

# Tumor Suppression by the *Prohibitin* Gene 3'Untranslated Region RNA in Human Breast Cancer<sup>1</sup>

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## ABSTRACT

*Prohibitin* is a candidate tumor suppressor gene located on human chromosome 17q21, a region of frequent loss of heterozygosity in breast cancers. We showed previously that microinjection of RNA encoded by the *prohibitin* gene 3'untranslated region (3'UTR) blocks the G<sub>1</sub>-S transition causing cell cycle arrest in several human cancer cell lines, including MCF7. Two allelic forms (*C* versus *T*) of the *prohibitin* 3'UTR exist, and carriers of the less common variant (*T* allele) with a family history of breast cancer exhibited an increased risk of breast cancer. In the present study, we examined the tumor suppressor activity of the *prohibitin* 3'UTR in human breast cancer cells. Stable clones of MCF7 cells expressing either the *C* allele or the *T* allele RNA under the control of the cytomegalovirus promoter were isolated and compared with empty vector clones. Clones expressing the *C* allele RNA (UTR/*C*) exhibited significant suppression of growth in cell proliferation assays, inhibition of colony formation in soft agar assays, and suppression of xenograft tumor growth when implanted on nude mice, compared with either *T* allele expressing or empty vector clones. Immunohistochemical analyses with Ki67 staining confirmed a significant reduction in proliferation of UTR/*C* tumors. Thus, the *C* allele of *prohibitin* 3'UTR produces a functional RNA, whereas a single nucleotide polymorphism creates a null allele (*T* allele) of which the RNA product has lost activity. Our data demonstrate for the first time that an RNA molecule functions as a tumor suppressor in human breast cancer.

## INTRODUCTION

The cDNA coding for prohibitin was originally identified and cloned in a screen to discover senescence regulating mRNAs highly expressed in normal resting but not regenerating rat liver (1, 2). Full-length prohibitin mRNA arrested cell cycle progression between G<sub>1</sub> and S phase, and inhibited DNA synthesis. Prohibitin is an evolutionarily conserved M<sub>r</sub> 32,000 protein, the majority of which is found on the inner membrane of mitochondria (3–5). Its function remains unclear; however, roles in diverse processes such as cellular aging in yeast (6, 7), development and viability in *Drosophila* (8), granulosa cell proliferation in mammals (9), and the *ras* signaling pathway in yeast (6), trypanosomes (10), and *Pneumocystis* (11) have been reported. Although very little prohibitin protein is found in the nucleus, it has been reported to bind the retinoblastoma protein and function as a novel regulator of E2F activity (12, 13).

The human homologue of rat *prohibitin* was localized distal to *BRCA1* on chromosome 17q21 (14), in a region that frequently undergoes loss of heterozygosity in breast and ovarian cancer (15, 16). This proximity to *BRCA1* prompted a search in *prohibitin* gene

protein-coding regions for mutations in familial and sporadic breast cancers (17–19). Extensive searches failed to identify protein coding region mutations in familial cancers. Only 5 of 120 sporadic breast cancers had mutations, and these were confined to regions in or around exon 4. In addition, no mutations were found in primary tumors of the ovary, liver, lung, or bladder (19–21).

Despite the lack of mutations in protein coding regions, our studies confirmed the antiproliferative activity of full-length prohibitin transcripts in normal human diploid fibroblasts and in certain immortalized cancer cell lines (22). Subsequent functional and mutational studies characterizing the *prohibitin* gene in a panel of immortalized cancer cell lines (23) and breast cancer cell lines (24) demonstrated for the first time that the antiproliferative activity of the transcript was localized to the 3'UTR.<sup>3</sup> In agreement with previous studies, we also did not find mutations in the *prohibitin* protein-coding region; however, mutations were identified in the 3'UTR in a number of human cancer cell lines including cervical carcinoma (HeLa), glioblastoma (T98G), bladder carcinoma (J82), and transformed skin fibroblasts (GM2096SV9) in which the cell cycle progression could be inhibited by introduction of prohibitin 3'UTR (23). This correlation was observed in several human breast cancer cell lines including SK-BR-3, BT-20, and MCF7 (24). Together these results suggested that loss of function in the prohibitin 3'UTR RNA may play a role in variety cancers. More recently, we found that the change originally reported as a mutation in MCF7 cells (C→T, position 729) actually represents a human allelic variant with potential clinical significance (25). In a case-control study, *T* allele carriers that reported a first-degree relative with breast cancer were found to have a significantly increased risk of getting breast cancer before the age of 50. These studies point to the importance of developing a better understanding of the prohibitin 3'UTR. Studies on the 3'UTR of  $\alpha$ -tropomyosin (26, 27) and ribonucleotide reductase (28, 29) reinforce the concept that *trans* acting 3'UTR subfragments represent a distinct class of novel regulatory RNAs (riboregulators) with important roles in controlling growth, differentiation, and tumor suppression.

Clearly, the regulatory RNA encoded by the prohibitin 3'UTR functions as a cell cycle inhibitor and is a candidate tumor suppressor, the inactivation of which plays an important role in the pathogenesis of human cancer, especially breast cancer. In the present study, we have investigated the tumor suppressor activity of the prohibitin 3'UTR in human breast cancer using the MCF7 cell line as a model system. Stable clones of MCF7 cells expressing either the *C* allele or the *T* allele RNA under the control of the cytomegalovirus promoter were isolated and compared with EV clones. Our results show that MCF7 clones expressing the prohibitin 3'UTR (*C* allele) transgene exhibited significantly reduced proliferation and tumorigenic potential in both *in vitro* (soft agar) and *in vivo* (growth in nude mice) tumorigenicity assays compared with either *T* allele or EV clones. Taken together, the data provide fundamental proof that the prohibitin

Received 1/15/03; revised 4/28/03; accepted 6/9/03.

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<sup>1</sup> Supported by funding from the United States Army Breast Cancer Research Program (DAMD17-99-1-9343), American Cancer Society (RPG-97-167-01-MGO), Oklahoma Center for the Advancement of Science and Technology (HN5-022, HR99-068), Presbyterian Health Foundation of Oklahoma City (to E. R. J.), InterGenetics, Inc. (E. R. J., S. M.), and Veterans Affairs Merit Review (to D. J. B.).

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<sup>3</sup> The abbreviations used are: UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; UTR/*C*, *C* allele 3' untranslated region; EV, empty vector; UTR/*T*, *T* allele 3' untranslated region.

3'UTR regulatory RNA molecule functions as tumor suppressor in human breast cancer.

## MATERIALS AND METHODS

**Cells and Cell Culture.** We chose to develop a model system using the breast cancer cell line MCF7 to characterize the tumor suppressor activity of the prohibitin 3'UTR for the following reasons: (a) MCF7 is a well-established human breast cancer cell line that is widely used for the study of human breast cancer; (b) microinjection of prohibitin 3'UTR C allele into MCF7 cells was able to effectively inhibit the G<sub>1</sub>-S phase transition; and (c) MCF7 cells carry a single nucleotide change in the 3'UTR (nucleotide 729) that leads to loss of antiproliferative activity. MCF7 cells were obtained from American Type Culture Collection (Gaithersburg, MD). Cells were cultured in DMEM (Fisher Scientific) plus 10% fetal bovine serum (HyClone, Logan, UT) and 4 mM L-glutamine (Sigma, St. Louis, MO), and maintained by weekly passage. After transfection, stable clones were isolated and maintained in the same medium with the addition of 1 mg/ml G418 (Life Technologies, Inc., Rockville, MD).

**Construction of Vectors.** The DNA coding for the 852 base subfragment (23, 24) of prohibitin C allele 3'UTR RNA (UTR/C) was PCR amplified from normal human diploid fibroblasts (CF3) and cloned into the *XhoI/XbaI* sites of the expression vector pCR3.1 (Invitrogen, Carlsbad, CA) under the control of the cytomegalovirus promoter. The DNA coding for the same subfragment except for a single alteration (C to T change at nt729, nt1630 in full-length transcript; UTR/T) was isolated from a human breast carcinoma obtained from the Cooperative Human Tissue Network (Cleveland, OH) and similarly cloned into the pCR3.1 vector. These DNAs were tagged with the T7 promoter sequence to provide a unique marker for detecting expression of the transgene in the presence of endogenous 3'UTR transcript.

**Generation and Isolation of Stable Clones.** For transfection, exponentially growing MCF7 cells ( $6 \times 10^5$ ) were seeded into 10-cm dishes and allowed to grow overnight. The UTR/C, EV, or UTR/T plasmids were transfected into MCF7 cells using TransIT LT1 reagent (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Clones were selected in the presence of 1 mg/ml G418 (Life Technologies, Inc.) for 2 weeks and expanded. DNA was isolated from the clones using the PureGene kit (Gentra Systems, Minneapolis, MN). The presence of the 3'UTR transgene in DNA of MCF-UTR clones was confirmed by PCR using the conditions described below. Clones that carried the 3'UTR transgene by the DNA-PCR screen were selected for analysis of transgene expression.

**RT-PCR.** RNA was prepared using the Atlas RNA purification kit (Clontech, Palo Alto, CA), and the integrity was checked by agarose gel electrophoresis. The expression of the transgene transcript was confirmed by two-step RT-PCR with 2  $\mu$ g RNA using the ThermoScript RT-PCR kit (Life Technologies, Inc.) according to the manufacturer's instructions. One-tenth of the generated cDNA was used for PCR amplification using gene-specific primers under the following conditions: denaturation at 94°C for 1 min followed by 35 cycles of 94°C, 58°C, and 72°C for 1 min each in a Perkin-Elmer 9600 Thermocycler. For detection of the 3'UTR transgene (both UTR/C and UTR/T clones), a 395-bp product was amplified using the T7 sequence (Sense: 5'-TAATACGACTCACTATAGGG-3') and a 3'UTR-specific sequence (Antisense: 5'-CTCCAGCAGGCCCCGAATTG-3') as primers. GAPDH RNA was amplified as a control using the primers (Sense: 5'-ACCACAGTCCATGCATCAC-3' and Antisense: 5'-TCCACCACCCTGTTGCTGTA-3') to produce a 452-bp product. Relative amounts of the 3'UTR transgene were determined in the clones by removing aliquots of the PCR reaction from each sample at 3-cycle intervals in the linear range (cycles 20–30) of the reaction. These were run on a gel, and the band intensities for both 3'UTR and GAPDH were measured by video densitometry using a video imaging system (Fotodyne, Inc., Hartland, WI).

**Cell Proliferation Assays.** Growth of clones from each construct was measured using the CellTiter96 cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. The assay measures the conversion of a novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium, to a soluble formazan product by metabolically active cells. The quantity of formazan product, as measured by the amount of 570-nm absorbance, is directly proportional to the number of living cells in culture. Briefly, exponentially growing cultures of

each clone were seeded (2000 cells/well) in triplicate for each time point in 96-well plates in complete medium and allowed to attach overnight. Cells were fed with fresh medium every 2 days. For each time point, cells were incubated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent at 37°C for 3 h, and the absorbance was read at 570 nm (with a reference absorbance at 690 nm) in a  $\mu$ Quant plate reader (BioTek Instruments Inc., Winooski, VT).

**In Vitro Tumorigenicity Assays.** EV, UTR/C clones, and UTR/T clones were subjected to soft agar assays essentially as described elsewhere (30) with minor modifications as established in our laboratory. Briefly, 0.5% agar in medium was layered on the bottom of a six-well plate (Corning Costar, Corning, NY). Each clone was plated on top of this layer in triplicate at 3000 cells/well in 0.3% agar-medium. Cells were fed every 3 days for 2 weeks, and colonies having >50 cells were counted as positive under a phase contrast microscope between days 18 and 21.

**Growth of MCF7 Xenografts in Nude Mice.** To measure *in vivo* tumorigenicity, 5-week-old female NCR homozygous nude mice (Taconic Farms, Germantown, NY) were implanted with a sustained release, estrogen pellet (17 $\beta$ -estradiol; 0.5 mg; Innovative Research of America, Toledo, OH), 48 h before cell line transfer. Clones ( $5 \times 10^6$  cells/mouse) were mixed (1:1, v/v) with Matrigel (Becton Dickinson, San Jose, CA) and injected s.c. in the flank as described (31). Tumor xenografts were measured weekly beginning at day 21 using digital calipers. Tumor volume was calculated using the formula  $4/3\pi R_1 \times R_2 \times R_3$ . All of the animal studies were performed in accordance with Institutional Animal Care and Use Committee guidelines and approved by the Oklahoma Medical Research Foundation Review Board.

**Immunohistochemistry.** At the end of the experiment animals were euthanized, and tumors were excised and weighed. Tumors were fixed in 10% formalin and paraffin embedded for immunohistochemical analyses using standard procedures. Five- $\mu$ m sections of each tumor were subjected to H&E staining. The slides were first scanned under low power in a Polaroid Sprint Scan35 to show differences in tumor size, and then high power photomicrographs ( $\times 40$  magnification) were taken using an Olympus Microscope. In addition, sections from each tumor were also subjected to antigen retrieval procedures and stained with Ki67 antibody (predilute; Zymed, South San Francisco, CA) as described by the manufacturer, followed by detection with 3, 3'-diaminobenzidine (Invitrogen). Unlabeled cells were counterstained with methyl green. Photographs were taken at  $\times 40$  magnification on a BLISS imaging system (Bachus Laboratories, Evanston, IL), and the Ki67 labeling index was determined as the percentage of the total cells labeled.

## RESULTS

### Expression of Prohibitin 3'UTR Transgenes in MCF7 Cells.

The tumor suppressor potential of prohibitin RNA in human breast cancer was investigated by determining whether expression of exogenous 3'UTR C allele would inhibit the transformed, tumorigenic phenotype of MCF7 cells. MCF7 cells were transfected with plasmids containing UTR/C, UTR/T, and the EV plasmid. Transfectants were selected in G418, and 10–12 clones of each construct were screened for expression of transgene using RT-PCR. Fig. 1a shows the expression of the 3'UTR in representative clones, UTR/C 2–1, UTR/C 2–2, UTR/C 2–4, UTR/C 2–5, and UTR/C 2–8. A 395-bp fragment, which includes the T7 sequence, is amplified in the positive clones and in the plasmid positive control. As expected, the EV clone EV3, used as a negative control, did not amplify a 3'UTR sequence. Similarly, Fig. 1b shows the expression in representative clones expressing the variant allele of the human 3'UTR (UTR/T). Clones UTR/T 2–4, UTR/T 2–5, UTR/T 2–14, UTR/T 2–15, and UTR/T 2–17 were positive for expression of the UTR/T transcript. We next determined the level of 3'UTR transgene expression in the positive clones relative to endogenous GAPDH expression. Fig. 1c summarizes these data. Clones (UTR/C 2–2, UTR/C 2–8, UTR/T 2–5, and UTR/T 2–14) that showed high levels of transgene expression, along with two EV clones (EV3 and EV4), were used for all of the additional experiments described in this study.

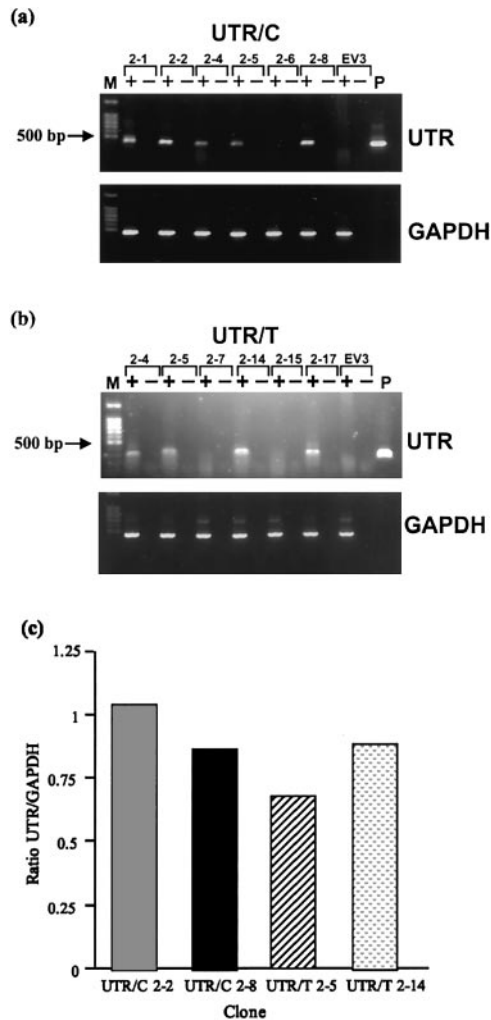


Fig. 1. RT-PCR analysis of 3'UTR expression in stable transfectants. Two  $\mu$ g RNA from UTR/C, UTR/T, and an EV clone (EV3) was subjected to RT-PCR analysis using the ThermoScript RT kit and run on 1% agarose gels. *a*, analysis of C allele transcript levels (top) in 6 isolated UTR/C clones along with GAPDH transcript levels (bottom) in 6 isolated UTR/C clones along with GAPDH transcript levels (bottom) in 6 different clones. M, marker lane loaded with 100-bp ladder; P, plasmid (used as a positive control) and subjected to PCR using the same primers as the clones. The  $\pm$  refers to with and without reverse transcription, respectively. *c*, relative amounts of 3'UTR transgene in UTR/C and UTR/T clones determined by RT-PCR. PCR reactions were set up with cDNA from each clone (prepared in *a* and *b* above) and analyzed as outlined in "Materials and Methods." The data are represented as the ratio of relative amounts (arbitrary units) of 3'UTR to GAPDH for each clone.

#### Effect of 3'UTR Expression on Growth of MCF7 Cells *in Vitro*.

We examined the growth of UTR/C, UTR/T, and EV clones in 10% serum using the Cell Titer96 cell proliferation assay. This is a colorimetric assay for determining the number of viable cells in proliferation or cytotoxicity assays, and has been used for the measurement of cell growth and viability in several studies (32–34). The assay was performed first on the day of seeding (day 0) to ensure uniformity in cell plating. Thereafter, cell growth was measured every 2 days beginning day 1 up to day 7. Fig. 2 shows that both UTR/C clones grew significantly slower than either UTR/T or EV clones.

**Suppression of Tumorigenicity *in Vitro*.** One of the hallmarks of cellular transformation and tumorigenicity is the ability to form colonies in soft agar (30). Fig. 3 shows the results from a series of soft agar assays done on these clones. The clones expressing the 3'UTR C allele (UTR/C 2–2 and UTR/C 2–8) show a marked reduction (75–90%) in the number of colonies formed as compared with the EV clones EV3 and EV4. In contrast, the two UTR/T clones (UTR/T 2–5

and 2–14) formed similar numbers of colonies as the EV clones. In addition, EV clones formed colonies comparable with the parent MCF7 cell line (data not shown). These results show that expression of 3'UTR C allele RNA inhibited the transformed phenotype of MCF7 breast cancer cells and that a single nucleotide (C→T) polymorphism produces a functionally compromised RNA that is not capable of inhibiting the tumorigenic phenotype.

**Suppression of Tumor Growth in Nude Mice.** To determine whether the results observed *in vitro* could be translated *in vivo*, we transplanted 1 EV clone (EV3), 1 UTR C allele clone (UTR/C 2–2), and 1 UTR T allele clone (UTR/T 2–5) onto athymic nude mice. Palpable tumor xenografts generally emerged at 21 days after inoculation, and tumor volumes were measured weekly until day 63 (duration of the estrogen pellet). Fig. 4A shows that the mice injected with the UTR/C 2–2 clone formed significantly smaller tumors compared with those formed by the UTR/T 2–5 or EV3 clones. A second xenograft study was conducted to determine whether the prohibitin

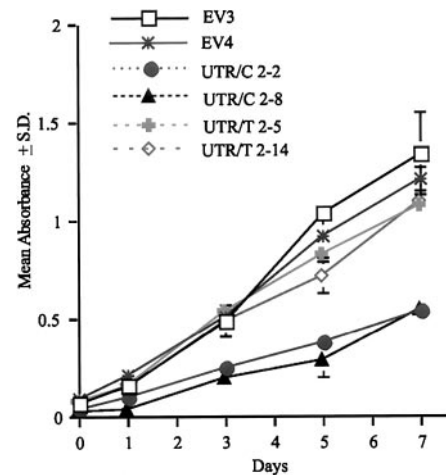


Fig. 2. Growth of clones expressing prohibitin 3'UTR. Two clones of each construct, 3'UTR C allele overexpressing clones (UTR/C 2–2 and UTR/C 2–8), clones overexpressing 3'UTR T allele (UTR/T 2–5 and UTR/T 2–14), and two EV clones (EV3 and EV4) were seeded in triplicate at a density of 2000 cells/well in 96-well plates for each time point. Cell growth was assayed at the time points indicated using the Cell Titer96 kit. The data are represented as the mean absorbance units of triplicate wells; bars,  $\pm$ SD. All of the experiments were repeated three times with similar results.

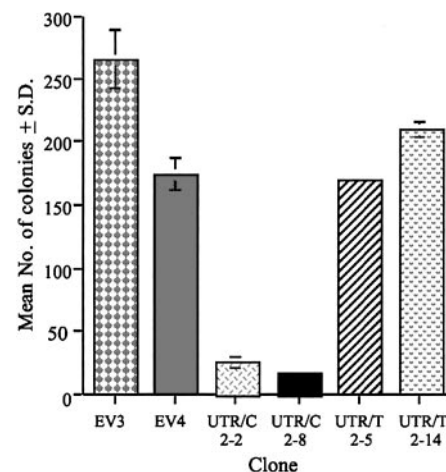


Fig. 3. Soft agar colony formation by prohibitin 3'UTR clones. EV clones (EV3 and EV4), UTR/C clones (UTR/C 2–2 and UTR/C 2–8), and UTR/T clones (UTR/T 2–5 and UTR/T 2–14) were subjected to soft agar assays in triplicate. Colonies having >50 cells were counted between days 18 and 21. The results are represented as the mean number of colonies; bars,  $\pm$ SD. The experiments were repeated at least three times with similar results.

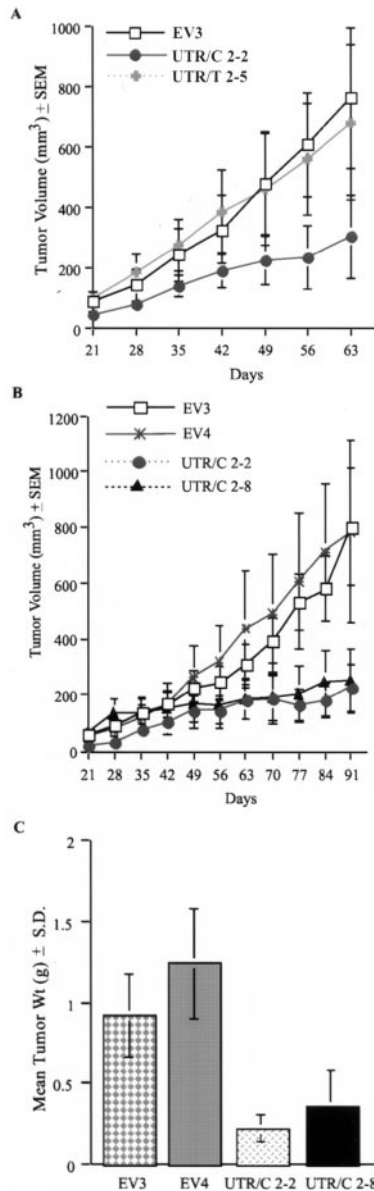


Fig. 4. Growth of clones expressing prohibitin 3'UTR on nude mice. A, one EV clone (EV3), one UTR/C clone (UTR/C 2-2), and one UTR/T clone (UTR/T 2-5) were injected separately ( $5 \times 10^6$  cells/mouse) into nude mice implanted with a sustained release estrogen pellet (17 $\beta$ -estradiol; 0.5 mg). Beginning on day 21, tumors were measured weekly for the duration of the experiment. Each time point represents the mean tumor volume  $\pm$  SE of 5 animals for each clone; bars,  $\pm$ SD. B, two UTR/C (UTR/C 2-2 and UTR/C 2-8) and two EV clones (EV3 and EV4) were injected into nude mice as outlined above. Tumor volumes were measured and are represented as the mean tumor volumes of 5 animals/group; bars,  $\pm$ SD. C, animals from the experiment in B above were euthanized at day 91, and tumors were excised and weighed. The data are represented as the mean tumor weight of each group in grams; bars,  $\pm$ SD.

3'UTR C allele could suppress tumor growth over a prolonged period of time. Two EV clones (EV3 and EV4) and 2 of the UTR/C clones (UTR/C 2-2 and UTR/C 2-8) were transplanted onto athymic NCR nude mice. Tumor xenograft growth was monitored by measuring volumes as described above. On day 60, animals were implanted with a second 60-day release estrogen pellet. The experiment was ended at day 91, and tumors were excised, weighed, fixed in 10% formalin, and paraffin embedded for immunohistochemical studies. Fig. 4B shows the tumor volumes measured in this extended experiment. Constitutive expression of the 3'UTR in both UTR/C clones UTR/C 2-2 and UTR/C 2-8 significantly inhibited the growth of tumors in nude mice even up to day 91. Fig. 4C shows that end point tumor weight in UTR/C tumors was reduced by 60–70% compared with EV tumors.

UTR/C and EV tumors from the experiment shown above (Fig. 4, B and C) were stained with H&E and Ki67 to examine the level of cell proliferation. Fig. 5 shows photomicrographs of representative tissues from one control tumor (EV4; Fig. 5, a, c, and e) and one tumor expressing *prohibitin C* allele (UTR/C 2-2; Fig. 5, b, d, and f). Fig. 5, a and b, show a low power path scan to show differences in tumor diameter. H&E staining (Fig. 5, c and d) shows that the histological features are generally similar except for the higher mitotic rate (Fig. 5, indicated by arrows) in EV4. Ki67 staining (Fig. 5, e and f) confirmed a higher number of labeled cells in EV4. Quantitative data on the percentage of Ki67 labeling observed in these tumors is summarized in Table 1. Tumors from both UTR/C 2-2 and UTR/C 2-8 clones showed an  $\sim$ 50% reduction in Ki67-positive cells as compared with EV3 or EV4 tumors. These data additionally indicate

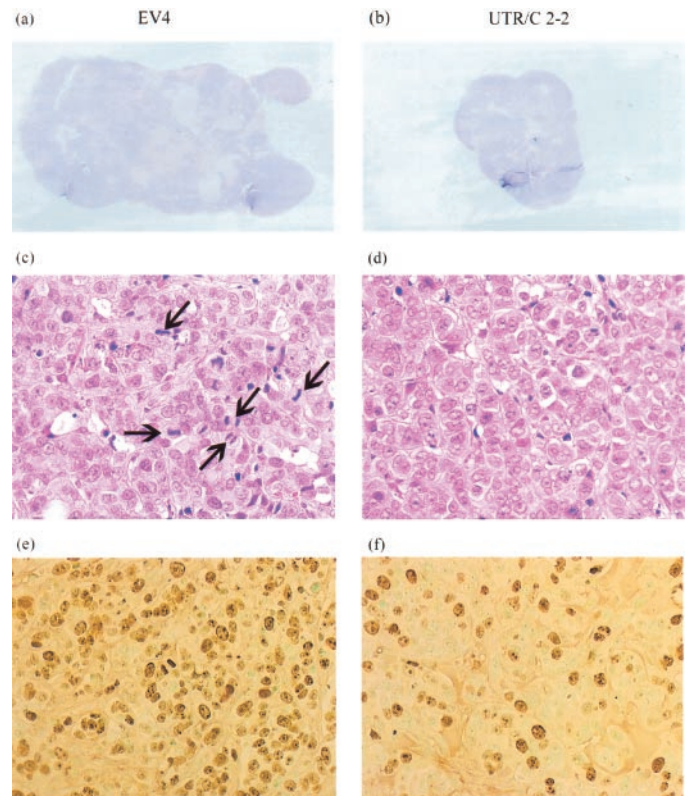


Fig. 5. Inhibition of cell proliferation in UTR/C tumors. Five- $\mu$ m sections of tumors from one EV (EV4; a, c, and e) and one UTR/C clone (UTR/C 2-2; b, d, and f), harvested from the experiment in Fig. 4, B and C, were analyzed by H&E as well as Ki67 antibody staining as described in "Materials and Methods." H&E stained sections were first scanned under low power in a Polaroid Sprint Scan35 (a and b), and then high power photomicrographs of H&E (c and d) and Ki67 stained sections (e and f) were taken under  $\times 40$  magnification using an Olympus Microscope (a–d) and a BLISS imaging system (e and f).

Table 1 Cell proliferation in UTR/C tumors by Ki67 staining

Sections stained with Ki67 in Fig. 5 were used to determine the percentage of Ki67-positive cells. Three to four randomly selected fields per slide and 300–400 cells (both Ki67-positive and -negative) per field were counted using  $\times 20$  magnification. The percentage of Ki67-positive cells was determined from the total cells counted. The percentage decrease in Ki67-positive cells in the UTR/C tumors represents the decrease in each UTR tumor compared with the average of the percentage positive cells in the two EV tumors.

Clone	% Ki67-positive cells	% decrease
EV3	62.5 $\pm$ 1.96	
EV4	66.0 $\pm$ 1.76	
UTR/C 2-2	35.0 $\pm$ 5.0	45.5
UTR/C 2-8	30.6 $\pm$ 2.74	52.4

that the prohibitin 3'UTR effectively inhibited cell proliferation of breast cancer cells *in vivo*.

## DISCUSSION

Our results clearly show that expression of prohibitin 3'UTR RNA from the *C* allele significantly inhibits the proliferation and tumorigenic phenotype of MCF7 cells. In *in vitro* assays, UTR/*C* clones exhibited reduced growth in culture and a 70–90% reduction in anchorage-independent growth in soft agar. These results were reinforced by *in vivo* studies in which UTR/*C* clones formed significantly smaller tumors when transplanted in nude mice. These tumors exhibited an ~50% reduction in cell proliferation as measured by Ki67 staining. In contrast, our results showed that the 3'UTR *T* allele produces a functionally compromised RNA product. The UTR/*T* clones were unable to suppress growth of cells in culture or suppress colony formation in soft agar. Moreover, expression of the *T* allele in UTR/*T* clones did not inhibit tumor growth in animals, because these clones formed tumors similar in size to the EV clones. Overall, the results of this study provide fundamental proof that the *C* allele of the prohibitin 3'UTR codes for an antiproliferative regulatory RNA that acts as a tumor suppressor in human breast cancer.

Our studies have recently shown that the C→T change in the prohibitin 3'UTR is a germ-line polymorphism with the *C* allele being the most common form, whereas the *T* allele is a less prevalent variant (25). A case-control study showed that carriers of the *T* allele reporting a first-degree relative (mother or sister) with breast cancer exhibit a significantly increased risk (~5-fold) of getting breast cancer before the age of 50. The inability of the *T* allele to function as a tumor suppressor in the present study provides a molecular explanation for these case-control results and reinforces the idea that inactivation of the prohibitin 3'UTR plays an important role in the pathogenesis of some breast cancers. It may also be important in the development of a number of other types of cancers (23).

The prohibitin 3'UTR is not the only RNA shown to function as a *trans*-acting regulator of biological processes. Initially, an isolated fragment of  $\alpha$ -tropomyosin, a structural muscle gene, was found to be a regulator of growth and differentiation (26). Subsequently, it was demonstrated that the normal tropomyosin 3'UTR could complement the deficiencies of a mutant muscle line unable to complete differentiation, suppress cell proliferation in 10T1/2 fibroblasts and reverse the phenotype of a transformed cell line, restoring anchorage dependence and suppressing tumor formation in mice (27). In other studies, the 3'UTR of ribonucleotide reductase, a key rate-limiting enzyme in DNA synthesis, significantly suppressed the tumorigenic properties and the metastatic phenotype of transformed fibroblasts, as well as HeLa cells (28, 29).

The mechanism by which these and other regulatory RNAs exert their biological functions is poorly understood, but some examples have been described. The  $\alpha$ -tropomyosin RNA appears to bind and activate a protein kinase that triggers inhibition of translation, including viral translation (35). Other regulatory RNAs have been described that bind to antisense RNA (36) or promoters resulting in repression of gene transcription (37). In addition, Ishizaki *et al.* (38) have isolated synthetic RNA molecules that avidly bind the E2F family of proteins to block the induction of S phase in quiescent cells. The development of the MCF7 model described here should enable us to begin to address the specific mechanism by which the prohibitin 3'UTR suppresses growth and tumor formation.

Finally, our results suggest that the prohibitin regulatory RNA might be of therapeutic value for breast cancer. In support of this idea we have found that, in a rat mammary tumor model, direct injection of prohibitin 3'UTR RNA resulted in complete regression of tumors

as well as the metastases (39). The administration of prohibitin RNA effectively controls tumor cellular proliferation *in vivo* and induces a systemic antitumor immunity in this rat model. The efficacy of the prohibitin 3'UTR RNA in this rat cancer model additionally illustrates its importance as a tumor suppressor in breast cancer. Our studies showing the cell cycle inhibitory activity of the prohibitin 3'UTR in a panel of immortalized cell lines certainly suggests that its loss of function may play a role in a number of cancers. The prohibitin RNA may represent a candidate targeted therapeutic for tumors that are genetically identifiable by mutation or polymorphism in the 3'UTR (23, 24). Taken together our data show that the prohibitin 3'UTR is a member of a novel and underappreciated class of RNA molecules that play important roles in cellular growth and that these molecules may be effective as therapeutic agents to control proliferation of cancer cells.

## ACKNOWLEDGMENTS

We thank Dr. Stan Lightfoot for examining tumor slides, Drs. Linda Thompson and Justin Van De Wiele for assisting with vector constructs, and Drs. Dittakavi S. R. Sarma, Prema Rao, John Mulvihill, Eric Howard, and Ann Thor for critical comments on the manuscript. The Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center provided oligonucleotide synthesis services.

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