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Transgenic mouse line overexpressing the cancer-specific tNOX protein has an enhanced growth and acquired drug-response phenotype☆

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Abstract

tNOX, a novel cell surface protein related to unregulated growth and drug response of cancer cells, has been proposed as the cellular target for the anticancer action of various quinone site inhibitors with anticancer activity including the polyphenol (−)-epigallocatechin-3-gallate (EGCg). A transgenic mouse line overexpressing tNOX was generated to determine its overall growth phenotype and susceptibility to EGCg. Cultured noncancer cells lack tNOX and are unresponsive to EGCg. Overexpression of tNOX in cultured noncancer cells through transfection resulted in both enhanced growth and an acquired inhibitory response to EGCg. The tNOX transgenic mouse line was developed using a phCMV2 vector with the hemagglutinin (HA) tag. Transgenic mice exhibited both an enhanced growth rate and a response to EGCg not observed with wild-type mice. Female transgenic mice grew twice as fast as wild type, and growth was reflected in an overall increased carcass weight. Administration of EGCg in the drinking water [500 mg/kg body weight (BW)] reduced the growth rate of the transgenic mice to that of wild-type mice. The findings provide in situ validation of the hypothesis that tNOX represents a necessary and sufficient molecular target as the basis for the protective and potential cancer therapeutic benefits of EGCg.
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Keywords: ECTO-NOX; tNOX; NADH oxidase; Transgenic mouse; (−)-Epigallocatechin-3-gallate (EGCg); Growth

1. Introduction

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 (*t*umor-associated *N*ADH *ox*idase) (GenBank Accession No. AF207881) [1]. A member of a family of cell surface (ECTO) NOX proteins, tNOX has been suggested to be the molecular target to explain the anticancer activity of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCg) [2,3]. The major catechin in green tea, EGCg [4], uniquely has the ability to selectively block the growth of cancer cells in culture [5], suggestive of a cancer-specific target [6] independent of its antioxidant properties [7]. Not only does EGCg selectively block the growth of tumor cells, but tumor cells also are more sensitive to inhibition by EGCg than their normal counterparts [2,5,8,9].

The activity of ECTO-NOX proteins is defined by enzymatic transfer of protons and electrons from cytosolic NAD(P)H via the membrane pool of reduced coenzyme Qs to molecular oxygen [10] or to protein disulfides [11] as terminal electron acceptors. The protein disulfide-thiol interchange activity, also a general function of ECTO-NOX proteins [12,13], correlates with cell enlargement [13]. They are ectoproteins, reversibly bound at the outer leaflet of the plasma membrane [14]. They appear in soluble form in conditioned media of cultured cells [15] and in patient sera [16,17]. The oxidative activity of ECTO-NOX proteins is distinguished from that of other oxidases and oxidoreductases of both organelles and internal membranes and from other oxidoreductases of the plasma membrane by its resistance to inhibition by cyanide [13].

tNOX is both cancer specific and pancancer [16–19]. Cancer cells expressing tNOX such as HeLa (human cervical carcinoma) and BT-20 (human mammary carcinoma) cells not only grow in an unregulated manner but their growth is inhibited by EGCg [2] and other quinone site inhibitors with anticancer activity [13–19]. When tNOX cDNA was overexpressed in COS or MCF-10A (noncancer

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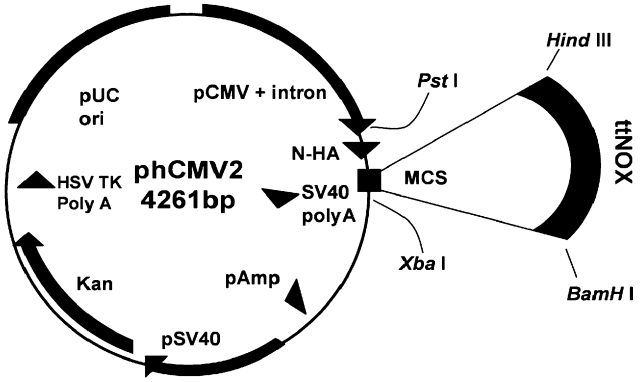

Fig. 1. Construct of tNOX plasmid. The tNOX cDNA was subcloned into a mammalian phCMV2-HA expression vector. The vector included the N-terminus HA tag.

mammary epithelial) cells, the transfected cells exhibited an accelerated rate of cell enlargement, a larger cell size at confluence and a heightened susceptibility to growth inhibition by EGCg [3].

Antisense results and the transfection and overexpression experiments taken together showed that functional cell surface expression of tNOX was both necessary and sufficient for the cancer-specific cell growth inhibitions attributed to EGCg [3]. As an extension of these observations, transgenic animals overexpressing tNOX might be expected to exhibit the same level of unregulated cell enlargement and sensitivity to EGCg as cancer cells. The experiments with transgenic mice reported here test further the hypothesis that tNOX represents both a necessary and sufficient molecular target for EGCg to explain its anticancer preventative and therapeutic potential.

2. Materials and methods

2.1. Preparation of tNOX plasmid construct

The truncated form of tNOX cDNA was amplified using primers 5′-GAGTGCAAGCTTATGCTAGCCAGAGAG-3′ (forward) and 5′-TCTTCAGGATCCCAAGTTGTTAGG-CAAAGA-3′(reverse) to construct *Hin*dIII and *Bam*HI (New England Biolabs, Beverly, MA, USA) restriction sites. The tNOX cDNA was then subcloned into the mammalian phCMV2-HA expression vector (Gene Therapy Systems, San Diego, CA, USA) (Fig. 1) and transfected in *E. coli*. The transformants were plated on LB plates containing 50 μg/ml kanamaycin. Antibiotic-resistant transformants were analyzed for the presence of inserts by restriction digestion analysis. A transformant with the correct restriction pattern was selected and sequenced to confirm that the gene was cloned in the proper orientation. The plasmid DNA was prepared using the EndoFree plasmid Midi kit (Qiagen, Valencia, CA, USA). Transfection of COS cells with purified tNOX containing plasmid cDNA was with a Calcium Phosphate Transfection Kit (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was monitored by Western blot analysis (Fig. 2) using the hemagglutinin (HA)-tag monoclonal antibody as the first antibody (sc7932; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. Production of tNOX transgenic mice

Transgenic mice were generated using the standard pronuclear injection of egg donors from the FVB/N (Harlan Industries, Indianapolis, IN, USA) strain [20]. Animals were housed in the Purdue University Transgenic Mouse Core Facility. The following protocols were reviewed and approved by the university-wide animal care committee. For microinjections, the tNOX transgene was separated free from the backbone of phCMV2-HA by *Afl*III, *Ssp*I and *Fsp*I restriction enzymes (New England Biolabs, Beverly, MA, USA). The restriction enzyme reactions were separated on 0.8% agarose gel electrophoreses in 1 mM Tris–acetate plus 1 mM EDTA, pH 8.5 [21], with ethidium bromide (Sigma, St. Louis, MO, USA) (1 μg/ml) at 30–40 mA. The injected fragments of tNOX transgene were isolated and purified by the Purdue University Transgenic Mouse Core Facility.

2.3. Genotyping by Southern blotting

To confirm the presence of tNOX in the transgenic mouse genome, Southern blots were performed by standard techniques [21]. Mouse-tail genomic DNA was prepared using a standard protocol [22]. Briefly, 10 μg of tail genomic DNA was digested overnight with *Hin*dIII and *Bam*HI restriction enzymes, and the restriction fragments were fractionated by electrophoresis through 0.8% agarose gels in Tris–borate–EDTA (90 mM Tris–borate, 2 mM EDTA, pH 8.0), transferred onto a positively charged nylon membrane (Schleicher and Schuell, Keene, NH, USA) [23] by alkaline transfer, and subjected to prehybridization and then hybridization with a probe of 1.2-kb [^{32}P]-labeled tNOX. After stringent washing, the membranes were exposed to X-ray film (Sigma Kodak, Rochester, NY, USA) at −80°C. For Southern blots, genomic DNA from a

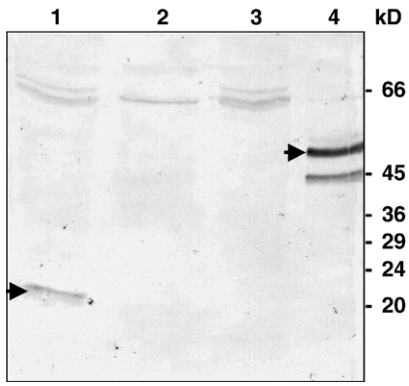

Fig. 2. Overexpression of tNOX. Lane 1: Plasmid with HA tag only (arrow). Lane 2: pHCMV2 vector alone. Lane 3: COS cells. Lane 4: COS cells transfected with tNOX plasmid. The band corresponding to the tNOX plasmid construct with the HA tag is indicated by the arrow. Detection was with the anti-HA tag antibody.

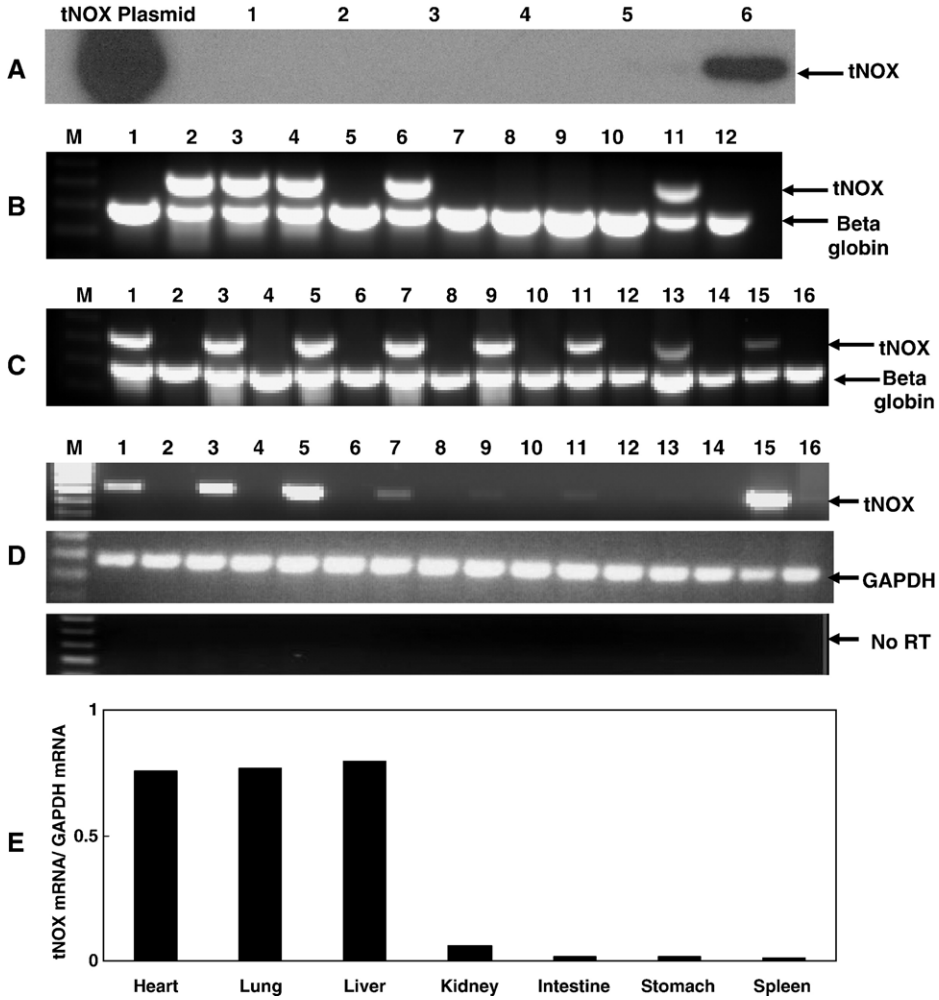

Fig. 3. (A) Southern blot analysis of DNA from mice tail tissues. Mouse no. 6 was selected as the founder mouse. (B) Genotyping by PCR analysis. M: Marker; Lanes 1, 5, 7, 8, 9, 10: nontransgenic mice; Lanes 2, 3, 4, 6: transgenic mice; Lane 11: positive control (founder mouse); Lane 12: negative control (wild-type FVB mouse). (C) Tissue distribution of tNOX transgene. Tissue samples were analyzed by PCR. Lane 1: transgenic heart; Lane 2: wild-type heart; Lane 3: transgenic lung; Lane 4: wild-type lung; Lane 5: transgenic liver; Lane 6: wild-type liver; Lane 7: transgenic kidney; Lane 8: wild-type kidney; Lane 9: transgenic intestine; Lane 10: wild-type intestine; Lane 11: transgenic stomach; Lane 12: wild-type stomach; Lane 13: transgenic spleen; Lane 14: wild-type spleen; Lane 15: positive control; Lane 16: negative control. (D) Tissue expression of tNOX mRNA. RT-PCR with tNOX specific primers (top gel). Mouse GAPDH as internal control (middle gel). No RT-PCR control (bottom gel). Lane 1: transgenic heart; Lane 2: wild-type heart; Lane 3: transgenic lung; Lane 4: wild-type lung; Lane 5: transgenic liver; Lane 6: wild-type liver; Lane 7: transgenic kidney; Lane 8: wild-type kidney; Lane 9: transgenic intestine; Lane 10: wild-type intestine; Lane 11: transgenic stomach; Lane 12: wild-type stomach; Lane 13: transgenic spleen; Lane 14: wild-type spleen; Lane 15: positive control; Lane 16: negative control. (E) Quantification by densitometry of (D). tNOX mRNA was found in different tissues of transgenic mice but not in tissues of wild-type mice.

normal FVB/N mouse was used as a negative control, while the tNOX plasmid was used as the positive control (Fig. 3A).

2.4. Genotyping by polymerase chain reaction analysis

Polymerase chain reaction (PCR) analysis was performed on tail genomic DNA preparations to determine which mice had tNOX integrated into their genome. Two primers [5′-TACCCATACGATGTTCCGGAT-3′(forward) and 5′-CAGAGGTTCTGCCTGTGATAC-3′(reverse)] were designed to be transgene specific. PCR amplifications were in a total volume of 25 μl. Amplified products were analyzed using 1% agarose gels. A beta-globin primer pair (Genbank Accession No. J00413) was used as an internal positive control to ensure that the DNA sample was amplifiable (Fig. 3B). This prevented false-negative results and insured that positive animals were not accidentally discarded.

2.5. Reverse transcriptase PCR

Total RNA was isolated from different tissues using TRI-REAGENT (Molecular Research Center, Cincinnati, OH, USA) 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 reverse transcriptase PCR (RT-PCR) with primers for the mouse GAPDH primers as an internal standard (Fig. 3D, middle gel). Equal quantities (2 μg) of total RNA were tested in each reaction. The two transgene-specific primers were 5′-ACCCATACGATGTTCCGGATT-3′(forward) and

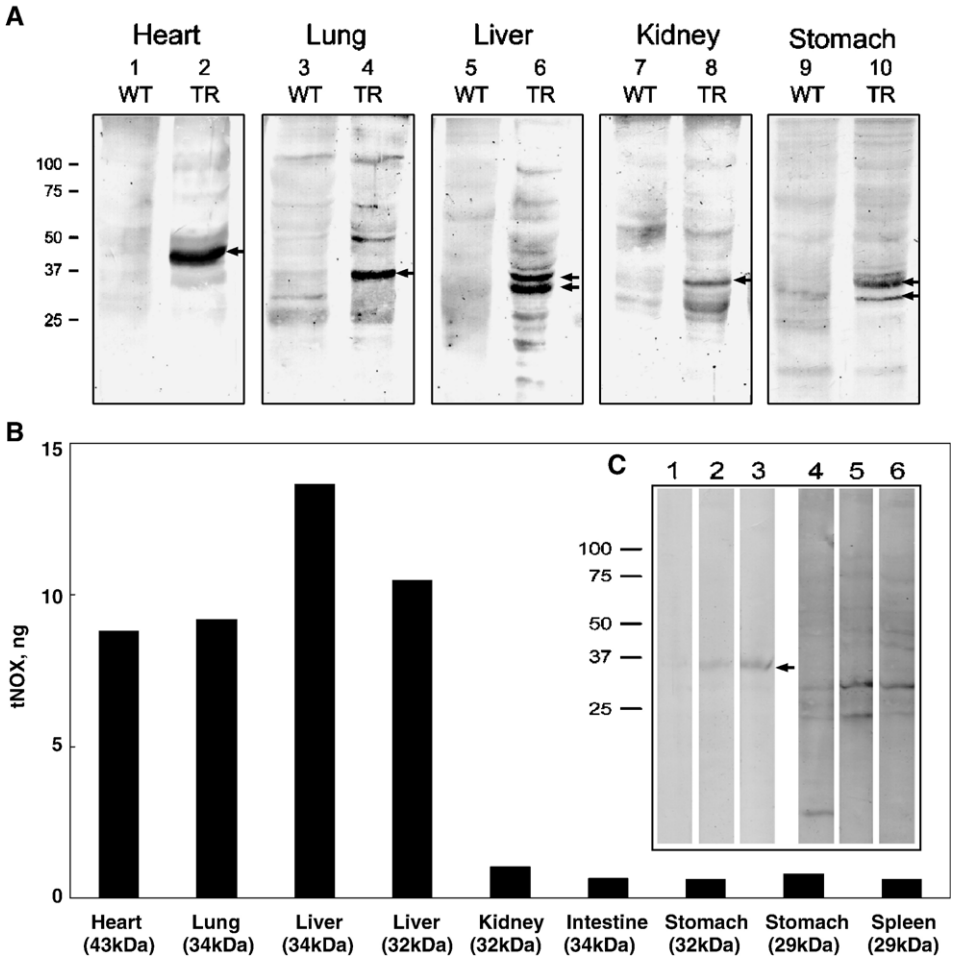

Fig. 4. (A) Western blot analysis of tNOX presence in representative tissues comparing microsomes of wild-type and transgenic mice. Microsomes (50 μg of total protein) from tissues of transgenic animals contained protein bands reactive with anti-tNOX antibody that were absent from tissues of wild-type animals (arrows). Also analyzed were intestine and spleen (not shown). Transgenic tissues showed specific bands corresponding to the processed molecular weights of tNOX of 43 (heart), 34 (lung, liver and intestine), 32 (liver, kidney and stomach) or 29 (stomach and spleen) kDa (see text). All lanes were from the same blot except for Lanes 1, 2, 9 and 10, which were from blots processed in parallel. (B) Quantitation of (A) by densitometry of the designated lanes (minus background) from the original Western blots. Arbitrary units were converted to nanogram tNOX from a series of recombinant tNOX standards analyzed under comparable conditions. (C) Western blot analysis to show plasma membrane expression of 34 kDa of tNOX (arrow). Samples were from liver tissue. Each lane was loaded with 25-μg microsomes or plasma membrane proteins. Lanes 1–3, anti-tNOX 2281.1; Lanes 4–6, anti-HA; Lanes 1 and 4, wild-type plasma membranes; Lanes 2 and 5, transgenic plasma membranes; Lanes 3 and 6, transgenic microsomes. All lanes were from the same blot cut to permit incubation with the different antisera indicated.

5′-GGTCAGCTTCAAGCCTCGAAGC-3′(reverse) (Fig. 3D, top gel). The negative control reactions were included without reverse transcriptase to ensure that RT-PCR was RNA-dependent (Fig. 3D, bottom gel). All experiments were in duplicate.

2.6. Western blot analysis of tNOX expression

The tissue samples were prepared as described [23]. Proteins were separated on 10% SDS-PAGE and then transferred by electroblotting onto nitrocellulose membranes [24] and detected as described [1]. 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 adenine nucleotide binding region. The secondary antibody was either goat antimouse linked to the alkaline phosphates for anti-HA tag or monoclonal antipolyhistidine clone His-1 (Sigma) for anti-tNOX 2281.1 (Fig. 4A,C).

2.7. Purification of plasma membranes

The plasma membranes of transgenic and wild-type mouse tissue samples were prepared as described [23]. The membranes were stored in 50 mM Tris–MES (pH 7.5).

2.8. Spectrophotometric assay of NADH oxidase

NADH oxidase activity of plasma membranes was determined from the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris–MES

Table 1
Total plasma membrane NADH oxidase activities of liver and intestine from transgenic mice compared to wild-type mice and inhibition by EGCg

Tissue	1 μM EGCg	nmol/min per mg protein	
		Constitutive ECTO-NOX (CNOX)	Tumor-specific ECTO-NOX (tNOX)
Wild type liver	–	2.2±0.4	–
	+	2.0±0.2	–
Transgenic liver	–	1.4±0.2	1.5±0.3
	+	1.3±0.3	0.1±0.1
Wild type intestine	–	3.1±0.3	–
	+	3.0±0.2	–
Transgenic intestine (normal assay)	–	2.4±0.3	0.55±0.1
	+	2.2±0.2	0.4±0.3
Transgenic intestine (enhanced assay)*	–	2.3±0.6	2.1±0.6
	+	2.2±0.7	0.4±0.4

Average±standard deviations from three experiments.
* Enhanced assay conditions were in the presence of 0.2% Triton X-100 and overnight incubation with 300 mM NADH in which tNOX activity was enhanced fourfold. CNOX was unaffected by these assay conditions.

buffer (pH 7.5), 1 mM KCN to inhibit mitochondrial oxidase activity and 150 μM NADH at 37°C with a Hitachi U-3210 spectrophotometer (Hitachi, Japan) and continuous recording over 1.0 min at intervals of 1.5 min each. EGCg (Sigma) (1 μM) was added as a tNOX inhibitor (Table 1). Where indicated, addition of 0.2% Triton X-100 and overnight incubation in the presence of 300 mM NADH were utilized to enhance low levels of tNOX activity. This treatment did not alter CNOX activity. Proteins were estimated by the bicinchoninic acid method with bovine serum albumin as standard [25].

2.9. EGCg response

Four experimental groups of 8- to 10-week-old mice were used to compare the growth phenotype and response to EGCg of the transgenic and wild-type mice. Each group contained three female and three male mice housed individually in separate cages. EGCg-treated mice were provided with 500 mg/kg body weight (BW) fresh EGCg in drinking water daily for 3 months. The water bottles were covered with aluminum foil to reduce EGCg degradation. The body weights of the mice and the amounts of water consumed were recorded daily. The experiment was repeated a total of three times.

2.10. Acute toxicity evaluations

For the evaluation of dose response, 8- to 10-week-old transgenic or wild-type male and female mice were treated with accelerated EGCg doses (0, 25, 50, 75, 100, 125 and 250 mg/kg BW) delivered by sequential intraperitoneal injections at 24-h intervals. Control groups received vehicle alone. Three mice were included in each group. Treated mice were monitored every 2 h during the light period for evidence of physical disability or discomfort (Table 3). These experiments were repeated a total of three times.

2.11. Statistical analysis

Statistical analyses were based primarily on paired *t*-tests. Differences where treatments differed from controls by more than two standard deviations were highly significant. Individual *P* values are reported.

3. Results

3.1. Construction of tNOX phCMV2-HA expression vector

Truncated tNOX corresponding to the 34-kDa tNOX form expressed at the cancer cell surface (1.2 kb) was amplified by PCR, subsequently inserted into phCMV2-HA expression vector (Fig. 1) and thereafter sequenced. The transgenic construct contained the N-terminal HA-tag sequence and the tNOX gene followed by a poly (A) signal. After sequence confirmation, the tNOX plasmid construct was overexpressed in COS cells in order to verify that the gene was cloned in the proper orientation. After Western blotting with the HA-tag antibody, a protein band corresponding to the overexpressed tNOX band was observed in the tNOX transfectants (Fig. 2, Lane 4), but was absent from vector alone (Fig. 2, Lane 2). Control plasmid with the HA tag was also used to establish the specificity of the HA-tag antibody (Fig. 2, Lane 1). The tNOX-HA fragment removed from the vector using *Afl*III, *Ssp*I and *Fsp*I restriction enzymes was isolated and purified for microinjection.

3.2. Generation and genotyping tNOX-HA transgenic mice

The ~2.5-kb tNOX-HA transgene was transferred into mice by pronuclear injection. Embryos that survived the pronuclear injection were transferred to recipient FVB/N females and 55 embryos developed to term. Genomic DNA (10 μg) of these F_0 pups and a negative control normal mouse were digested with *Bam*HI and *Hin*dIII restriction enzymes and used for Southern blot analysis with a tNOX probe (Fig. 3A). Mouse no. 6 was determined to be transgenic and was selected as one of the founders. The findings were confirmed by PCR analysis by using the HA tag-specific (forward) and the tNOX-specific (reverse) primer (Fig. 3B, tNOX band). Beta globin primers were used as an internal positive control (Fig. 3B, beta globin band). Among 55 potential founders, PCR analysis revealed five, which carried the tNOX-HA transgene. Fifty-five offspring were individually analyzed by PCR for the genomic integration of the transgene from tail biopsy-derived DNA. The results were compared with those obtained with DNA from a negative control wild-type mouse and a positive control DNA. Lanes 2, 3, 4, 6 and 11 of the genomic DNA of the representative five animals were analyzed out of 55 F_0 pups in a PCR reaction (Fig. 3B). The data on the remaining nontransgenic littermates are not shown. The molecular weight of the amplified tNOX-HA fragment band was calculated around 600 bp by the

Table 2
EGCg (1–1.5 mg/ml, adjusted to 500 mg/L per kg BW) provided in drinking water

	Growth, mg/day±S.D.	
	Wild type	tNOX Transgenic
Control	26±4[a]	60±20[b]
EGCg	33±6[a]	33±5[a]

Average consumption of 4 ml (5.5 mg/day)=22 mg/kg body weight. Addition of EGCg to drinking water reduced growth rate of the transgenic female mice to wild-type mice rates. EGCg had no effect on growth rate of wild-type mice (which lack tNOX). Each group within each experiment contained five mice. Averages are ±standard deviations from three experiments. Values not followed by the same letter are statistically different (a,b: $P<.04$).

amplified band and by the migration of standard DNA molecular weight markers (Fig. 3B, Lane M).

3.3. Reverse transcriptase PCR

Comparing different tissue samples, we found the presence of the overexpressed tNOX gene in all tissues examined (heart, lung, liver, kidney, intestine, stomach and spleen) of transgenic mice, but not in the comparable tissues of wild-type mice (Fig. 3C). tNOX mRNA was expressed in heart, lung, liver, kidney, intestine, stomach and spleen tissues of transgenic mice, but not in tissues of wild-type mice. RT-PCR primers were designed to be specific to the HA tag (forward) and the tNOX gene (reverse) (Fig. 3D, top gel). GAPDH transcripts were monitored as a control to quantify the transcripts of the genes in each tissue sample (Fig. 3D, middle gel). Amounts of tNOX and GAPDH mRNAs were determined by densitometry, and ratios of tNOX/GAPDH mRNA were calculated. Compared to RT-PCR products using mouse GAPDH primers as internal controls, tNOX mRNA was expressed in heart ≈ lung ≈ liver > kidney > intestine ≈ stomach ≈ spleen (Fig. 3E).

3.4. Spectrophotometric assay of NADH oxidase

The tNOX activity in liver and intestine of the transgenic mice was quantitated by enzymatic assays. Plasma membranes from tissues of wild-type mice carried only the EGCg-resistant constitutive ECTO-NOX (CNOX) (Table 1). Plasma membranes from livers and intestines of the tNOX transgenic mice also contained the EGCg-resistant constitutive CNOX. In addition, a second set of activities subject to >90% inhibition by 1 μM EGCg was observed with plasma membranes from livers of transgenic mice. However, with plasma membranes from intestines of transgenic mice analyzed in parallel, the tNOX activity was near the level of detection of the method. However, subsequent assay after an overnight incubation with Triton X-100 in the presence of 300 mM NADH to effect a fourfold enhancement of tNOX activity did reveal a statistically significant tNOX component. Total NADH oxidation, the sum of CNOX+tNOX, was 1.4+(1.5−0.1)=2.45 for plasma membranes of liver, and 2.3+(0.55−0.4)=2.45 for plasma membranes of transgenic intestine.

In the presence of 1 μM EGCg, the rate of NADH oxidation by plasma membranes of tissues from transgenic mice was reduced to values approximating those of membranes of tissues from wild-type mice as enzymatic confirmation that the elevated values were the result of the overexpression of tNOX.

3.5. Western blot analysis of tNOX expression

Immunoreactive bands, absent from tissues of wild-type mice, were detected in all tissues from transgenic mice examined (Fig. 4A). Using liver as an abundant tissue source, we found the expressed tNOX in microsomes (Fig. 4A and C, Lanes 3 and 5) and purified plasma membranes (Fig. 4C, Lanes 1, 2, 4 and 5). The HA tag was no longer evident either in microsomes or plasma membrane-associated tNOX (Fig. 4C, Lanes 4–6).

The amount of tNOX present at the plasma membrane correlated approximately with plasma membrane levels determined spectrophotometrically. From the turnover number of recombinant tNOX (200–500) [26], the amount of tNOX present in plasma membranes of liver from transgenic mice was calculated to be about 0.1 μg/mg plasma membrane protein (Table 2). For plasma membrane of intestines from transgenic mice prior to enhancement, the amount was 0.1 that amount, very near the limit of detectability either by enzymatic assays or on Western blots. Densitometry analysis of the band of Lane 2 for plasma membranes of livers from transgenic mice (Fig. 4C) revealed a tNOX amount of 2.5 ng/25 μg plasma membrane protein, which is approximately the expected amount based on measured enzymatic activity.

That the immunoreactive bands exhibited different molecular weights can be explained due to tissue-specific differences in processing of tNOX. Experiments with processing enzymes from endoplasmic reticulum and Golgi apparatus of HeLa cells demonstrated that the recombinant transgene product expressed in bacteria is cleaved into intermediates of 43, 34, 32 and 29 kDa (Xiaoyu Tang and Sshuen Chen, Purdue University, unpublished data). In heart, the 43-kDa peptide generated after N-terminal cleavage of the signal peptide appears not to be processed further. In lung, a 34-kDa species is represented. The liver shows both 34- and 32-kDa species. In kidney and stomach, a 32 kDa band is represented. Also present in stomach is an immunoreactive 29-kDa band. An immunoreactive 29-kDa protein isolated from human sera exhibited drug-responsive NADH oxidase activity (D.J. Morré, Purdue University, unpublished results). A 34-kDa species in intestine and a 29-kDa species in spleen are present (not shown). Given that information, the protein expression levels correlate with mRNA contents, being most abundant in heart, liver and lung, and less abundant in kidney and stomach and least abundant in intestine and spleen. These values have been quantitated from the gels by densitometry and compared to a series of recombinant tNOX standards analyzed in parallel (Fig. 4B).

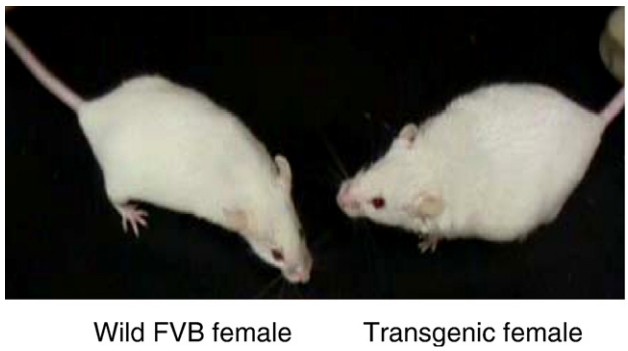

Fig. 5. Photograph of a representative female tNOX transgenic mouse 9 months old compared to a female wild-type mouse of the same age.

3.6. tNOX and growth

The most evident phenotypic characteristic of tNOX overexpression was an increased rate of growth. Based on body weight, the female tNOX transgenic mice grew to be approximately 50% heavier than wild-type mice (Fig. 5). Male transgenic mice also grew faster but exhibited a correspondingly lower rate of increase relative to wild type (Fig. 6). Between 3 and 9 months of age, the growth rate of female transgenic mice was twice that of wild-type mice (Table 2 and results not shown). The increase in size of the transgenic mice was reflected as well in an overall increase in carcass weight (ca. 20%).

3.7. EGCg response

The average body weight of mice was ~25 g for females and ~30 g for males. The mice consumed ~4 ml/day of drinking water. The daily intake of EGCg was ~5.5 mg/kg BW. The amount of EGCg consumed by the mice corresponded to ~330 mg intake of EGCg in humans, assuming a body weight of 60 kg. In general, green tea contains 200–700 mg/L EGCg. Therefore, ~330 mg EGCg given to mice in drinking water corresponded to ~470 to 1600 ml of green tea per day in humans. During the experimental time period, no EGCg toxicity was observed in any of the groups.

The EGCg response seen in the transgenic mice was not exhibited in the wild-type mice. Female mice given 500 mg/kg fresh EGCg in the drinking water daily for 3 months grew at precisely the same rate as wild-type mice (Table 2). Female transgenic mice not receiving EGCg in the drinking water showed the accelerated growth rates characteristic of tNOX overexpression.

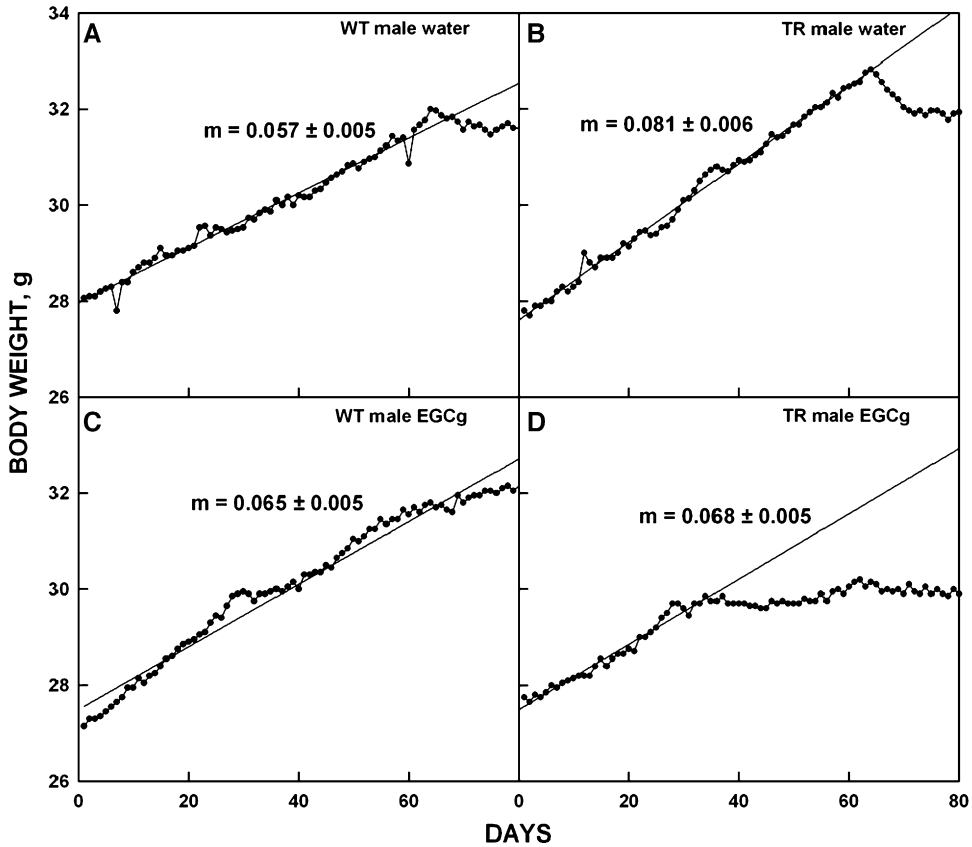

Fig. 6. Total body weight of transgenic male mice (B) compared to wild-type (A) and response to EGCg in the drinking water (C, D). Results are from three mice per treatment. Growth rates (m) in g/day were determined individually for each mouse by linear regression analysis. Growth rates of transgenic male mice in the absence of EGCg were significantly different from the growth rate of wild-type mice ($P = .007$) and from the growth rates of both transgenic and wild-type mice receiving EGCg in the drinking water ($P = .004$). Values are averages±standard deviations among the three mice. The experiment was repeated a total of three times with consistent results.

Table 3
Toxicity of EGCg comparing wild-type and tNOX transgenic mice

	EGCg toxicity (mg/kg BW)	
	Wild type	tNOX Transgenic
25 mg/kg	–	–
50 mg/kg	–	+
75 mg/kg*	–	+
100 mg/kg	+	+
125 mg/kg**	+	+
250 mg/kg	+	+

–, No toxicity observed; +, animals showed discomfort or dead in 24 h. The dose to achieve discomfort or death within 24 h was decreased from 100 to 50 mg/kg BW EGCg with the transgenic animals.

* EC50 for 24-h survival of transgenic animals.

** EC50 for 24-h survival of wild-type animals.

With male transgenic mice, the response to EGCg differed from that of female transgenic mice (Fig. 6). Male transgenic mice grew at an accelerated rate (1.4-fold) between 30 and 60 days of treatment in the absence of EGCg (Fig. 6B). In male transgenic mice receiving EGCg in the drinking water, the growth ceased abruptly after the first 30 days (Fig. 6D). The growth of wild-type mice with both male (Fig. 6A,C) and female wild-type mice was unaffected by EGCg in the drinking water (Table 2).

3.8. Acute toxicity evaluations

When an amount of 150 or 250 mg/kg BW was injected intraperitoneally, EGCg was toxic to both transgenic and wild-type mice (Table 3). Mice from both groups were dead within 24 h. The EC_{50} for 24-h survival of wild-type mice was about 125 mg/kg BW of EGCg, but even at 100 mg/kg BW the animals died within 48 to 72 h. The transgenic mice were only somewhat more susceptible to the administration of EGCg. The EC_{50} for 24-h survival was about 75 mg/kg BW. At 25 mg/kg BW of EGCg, neither transgenic nor wild-type mice showed evidence of toxicity or discomfort.

4. Discussion

Results with tNOX (GenBank Accession No. AF207881) overexpression in noncancer cell lines and results with antisense in cancer cell lines have been consistent with the 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 cell lines [3]. The results of experiments reported here where transgenic mice overexpressing tNOX were examined for overall growth phenotype and response to EGCg extend those results. The deduced amino acid sequence of the tNOX protein showed homology over part of its length, with the deduced amino acid sequence of cDNA previously designated as APK1 antigen (from K357 to T610 of tNOX) (GenBank Accession No. 572094) [1].

That the tNOX transgenic mice grew at an accelerated rate up to 2-fold for females and 1.4-fold for males compared to wild-type mice is consistent with observations from tNOX transfected cells that tNOX facilitates the uncontrolled growth rate exhibited by cancer tissues and cell lines [26]. This increased growth rate correlated with the tNOX overexpression in these mice since EGCg in the drinking water completely restored growth to normal wild type growth rates. These data provided important in situ validation of tNOX as the EGCg target for the low-dose growth inhibitions observed for EGCg both in vivo and in vitro.

EGCg inhibits growth of cancer cell lines in culture [2,27–29]. Moreover, Kavanagh et al. [30] reported that female rats given green tea as their drinking fluid displayed a significant decrease in carcinogen-induced mammary tumor burden and invasiveness and significantly increased latency to first tumors. In our study, it was clear that EGCg consumption had a growth inhibitory effect against tNOX overexpression in transgenic mice. Also validated in these studies was the expectation that wild-type mice that lack tNOX would be unaffected by low doses of EGCg.

EGCg had no effect on the NADH oxidase activity of plasma membranes from liver and intestine of wild-type mice yet did inhibit the overexpressed NADH oxidase levels attributed to tNOX in transgenic mice back to basal levels. Growth of wild-type mice was unaffected by EGCg in the drinking water at the same doses as those that completely inhibited the tNOX-stimulated growth of the tNOX transgenic mice. The marked difference in response of female (return to wild type rate of growth) and male mice (abrupt cessation of growth) was not explained by our findings.

Since EGCg is easily oxidized, the EGCg in the drinking water was routinely monitored by HPLC analysis. Based on samples collected at 0, 2, 4, 6 and 24 h and stored in darkness, approximately 50% of the EGCg had degraded within 24 h. These observations were utilized in the calculation of EGCg consumption of ca. 2.5 mg/kg BW of EGCg based on 24-h water intake.

Catechin responses depend on their bioavailability. The chemical structure of catechins determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma. Also, the formation of conjugates can dramatically alter the biological properties of the circulating metabolites [31]. Kim et al. [32] showed that the level of EGCg in plasma and tissues was higher than the levels of EGC and EC, suggesting a higher bioavailability of EGCg in mice. The dose also determines the primary site of metabolism. Large doses are metabolized primarily in the liver. Small doses are metabolized by the intestinal mucosa, with the liver playing a secondary role to further modify the conjugates from the small intestine [31]. The intestinal absorption of catechins can be high. However, the plasma concentration of any individual catechins rarely exceeds 1 μM after the consumption of 10–100 mg of a single compound [33–36]. Such levels are more than sufficient to inhibit cell surface tNOX. The EC_{50} for inhibition of tNOX in

cancer cells is in the order of 5 nM, and concentrations in the order of 100 nM (10 times less than the plasma levels of 1 μM) are usually sufficient to completely inhibit the activity [2]. This makes the tNOX protein unique among various target proteins postulated to explain the anticancer responses to EGCg. The majority of targets other than tNOX require micromolar or even millimolar levels of EGCg for inhibition. Except for tNOX, none is both pancancer (all forms of cancer) and cancer specific (absent from non-cancer cells and tissues).

Transgenic technologies such as those utilized in this study have considerable value for modeling genetic disorders and answering specific questions of developmental biology [37–39]. Genetically engineered mice have been utilized to assess the importance of numerous signaling pathways potentially involved as therapeutic targets for anticancer intervention. Andrechek and Muller [40] reported that mammary epithelial expression of activated receptor tyrosine kinases such as ErbB2/neu in transgenic mice resulted in the efficient induction of metastatic mammary tumors. They addressed the possible use of tea, especially green tea, for the chemoprevention of prostate cancer based on results with the TRAMP mouse model.

Transgenic mouse studies allow transgene expression to be monitored in all cell lineages and at any stage of development. In conventional transgenic mice models, the DNA is integrated in a random fashion as a result of injection into the pronucleus of the fertilized ovum. The DNA can and does integrate anywhere in the genome, and multiple copies may integrate in a head-to-tail fashion. However, many factors affect whether or not a promoter/transgene construct will be expressed in the transgenic mice. At some chromosomal locations, transgenes can be transcriptionally silent or disturb the functions of other genes. Therefore, the phenotypic characteristic of specific transgenic models depends on the function of the transgene as well as the insertion site in the chromosome. Since our transgene was introduced into the mouse genome randomly, we presently have no information on the location of its insertion in the genome. However, functional expression is evidenced by the presence of EGCg-inhibited NADH oxidase activity in the plasma membranes isolated from liver and intestine, and in the accelerated growth rates, especially in female mice inhibited by EGCg and the presence of an immunoreactive band corresponding to the processed molecular weight of tNOX in microsomes and plasma membranes of the several tissues examined. Various targets have been proposed to explain the chemopreventative, antioxidant, antiproliferative, anticancer and pro-apoptotic actions of EGCg and green tea catechins in cancer [6]. However, target tNOX validated here emerges as being both necessary and sufficient to explain the absolute specificity of EGCg for selective inhibition in vitro of cancer growth leading to failure of cancer cells to proliferate and the induction of apoptosis, not provided by other antioxidants of equal or more potent activity.

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