# Distinct Effects of Annexin A7 and p53 on Arachidonate Lipoxygenation in Prostate Cancer Cells Involve 5-Lipoxygenase Transcription

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## Abstract

**Tumor suppressor function for Annexin A7 (ANXA7; 10q21) is based on cancer-prone phenotype in *Anxa7*(+/−) mouse and ANXA7 prognostic role in human cancers. Because ANXA7-caused liposome aggregation can be promoted by arachidonic acid (AA), we hypothesized that the phospholipid-binding tumor suppressor ANXA7 is associated with AA cascade. In a comparative study of *ANXA7* versus canonical tumor suppressor *p53* effects on AA lipoxygenation pathway in the p53-mutant and androgen-insensitive DU145 prostate cancer cells, both tumor suppressors altered gene expression of major *5-lipoxygenase* (*LOX*) and *15-LOXs*, including response to T helper 2 (Th2)-cytokine [interleukin-4 (IL-4)] and endogenous steroids (mimicked by dexamethasone). Wild-type and mutant *ANXA7* distinctly affected expression of the dexamethasone-induced *15-LOX-2* (a prostate-specific endogenous tumor suppressor) as well as the IL-4-induced *15-LOX-1*. On the other hand, wild-type *p53* restored *5-LOX* expression in DU145 to levels comparable to benign prostate epithelial cells. Using mass spectrometry of DNA affinity–enriched nuclear proteins, we detected different proteins that were bound to adjacent p53 and estrogen response elements in the *5-LOX* promoter in DU145 cells introduced with *ANXA7* versus *p53*. Sex hormone regulator 17-β hydroxysteroid dehydrogenase 4 was identified under p53 introduction, which induced the *5-LOX* expression. Meantime, nuclear proteins bound to the same *5-LOX* promoter site under introduction of *ANXA7* (that was associated with the repressed *5-LOX*) were identified as zinc finger proteins ZNF433 and Aiolos, pyrin domain–containing NALP10, and the p53-regulating DNA repair enzyme APEX1. Thus, ANXA7 and p53 can distinctly regulate LOX transcription that is potentially relevant to the AA-mediated cell growth control in tumor suppression.** (Cancer Res 2006; 66(19): 9609-16)

## Introduction

Tumor suppressor gene Annexin A7 (ANXA7) or synexin is a member of the Annexin family of calcium-dependent phospholipid-binding proteins and codes for a $Ca^{2+}$-activated GTPase. Involved in exocytotic secretion and aggregation of chromaffin granules (1), ANXA7 also causes liposome aggregation, which can be drastically promoted in the presence of arachidonic acid (AA; ref. 2). Regulating innate immunity and apoptosis, major AA oxidation pathways are intimately linked to cancer. Inhibitors of AA metabolic enzymes have shown anticarcinogenic effects *in vivo* and induced apoptosis in cancer cell lines *in vitro* (3). Lipid metabolites derived from major cyclooxygenase and lipoxygenase (LOX) pathways of AA oxidation function as critical regulators of cell survival and apoptosis in both homeostasis and carcinogenesis.

ANXA7 has also been implicated in cell proliferation as a ligand for galectin-3, interfering with its regulatory role in cell cycle progression and survival (4). Located on human chromosome 10q21, a site long hypothesized to harbor a tumor suppressor gene(s), ANXA7 exhibits many biological and genetic properties of a tumor suppressor gene and is implicated in carcinogenesis through discrete signaling pathways involving other tumor suppressors, DNA-repair and apoptosis-related genes (5, 6). Interestingly, ANXA7 protein that is predominantly distributed in cytosol can be also found in the nucleus (1) similar to the structurally homologous ANXA11 or the indicator of early apoptosis ANXA5 (4), suggesting a possible involvement of ANXA7 in transcriptional regulation.

We have recently found that a high proportion of metastatic and hormone-refractory prostate cancers in human were associated with the essential loss of ANXA7 expression (5). Meanwhile, 15-LOX metabolites were also shown to contribute to the hormone insensitivity in prostate cancer by mediating conversion of male sex hormones (7). Specifically, the prostate-specific *15-LOX-2* or *15-LOX-B* represents an endogenous prostate senescence gene of which the tumor-suppressing functions may be associated with its ability to induce cell senescence (8). Promoter activity as well as mRNA and protein levels of 15-LOX-2 with multiple splice variants were up-regulated in the serially passaged normal human prostatic epithelium, whereas all immortalized prostate epithelial and cancerous cells did not express 15-LOX-2 (8). Conversely, the counterpart 5-LOX can regulate the senescence-like cell growth arrest by promoting reactive oxygen species–dependent p53 activation (9). In addition, a 5-LOX metabolite, 5-oxoETE, can control viability of prostate cancer cells through its own G-protein–coupled receptor (10).

Thus, uncovering a possible role of the phospholipid-binding ANXA7 in the AA-mediated tumor suppression that involves transcriptional regulation of the major *5-LOX* can provide new targets in cancer therapy, especially in hormone-resistant cancers. In the current study, we showed for the first time that in the androgen-insensitive and Bax-deficient DU145 prostatic cancer cells, introduction of the wild-type (wt) *ANXA7* elicited a LOX response that was distinct from the response to a canonical tumor suppressor gene, *p53*, and was further modulated by cytokines and glucocorticoids. In particular, we showed that *p53*, but not *ANXA7*,

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is capable of inducing *5-LOX* in the p53-mutant DU145 cells. Using mass spectrometry of the DNA affinity–enriched nuclear proteins, we detected a number of proteins that were specifically bound to the p53-binding site adjacent to estrogen response elements in the *5-LOX* promoter in DU145 cells introduced with *wt ANXA7* versus *wt p53*.

## Materials and Methods

**Cell culturing, infection, and treatment.** Benign prostate epithelial cells and androgen ablation–insensitive DU145 prostatic cancer cells (American Type Culture Collection, Manassas, VA) were routinely grown at 37°C in a humidified incubator with 5% $CO_2$, prostate epithelial cells in PrEGM (Cambrex, East Rutherford, NJ) and DU145 in MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and penicillin-streptomycin (100 units/mL). Cells were seeded in flasks (75 $cm^2$) for nuclear extraction procedure and in six-well plates for RNA extraction. After replacing media the following day, subconfluent cells were infected with a panel (×4) of constructs independently expressing green fluorescent protein expression marker as well as containing ANXA7 or p53 DNA inserts: "empty" vector alone, full-length wt ANXA7, mutant ANXA7, or full-length wt p53. Adenoviral vector–based plasmids were constructed with the AdEasy System (a gift from Dr. Bert Vogelstein). Dominant-negative mutant ANXA7 construct contained triple mutations, which were intended to affect COOH-terminal residues in the Annexin repeats 2, 3, and 4 (E277→Q277, D360-E361→N360-Q361, and D435-D436→N435-N436, respectively). Uninfected parental cells were used as a control. After infection (2 hours), parental and infected cells were cultured until reaching confluence (18 hours) in the absence or presence of IL-4 (20 ng/mL), tumor necrosis factor-α (TNF-α; 20 ng/mL), or $10^{-6}$ mol/L dexamethasone (R&D Systems, Minneapolis, MN; Sigma, St. Louis, MO).

**RNA extraction, cDNA synthesis, PCR, and sequencing.** After RNA extraction (RNAqueous-4PCR Kit, Ambion, Austin, TX) from cultured cells, 2 μg of RNA were used for reverse transcription reactions (SuperScript First-Strand Synthesis System for reverse transcription-PCR; Invitrogen).

Multiplex PCR was done in a 50-μL reaction mixture containing 1 μL of reverse transcription product, AmpliTaq Gold with GeneAmp Buffer, and deoxynucleotide triphosphate mix, according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA), as well as following primer sequences: *5-LOX activating protein* (*FLAP*), forward 5′-CACGAAAGCAGGACCCAGAA-3′ and reverse 5′-CAGAGCACAGCGAGGAAAGT-3′; *5-LOX*, forward 5′-ACCATTGAGCAGATCGTGGACACGC-3′ and reverse 5′-GCAGTCCTGCTCTGTGTAGAATGGG-3′; *15-LOX-1*, forward 5′-GAGTTGACTTTGAGGTTTCGC-3′ and reverse 5′-GCCCGTCTGTCTTATAGTGG-3′; *15-LOX-2*, forward 5′-ACTACCTCCCAAAGAACTTCCCC-3′ and reverse 5′-TCCAATGCCGATGCCTGTG-3′; *LTA4 hydrolase* (*LTA4H*), forward 5′-CCACCATCCTTCCCTTAT-3′ and reverse 5′-AAACAATCGTCCGCAAAT-3′; *Bcl-x*(*L/S*), forward 5′-CAGACAGCCCCGCGGTGAATGG-3′ and reverse 5′-CTGTCGGCTGCTGCATTGTTCC-3′; *Bax*, forward 5′-CTATGCAGAATTCGGCGGTGATGGACGGGTCC-3′ and reverse 5′-GTACGTAGAATTCTCAGCCCATCTTCTTCCAGATGG-3′; and *β-actin*, forward 5′-CTGGCCGGGACCTGACTGACTACCTC-3′ and reverse 5′-AAACAAATAAAGCCATGCCAATCTCA-3′.

Primers for Bax flanked the entire coding region being capable of amplifying full-size Bax or any alternative transcripts (11). As an internal control, we used a housekeeping gene, *β-actin*. Different amplicons for *Bax* and *15-LOX-2* were purified (MinElute Gel Extraction Kit, Qiagen, Valencia, CA) and verified by sequencing (ABI Prism, nodel 3100, version 3.7).

PCR procedure and cycling conditions (annealing temperature, 58-60°C; ×30-38 cycles on GeneAmp PCR System 9600; Perkin-Elmer, Wellesley, MA) were standardized for the RNA amount and linear range of amplification. Current results represent replicate experiments (two or three) with different batches of RNA. PCR protocol composed an initial denaturation step at 94°C for 600 seconds and a final extension step at 72°C for 600 seconds. PCR products (10 μL) were mixed with 5× DNA Gel Loading Solution (Quality Biological, Gaithersburg, MD), electrophoresed at 200 V in polyacrylamide 4% to 20% Tris-borate EDTA gels (Invitrogen), and visualized by ethidium bromide staining. Using NIH ImageJ software available for public access (1.36b),[3] we assessed intensity of bands (means ± SD, arbitrary units) and did variation statistics analysis using *t* test or *t*-dist (two-tailed distribution for both and two-sample equal variance for *t* test).

**Preparation of 5-LOX promoter DNA probes.** Putative p53-binding 5′ half site (GCCC, +strand) with high core (1.0) and matrix similarity (0.942) was identified in the 5′-untranslated region (UTR) of the *5-LOX* promoter sequences using Genomatix Software (Genomatix Software GmbH, Munich, Germany). The COOH terminus of the identified p53 site included additional sites potentially relevant to estrogen receptor (GTCA, +strand) or canonical palindromic estrogen response element (GCCA, −strand). Corresponding double-stranded DNA fragment 5′-CGGCCCGCGCCA-TGCCCTCCTACACGGTCACCGTGGCCACT-3′ (which contained putative p53 and estrogen receptor/estrogen response element binding sites with flanking sequences) was synthesized with (3′ or 5′) or without biotinylation (Gene Probe Technologies, Gaithersburg, MD).

**Preparation of nuclear extracts.** Nuclear extraction from DU145 cells after infection with wt ANXA7 or wt p53 was done using the modified protocols initially described by Dignam et al. (12) and Dobi et al. (13). Cells (~$5 \times 10^7$) were resuspended in prechilled Eppendorf tubes in 1 mL of buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L 4-(2-aminoethyl)benzenesulfonylfluoride, 5 μg/mL leupeptin, 5 μg/mL aprotinin] and collected by centrifugation (1,500 × *g* for 10 minutes at 4°C). Then cells were rewashed and pelleted again (10,000 × *g* for 30 seconds). To release nuclei, cell pellets were resuspended in 300 μL of buffer A containing 30 μL of IGEPAL CA-630 nonionic detergent (Sigma), then vortexed for 15 seconds. Nuclear fractions were collected by centrifugation (10,000 × *g* for 30 seconds at 4°C). Nuclei were then dissolved in a 4-fold volume of buffer C [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 420 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L 4-(2-aminoethyl)benzenesulfonylfluoride, 5 μg/mL leupeptin, 5 μg/mL aprotinin], homogenized by pipetting, and incubated at 4°C for 45 minutes. Insoluble chromatin fraction was separated from the soluble proteins by centrifugation (10,000 × *g* for 30 seconds at 4°C). Nuclear protein extracts were then dialyzed for 3 hours through a 25,000 MW cutoff dialysis membrane against two changes of a 250-fold volume of buffer D [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 100 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 20% glycerol, 0.5 mmol/L 4-(2-aminoethyl)benzenesulfonylfluoride]. Remaining particulates were removed by centrifugation (10,000 × *g* for 30 minutes at 4°C) and the nuclear fractions were stored in aliquots (~2 μg/μL protein) at −80°C.

**DNA affinity purification of nuclear proteins.** Nuclear extracts (~400 μg) were dissolved in HEGED-0.5 buffer [50 mmol/L KCl, 10 mmol/L HEPES (pH 7.9), 27% glycerol, 1 mmol/L DTT, 1 mmol/L EDTA] containing 1 mmol/L Na-vanadate and Complete Mini Protease inhibitor (Roche Diagnostics, Indianapolis, IN). Then, 30 μg of poly(deoxyinosinic-deoxycytidylic acid) [poly(dI-dC)] as nonspecific competitor were added to the mixture in a total volume of 300 μL at 4°C. One hundred picomoles (33 μL) of gel-purified double-stranded nonbiotinylated 5-LOX promoter DNA fragment were added to the mixture as a specific competitor in the preclearing precompetition step, followed by the addition of 50 μL of 50% bead slurry of Ultra Link Immobilized Streptavidin Plus (Pierce, Rockford, IL) beads. After removing beads by brief centrifugation (750 × *g* for 30 seconds at 4°C), 10 pmol of premixed 5′- and 3′-biotinylated 5-LOX promoter DNA fragments (specific competition) were added to the transferred supernatants. After incubation on ice for 1 hour, the streptavidin beads were added to the samples for capturing biotinylated probes in head-to-head rotation at 4°C for 4 hours. After collecting the beads by a brief spin at 4°C and washing twice in 600 μL of ice-cold HEGED-0.5 buffer, the bound proteins were finally eluted with 100 μL of Tris-glycine SDS sample buffer (Invitrogen) in the presence of 50 mmol/L DTT. After denaturation at 80°C for 4 minutes and brief centrifugation (750 × *g* for 30 seconds), transferred supernatant samples (20 μL) were loaded into a 4% to 12% NuPAGE gel along with See-Blue Plus2 and Mark12 markers and electrophoresed at 200 V for ~40 minutes. Gel was

[3] http://rsb.info.nih.gov/ij/.

stained overnight using Colloidal Blue Stain Kit (Invitrogen) and then destained in Millipore distilled water for 4 hours at 24°C.

**Identification of proteins by mass spectrometry.** Corresponding to the competed-out by the specific DNA probe bands (which were visualized in nonspecific competition) were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). First, protein bands were excised from the gel and subjected to in-gel trypsin digestion. For analysis on the ThermoFinnigan LCQ (ThermoFinnigan, San Jose, CA) electrospray ion trap, digests were loaded onto a nano-liquid chromatography programmed to elute peptides by ramping a linear gradient from 5% to 60% solvent B in 60 minutes (mobile phase A: 95% water, 5% acetonitrile, 0.1% formic acid; mobile phase B: 5% water, 95% acetonitrile, 0.1% formic acid) at a flow rate of 200 nL/min. Four mass scans were processed as the gradient progressed: first full mass scan was followed by three tandem mass scans for three intense ions. Eluted peptides were quantified using the peak intensities of the mass spectral signals and analyzed with Sequest protein identification algorithm (ThermoFinnigan). For additional analysis on Voyager DE STR MALDI-TOF Workstation (Applied Biosystems), the peptides were mixed with matrix, dried onto the target plate, and ablated by laser for measurement of mass to four decimal places. Tandem mass spectra were analyzed for a best fit with the Agilent-supported human database (Agilent Technologies, Palo Alto, CA).

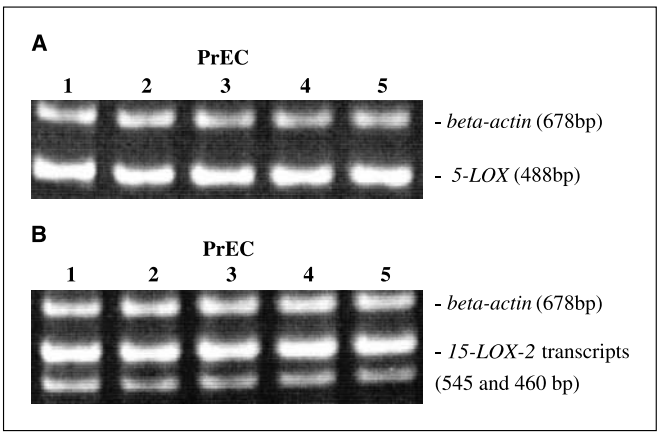


**Figure 2.** Comparison of gene expression profiles of a major *5-LOX* in response to ANXA7 or p53 in DU145 prostate cancer cells versus benign prostate epithelial cells. After seeding in six-well plates, the following day, subconfluent prostate epithelial cells and DU145 cells were infected with a panel of constructs containing ANXA7 or p53 DNA inserts. Parental and infected cells were further cultured (18 hours after infection) with no additional treatment. Each cell line set (×5) included parental uninfected cells (*1*) as a control as well as cells infected with vector alone (*2*), wt-*ANXA7* (*3*), mutant *ANXA7-J* (*4*), and wt-*p53* (*5*).

## Results

**Gene expression of the AA lipoxygenation–associated apoptotic regulators Bax and Bcl-x showed no response to ANXA7 or p53 in the Bax-deficient DU145 prostate cancer cells.** Several studies have shown that arachidonate oxidation is involved in the Bax/Bcl-x–mediated regulation of cell survival (14–16). To elucidate potential LOX-associated mechanisms of the ANXA7-promoted tumor suppression, we first examined the effects of ANXA7 or the well-known tumor suppressor *p53* gene on the transcription of major apoptotic regulators *Bax* and *Bcl-x* in subconfluent prostate cancer cells (DU145) in comparison with benign prostate epithelial cells. Unlike prostate epithelial cells with the full-length *Bax* intact, the DU145 cells aberrantly expressed two alternatively spliced *Bax* transcripts missing exon 3 alone or in conjunction with exon 2 (Fig. 1*A*). On the contrary, benign prostate epithelial cells and cancerous DU145 cells displayed similarly higher expression for a long *Bcl-xL* isoform, whereas the proapototic *Bcl-xS* was barely detectable (Fig. 1*B*). However, *Bax* and *Bcl-x* transcription in either benign prostate epithelial cells or cancerous DU145 cells did not respond to *ANXA7* or *p53* (Fig. 1*A* and *B*). These results suggest that to initiate cancer cell elimination, tumor suppressors ANXA7 and p53 may employ alternate routes to reinstate the AA oxidation–associated cell growth control.

**Differential ANXA7 and p53 effects on the basal and Th1/Th2 cytokine–induced AA lipoxygenation profile in the DU145 prostate cancer cells compared with benign prostate epithelial cells.** Because AA lipoxygenation metabolites are intimately linked to the cell survival in carcinogenesis, we examined basal as well as ANXA7- or p53-induced gene expression profiles of the major *5-LOX* with its counterpart *15-LOXs* (the prostate-specific *15-LOX-2* as well as the leukocyte-type *15-LOX-1*), the 5-LOX-activating upstream *FLAP*, and the downstream *LTA4H*. Dysregulation in the balance between type Th1 and type Th2 cytokines is implicated in carcinogenesis, including the development of prostate cancer (17). In particular, IL-4 stimulation may protect prostate tumor cells from CD95 and chemotherapy-induced apoptosis (18), whereas targeting IL-4 receptor may have a therapeutic potential in prostate cancer (19). Taking into account a role of LOXs in the cancer-related apoptosis and inflammatory response, we examined ANXA7 or p53 effects on LOX expression profile under Th1 (TNF-α) or Th2 (IL-4) cytokines as well as $10^{-6}$ mol/L dexamethasone that mimicked physiologic levels of endogenous glucocorticoids.

Benign untreated prostate epithelial cells expressed abundant levels of *5-LOX* (Fig. 2*A*), which controls formation of lipid mediators affecting cell survival (20) as well as both major

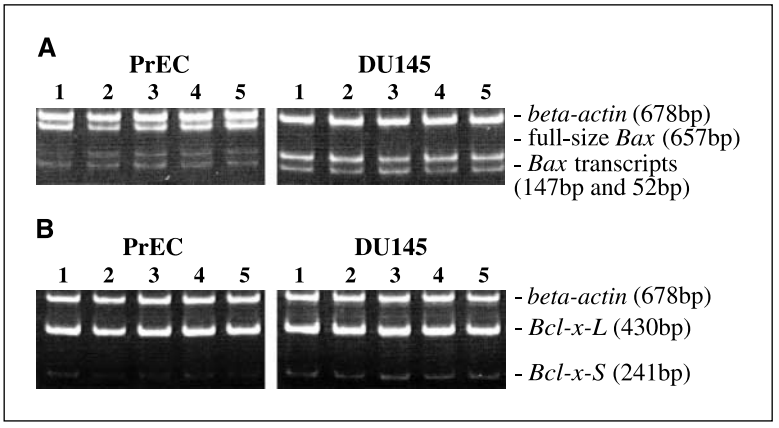


**Figure 1.** Transcriptional profile of major apoptotic regulators *Bax* and *Bcl-x* in DU145 prostate cancer cells compared with benign prostate epithelial cells (*PrEC*). After seeding in six-well plates, subconfluent prostate epithelial cells and DU145 cells were infected with a panel of constructs containing ANXA7 or p53 DNA inserts. Parental and infected cells were further cultured (18 hours after infection) with no additional treatment. Each cell line set (×5) included parental uninfected cells (*1*) as a control as well as cells infected with vector alone (*2*), *wt-ANXA7* (*3*), mutant *ANXA7-J* (*4*), and wt-*p53* (*5*).

transcripts of *15-LOX-2* (Fig. 2*B*), an endogenous prostate senescence gene with tumor-suppressing functions (8). On the contrary, the androgen-insensitive and p53-mutant (21) DU145 prostate cancer cells had reduced basal levels of *15-LOX-2* (Fig. 3*A*), which were up-regulated by dexamethasone (*t* test, 0.00214). Introduction of the mutant ANXA7 resulted in highest levels and of the wt ANXA7 in lowest levels of dexamethasone-induced *15-LOX-2* (*T*-dist, 2.07942e−18). Similarly, the wt ANXA7 (*T*-dist, 3.58409e−05), but not mutant ANXA7, reduced the Th2 cytokine IL-4–mediated increase (*t* test, 0.0436) of an initially barely detectable *15-LOX-1* expression in DU145 cells (Fig. 3*B*). Although 15-LOX-1 can mediate Bax accumulation and cell cycle arrest (16), positively correlated expression of 15-LOX-1 and mutant p53 was associated with the advanced malignancy degree in human prostate adenocarcinoma (22).

In addition to the loss of *15-LOX-2* expression, DU145 prostatic cancer cells did not express detectable levels of *5-LOX* (Fig. 3*C*), indicating a deep inactivation of AA lipoxygenation in general. Introduction of *p53* (but neither wt or mutant *ANXA7*) induced the *5-LOX* expression in cancerous DU145 (Fig. 3*C*) to the levels comparable to benign prostate epithelial cells (Fig. 2*A*). These results suggest alternative mechanisms of *5-LOX* transcriptional activation that was not accompanied by the upstream *FLAP* up-regulation (Fig. 3*C*). Although Th1-cytokine TNF-α is known to induce FLAP in some cells (23), both *5-LOX* and *FLAP* expression in DU145 did not change essentially under TNF-α treatment. Meantime, the *p53*-introduced cells maintained the *5-LOX* up-regulation under TNF-α, which alone can fail to induce apoptosis in prostate cancer cells including DU145 (24). Remarkably, canonical tumor suppressor *p53* did not affect the gene expression of *LTA4H* (Fig. 3*C*), a LOX-enzyme responsible for formation of leukotriene B4, which could delay apoptosis and enhance proliferation of malignant cells (25, 26).

Our results indicate that unlike *p53* restoring *5-LOX* expression in malignant cells (DU145) to normal levels (as in benign prostate epithelial cells), ANXA7 does not essentially affect the basal expression of 5-LOX or 15-LOX. On the other hand, ANXA7 can attenuate Th2-type responses from LOX pathway (*15-LOX-1* and *15-LOX-2*) potentially contributing to ANXA7 tumor suppressor effects.

**Distinct effects of ANXA7 and p53 on the transcriptional regulation of the major 5-LOX in DU145 prostatic cancer cells.** Because cell growth and survival in cancer are coregulated by 5-LOX-mediated mechanisms, the *5-LOX* transcriptional control can contribute to ANXA7 and p53 tumor suppressor effects. Our recent studies suggest that ANXA7 may regulate tumor suppression and progression. Although p53 is the primary driver of cell growth control, other proteins associated with the p53-binding site in the 5-LOX promoter may have a capacity to modulate the 5-LOX-mediated cell proliferation. Therefore, our goal has been to identify the transcriptional mechanism by which p53 activates 5-LOX and presumably controls the 5-LOX-mediated

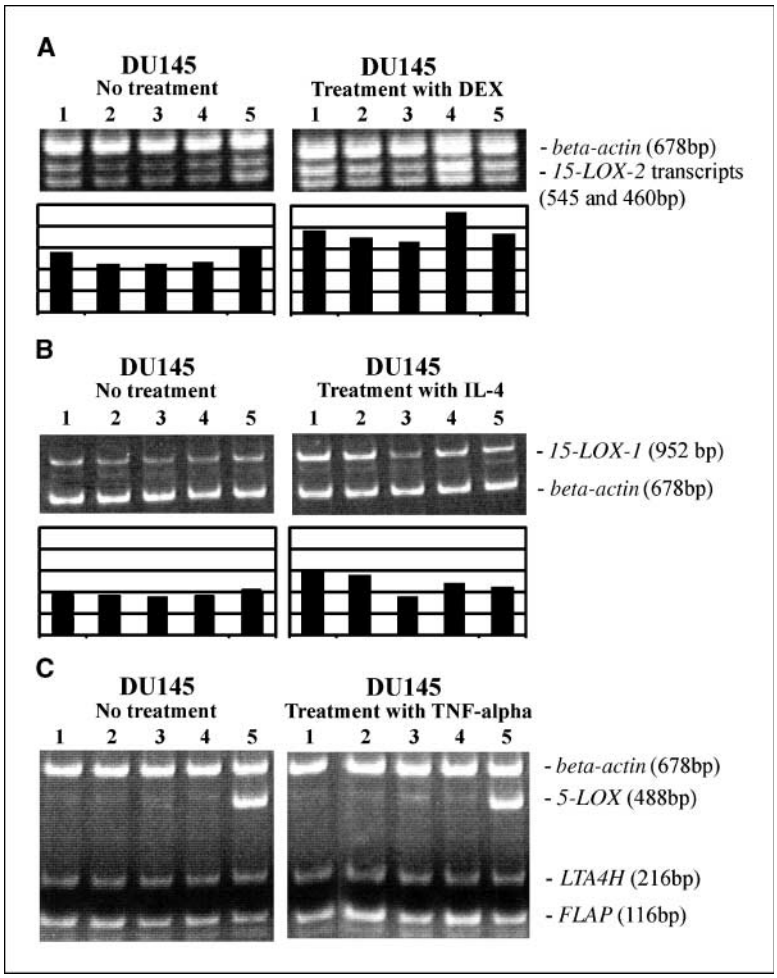


**Figure 3.** Comparison of Th1 and Th2 cytokine– as well as dexamethasone-induced gene expression profiles of major LOX enzymes under introduction of *ANXA7* or *p53* in the DU145 prostate cancer cells. After seeding in six-well plates, the following day, subconfluent prostate epithelial cells and DU145 cells were infected with a panel of constructs containing ANXA7 or p53 DNA inserts. Parental and infected cells were further cultured (18 hours after infection) in the absence or presence of IL-4 (20 ng/mL), TNF-α (20 ng/mL), or $10^{-6}$ mol/L dexamethasone (*DEX*). Each cell line set (×5) included parental uninfected cells (*1*) as a control as well as cells infected with vector alone (*2*), wt-*ANXA7* (*3*), mutant *ANXA7-J* (*4*), and wt-*p53* (*5*). Columns on the graphs under gel images represent corresponding bands for *15-LOX-2* (both bands) and *15-LOX-1*. Band intensity was assessed using the NIH ImageJ software, measured in arbitrary units and presented as means on the same scale (up to 100 in both graphs).

cell growth and survival in prostate cancer cells. Our approach has been to identify ensemble of nuclear proteins binding to the 5-LOX promoter (the 5-LOX "promoterome") in prostate cancer cells to determine which of these proteins might be responsible for the activation of *5-LOX* transcription and thereby modulation of cell growth and survival in cancer. As a rationale, such an approach may identify the dysfunctional upstream signaling pathways in prostate cancer cells and thereby generate novel molecular or pharmaceutical targets for cancer therapy. Part of the answer may be provided by proteins affecting the *5-LOX* promoter activity and expression.

Thus, through modulation of nuclear proteins at a potential binding site of p53 (a major transcriptional regulator in tumorigenesis) in the *5-LOX* promoter, p53 and ANXA7 may regulate both tumor suppression and progression.

**DNA affinity enrichment based isolation of multiprotein complexes bound to 5-LOX promoter in the presence of ANXA7 or p53.** Using the Genomatix Software to analyze human *5-LOX* promoter sequences for a putative p53-binding site, we found a p53 5′ half site (+strand) with high core and matrix similarity in the 5′-UTR sequences (−6 to −9 relative to the transcription initiation site). With an anchor position of 24 bp COOH-terminal from the p53-site anchor, we also identified binding elements relevant to female sex hormone responsiveness (estrogen receptor or estrogen response element).

To assess transcriptional modules and binding protein interactions responsible for the distinct *5-LOX* promoter activation effects of p53, we compared ensembles of nuclear proteins that specifically bound to the *5-LOX* promoter in DU145 cells overexpressing ANXA7 or p53. For the DNA affinity enrichment experiment, we first synthesized a double-stranded biotinylated DNA fragment (41 bp) containing the identified p53 and estrogen receptor/estrogen response elements with flanking sequences. Nuclear extracts from the DU145 prostate cancer cells infected with either wt ANXA7 or wt p53 were incubated with the synthetic 5-LOX promoter DNA, and then the formed DNA-protein complexes were captured by streptavidin-agarose. Nonspecific binding was blocked by poly(dI-dC), whereas specific binding was tested by including or omitting the 10× nonbiotinylated synthetic 5-LOX promoter DNA. Eluted proteins were separated on SDS-polyacrylamide gel.

Visualiazed in nonspecific competition (but not present in the specific competition) bands indicated specific binding proteins from nuclear fractions of the ANXA7- or p53-overexpressing DU145 cells. Three specific bands (two in ANXA7 and one in *p53, black arrows*; Fig. 4) were revealed by the DNA affinity enrichment of nuclear proteins from DU145 cells. The bands were excised from the gel and subjected to mass spectrometry for protein identification (Table 1).

**Mass spectrometry–based identification of nuclear proteins that were bound to 5-LOX promoter in the presence of p53.** Introduction of wt *p53* to the hormone-resistant and p53-mutant DU145 cells resulted in identification of 17-β hydroxysteroid dehydrogenase 4 (17-β-HSD4; ~80-kDa band, *arrow 1*, Fig. 4; Table 1). Also known as peroxisomal multifunctional enzyme type 2, or D-bifunctional protein, 17-β-HSD4 is an enzyme that inactivates estradiol by conversion to estrone and participates in peroxisomal β-oxidation of fatty acids. With 17-β-HSD4 highly expressed in prostate and testis, differential expression of 17-β-HSD enzymes is associated with the endocrine status of prostate cancer cells and dependent pathways of estrogen metabolism (27). In addition to being a major enzyme in the biosynthesis of sex hormones, 17-β-HSD4 is implicated in LOX metabolism: mutations in this gene are associated with a developmental neurologic disorder with the increased degradation of leukotrienes (28). Nuclear envelope localization of the 17-β-HSD enzymes that are responsible for the androgen metabolism in human hyperplastic prostate may control the amount of active testosterone that can bind to the nuclear androgen receptor (29). Thus, 17-β-HSD4 may be a potential transcriptional enhancer of the *p53*-induced *5-LOX* transcription in the androgen-insensitive prostatic cancer cells.

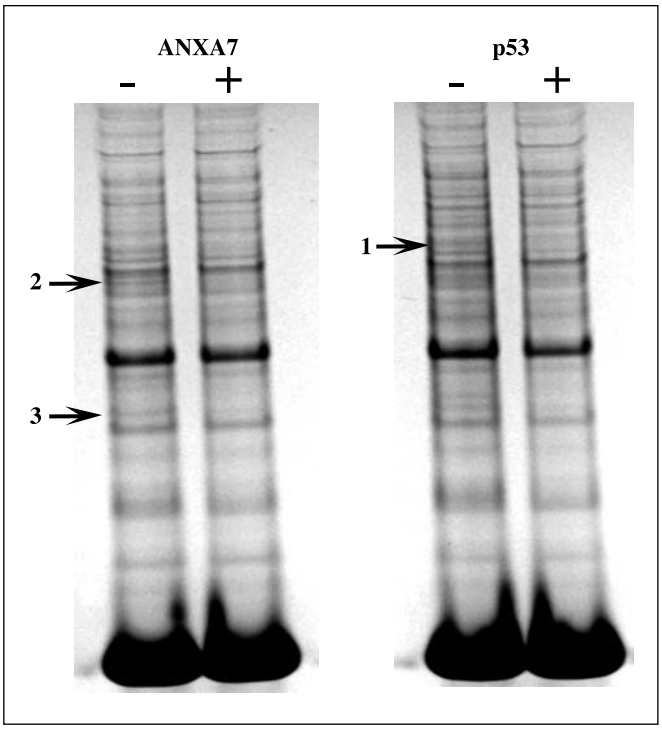


**Figure 4.** Comparison of specific DNA affinity of nuclear proteins at the 5-LOX promoter site with p53 and estrogen response elements under introduction of ANXA7 versus p53 in the androgen ablation–insensitive and p53-mutant DU145 prostate cancer cells. After seeding in 75-cm$^2$ flasks, subconfluent DU145 cells were infected with adenoviral vector–based constructs containing wt-ANXA7 or wt-p53 DNA inserts and then cultured without treatment (18 hours). ANXA7 and p53, gel images representing the DU145 prostate cancer cells infected with the wt-*ANXA7* or wt-*p53*, respectively. Signs over the lanes indicate inclusion (+) or exclusion (−) of the synthetic 5-LOX promoter site with p53 and estrogen response elements used as a specific DNA probe. Under introduction of wt-*p53* (~80-kDa band, *arrow 1*), a nuclear protein specifically bound to the 5-LOX promoter site was identified as 17-β-HSD4. Following specific nuclear proteins were identified under introduction of wt-*ANXA7* in nuclear extracts from DU145 cells [first three from a ~65-kDa band (*arrow 2*) and fourth from a ~40-kDa band (*arrow 3*)]: ZNF433. NALP10, Aiolos, and APEX1.

**Mass spectrometry–based identification of nuclear proteins that were bound to 5-LOX promoter in the presence of ANXA7.** On the other hand, proteins identified in the ANXA7-overexpressing DU145 cells suggest putative binding partners with repressor effects on the 5-LOX multiprotein regulatory complex. Under introduction of the wt *ANXA7* that did not induce *5-LOX* expression, four nuclear proteins [three from a ~65 kDa band (*arrow 2*) and a fourth from a ~40 kDa band (*arrow 3*)] were identified as specific DNA binding proteins at the 5-LOX promoter site (Fig. 4; Table 1).

ZNF433, a member of the Krueppel C2H2-type transcriptional regulator family, has been mapped to the breast and prostate

**Table 1.** Comparison of identified nuclear proteins bound to the 5-LOX promoter in DU145 cells introduced with p53 or ANXA7

| No. | Proteins identified under introduction of p53 (no. 1) or ANXA7 (nos. 2-5) | No. identified peptides | MOWSE score | % coverage (amino acids) |
|---|---|---|---|---|
| 1 | 17-β hydroxysteroid dehydrogenase [also known as D-bifunctional protein (DBP) or peroxisomal multifunctional enzyme type 2 (MFE-2)]; includes D-3-hydroxyacyl-CoA dehydratase and 3-hydroxyacyl-CoA dehydrogenase | 16 | 3.013e+08 | 27 |
| 2 | Zinc finger protein Aiolos, or ZNFN1A3 | 10 | 3.609e+04 | 26 |
| 3 | Zinc finger protein 433 | 8 | 2.176e+04 | 16 |
| 4 | NACHT, LRR, and pyrin (PYD) domain–containing protein 10 (also known as PYNOD) | 6 | 6.58e+04 | 15 |
| 5 | APEX nuclease [also known as DNA (apurinic or apyrimidinic site) lyase], AP endonuclease 1, APEN, or REF-1 protein | 4 | 4,599 | 16 |

NOTE: MALDI protein identification has been done as described in Materials and Methods. Identified proteins are compared using the number of identified peptides, sequence coverage (%) for digests, and the probability based MOWSE (MOlecular Weight SEarch) score.

cancer susceptibility locus *19p13.2* spanning *ICAM* genes (30). Another identified znf protein, transcription factor Aiolos was proposed to regulate the IL-4-mediated antipoptotic role of Bcl-xL (31). As a pyrin domain–containing member of NALP/PAN/PYPAF family, which is known to regulate nuclear factor-κB activation and induction of apoptosis (32), NALP10 may be also implicated in cancer cell elimination. APEX1, a multifunctional DNA repair enzyme 1, or redox factor 1, encodes the major DNA endonuclease (apurinic or apyrimidinic) in human cells. By repairing premutagenic lesions that can prevent normal DNA replication, APEX1 protects against cell lethality due to the DNA strand breaks generated by ionizing radiation or bleomycin. Implicated in breast and prostate tumorigenesis (33, 34), APEX1 is particularly involved in the p53 transcriptional control and p53 regulation by anticancer selenium compounds (35, 36). Identified along with transcriptional regulators (ZNF433 and Aiolos) involved in apoptosis and carcinogenesis, APEX1, a p53-regulator, may link the p53-mediated transcriptional control to the *5-LOX* repression under *ANXA7* in prostate cancer cells.

## Discussion

We showed for the first time that overexpression of *p53* directly induced *5-LOX*, whereas *ANXA7* affected the IL-4- or dexamethasone-induced gene expression of both *15-LOXs* in prostate cancerous cells. Consistent with tumor suppressor role that was previously suggested for the wt ANXA7 versus its haploinsufficiency promoting tumorigenesis in the *ANXA7*(+/−) murine model (6), in this study we identified distinct effects of wt ANXA7 versus mutant ANXA7 on arachidonate lipoxygenation potentially relevant to the regulation of cell death and proliferation in both homeostasis and carcinogenesis.

Expression and intracellular positioning of 5-LOX are determinants in the generation of LOX end products with opposing effects on cell survival (20, 37). Despite distinct patterns of *LOX* expression profile and *5-LOX* transcription in particular, ANXA7 and p53 tumor suppressor mechanisms can share a strictly constrained synthesis of powerful lipid mediators. LOX pathway can mediate the cationic liposome-induced apoptosis (38) with lipoxins involved in the nonphlogistic phagocytic clearance of apoptotic cells (39) and the inhibition of vascular endothelial growth factor–induced neoangiogenesis (40). Because liposome aggregation is induced by ANXA7 and promoted by AA, ANXA7 may potentially modulate phosphatidylserine externalization in apoptosis through the LOX pathway. Our recent studies showed that the dominant-mutant *ANXA7* (which in this study affected both *15-LOXs* distinctly from the wt *ANXA7*) failed to initiate apoptosis in DU145 cells as well as liposome aggregation in the phosphatidylserine membrane fusion assay. Thus, phospholipid-binding tumor suppressor ANXA7 can be involved in the cell proliferation and survival coregulated by endogenous glucocorticoids and lipid-relevant arachidonate cascade.

Our results of the DNA affinity enrichment–based MALDI-TOF MS analysis of nuclear proteins suggest that the *p53*- and *ANXA7*-mediated effects on LOX pathway in cancer cells may involve distinct regulatory protein complexes at the p53 and estrogen receptor/estrogen response element binding sites in the *5-LOX* promoter. Identification of a major sex hormone regulator, 17-β-HSD4, may imply sex hormone coregulation of the p53-driven *5-LOX* promoter activation. Our recent studies showed that *ANXA7* promoter activity is also affected by the female sex hormone nuclear receptors (estrogen receptor α and progesterone receptor) in coregulation with ribonucleoproteins including heterogeneous nuclear ribonucleoprotein K.

On the other hand, a heterogeneous nuclear ribonucleoprotein K–relevant Sp1/Sp3 transcriptional program may link p53 and ANXA7 through the LOX pathway. Heterogeneous nuclear ribonucleoprotein K can cause differential effects on the Sp1/Sp3-mediated transcription (41), coactivate p53 in response to DNA damage (42), as well as affect *in vitro* transcription of *15-LOX* through the LOX mRNA 3′UTR (43). In human prostate epithelial cells, transcription of a functional tumor suppressor *15-LOX-2* is differentially regulated by the Sp family, positively by Sp1 and negatively by Sp3 (44). In a proposed model of *5-LOX* transcription (45), Sp1 mediates basal levels of *5-LOX* transcription; however,

when EGR1 levels increase, Sp1 can be displaced, resulting in enhanced *5-LOX* transcription. Physically interacting with p53, the Sp transcription factors are essential for cell cycle arrest and other cellular responses to the p53 activation by genotoxic stress (46). Presenting another common link, mutant forms of p53 (which shares the same *17p13.1* locus with two *LOXs*, *12-LOX* and *15-LOX-2*) can affect the *15-LOX* promoter activity (47). On the other hand, closely located *ANXA7* (*10q21*) and *5-LOX* (*10q11.2*) may belong to tumor suppressor gene pathways affected by the 10q loss.

Consistent with a coregulatory role of Sp1/Sp3 family in p53- and LOX-relevant tumor suppressor gene pathways, we were able to identify Sp1 as a common element in the human, rat, and mouse *ANXA7* 5′ sequences using GEMS Launcher and FrameWorker[4] for promoter analysis (−500-100 bp to the transcription initiation site). Human *ANXA7* promoter sequences contained multiple Sp1 binding elements (at least four) similar to the Sp1 tandems recognized in *LTA4H* and major *LOXs* (more than 10 for *5-LOX* and *15-LOX-2*). Moreover, this matrix was incorporated in three (one of them included EGR) of four identified *ANXA7* transcriptional models (FrameWorker score, 1.00-0.75). In addition to the potential regulation of *ANXA7*, a transcriptional module reciprocally regulated by the Sp family and EGR can be implicated in the *LOX* pathway regulation (45, 48). As a compelling *in vitro* way of identifying multiprotein transcriptional modules, a novel "promoterome" approach can be used for further studies on the transcriptional dysregulation in cancer. Provided that nuclear proteins from cancer cells will bind in a sufficient amount to the promoter sequences used as a specific DNA affinity probe, they can be detected by gel electrophoresis and identified by mass spectrometry as potential coregulatory proteins in the transcription of a gene of interest, including its hormone or drug responses.

Thus, both tumor suppressors, p53 and ANXA7, can be linked to the LOX pathway that overlaps with coregulatory circuits of endogenous steroids and couples cell growth control with innate immunity. Uncovering an explicit role for the ANXA7- and p53-governed pathway in the AA-mediated tumor suppression can provide new targets in cancer therapy and in hormone resistance in prostate carcinogenesis in particular.

[4] http://www.genomatix.de.

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# Distinct Effects of Annexin A7 and p53 on Arachidonate Lipoxygenation in Prostate Cancer Cells Involve 5-Lipoxygenase Transcription

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