transgenic cells enlarge more rapidly, they will achieve a critical size where they are able to divide sooner than wild-type cells to contribute to accelerated cell division whereas the combination of enhanced rate of cell enlargement and enhanced cell division account for the doubling of cell mass observed in the 96-well plate assay based on optical density measurements.

Both EGCg [Liao et al., 1995; Stoner and Mukhtar, 1995; Hibasami et al., 1996; Ahmad et al., 1997, 2002; Chen et al., 1998; Katdare et al., 1998; Yang et al., 1998; Mukhtar and Ahmad, 1999; Morré et al., 2000] and phenoxodiol [Kamsteeg et al., 2003; Kelly and Husband, 2003; Alvero et al., 2006] have been shown previously to specifically inhibit the growth of cancer cells or at least inhibit the growth of cancer cells at concentrations one or two log orders lower than those required to inhibit growth of non-cancer cells. The tNOX target protein has been proposed as both sufficient and necessary for cancer cells to respond to these two substances [Morré et al., 2000, 2007]. A similar conclusion is reached from the findings reported here. Wild-type MEF cells were resistant to both EGCg and phenoxodiol over a wide range of concentrations. On the other hand, the transgenic MEF cells overexpressing tNOX responded to both EGCg and phenoxodiol concentrations in the nanomolar range. Not only was growth of transgenic MEF cells more susceptible to inhibition than growth of wild-type MEF cells, the transgenic MEF cells also underwent apoptosis in response to EGCg and phenoxodiol whereas wild-type MEF cells did not.

Studies of Hayakawa et al. [2001] with U937 cells suggested that the apoptosis triggered by EGCg was Fas-mediated. EGCg decreased the anti-apoptotic Bcl-2 and Bcl-xL and increased Fas and Fas-ligand [Masuda et al., 2001; Kuo and Lin, 2003; Weinreb et al., 2003]. Choi et al. [2003] reported that ERK activation plays an active role in mediating EGCg-induced apoptosis of U937 cells and functions upstream of caspase activation to initiate the apoptotic signal. In addition, EGCg treatment induced apoptosis of NPC cells via the mitochondrial signal transduction pathway [Yan et al., 2004]. Activity levels of caspases-3, -8, and -9 were elevated in EGCg-treated cells, suggesting that these caspases were involved in the apoptosis induced by EGCg [Horie et al., 2005]. Moreover, EGCg treatment triggered c-Jun N-terminal kinase and p38 kinase of the MAP kinase family to induce apoptosis in tumor cells [Saeki et al., 2002]. Another tNOX inhibitor, phenoxidiol [Morré et al., 2006], has been shown to induce apoptosis by the activation of the mitochondrial pathway through caspase-2 and Bid signaling, and the proteasomal degradation of the anti-apoptotic protein XIAP in epithelial ovarian carcinoma cells [Alvero et al., 2006].

The findings with the embryo fibroblasts from tNOX transgenic mice are unique in that they demonstrate in a simple single cell system stable expression of a single protein, tNOX, is both necessary and sufficient to impart drug sensitivity to EGCg and phenoxodiol as wild-type fibroblasts are normally unresponsive to these agents. Also consistent with our observations is the hypothesis that tNOX facilitates the uncontrolled growth exhibited by both cancer tissues and cancer cell lines [Morré and Morré, 2003]. As such tNOX may be expected to have an important future role as a molecular drug target in cancer.

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