# Role of Estrogen Receptor in the Regulation of Ecto-5′-Nucleotidase and Adenosine in Breast Cancer

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## ABSTRACT

***Purpose:*** **The purpose is to understand the expression of ecto-5′-nucleotidase (eN), an adenosine producing enzyme with potential roles in angiogenesis, growth, and immunosuppression, in estrogen receptor (ER)-negative and -positive breast cancer.**

***Experimental Design:*** **We investigated the regulation of eN expression at the mRNA and protein levels by α in a panel of breast cancer cell lines that differ in ER status and invasive and metastatic potential. We also determined rates of adenosine formation in cells with high and low eN expression and in ER+ cells treated with estradiol.**

***Results:*** **ER-negative cells express high eN protein and mRNA levels and produce up to 104-fold more adenosine from AMP and ATP. Estradiol and antiestrogen treatments confirm that eN mRNA and protein expression and adenosine generation are negatively regulated through the ER. Endogenous expression of eN in ER− cells transfected with ERα and phorbol ester-induced eN expression in ER+ cells was strongly suppressed by estradiol, suggesting a dominant function of ER. Finally, an examination of 18 clinical breast cancer samples that were analyzed for both ER status and eN expression by Martin *et al.* (Cancer Res., *60:* 2232–2238, 2000) revealed a significant inverse correlation between ER and eN status.**

***Conclusions:*** **Our results show for the first time that eN is negatively regulated by ERα in dominant fashion and suggests that eN expression and its generation of adenosine may relate to breast cancer progression. Additionally, increased expression of eN in a subset of ER-negative cells may serve as a novel marker for a subset of more aggressive breast carcinoma.**

## INTRODUCTION

Solid tumors frequently experience chronic hypoxia and necrosis that lead to release of adenine nucleotides. Degradation of nucleotides in the presence of an ecto-nucleotidase cascade that includes ecto-ATPase, ecto-ADPase, and ultimately ecto-5′-nucleotidase (eN, also described as CD73 or ecto-5′-NT) leads to the generation of extracellular adenosine (1–3). Adenosine, acting through G-protein coupled receptors, has been known to exert a multitude of physiological effects that are cardioprotective and cerebroprotective, including vasodilation, stimulation of angiogenesis, cytoprotection, and immunosuppression (reviewed in Ref. 2). Several of these activities, including vasodilation, angiogenesis, and growth promotion, have been reported in the context of cancer (4–8). In addition, anti-inflammatory and immunosuppressive activities of adenosine may also inhibit the immunologically mediated suppression of tumor growth. Thus, adenosine may be an important metabolite released by cancer cells that elicits physiological responses that promote tumor progression.

eN is an essential enzyme that generates extracellular adenosine from AMP. Because of the rapid metabolism of adenosine [$t_{1/2}$ of blood adenosine is $<1$ s (9)], most of its autocrine and/or paracrine effects are exerted locally and local expression of enzymes that regulate adenosine levels are critical determinants of adenosine homeostasis. However, the relationship of eN expression, activity, and adenosine generation to tumor cell biology and tumor progression has not been yet determined. Previously published data suggested increased expression of eN in breast cancer when compared with nonmalignant surrounding tissues (10–12). Our preliminary survey of a panel of cancer cell lines showed a highly variable expression of eN (2). However, more detailed analysis of breast cancer cell lines in this work reveals a striking inverse correlation of eN expression with estrogen receptor (ER) status that in turn may be predictive of a more invasive tumor phenotype, as determined in animal models (12–17). Here, we report that the ER negatively regulates the expression of eN in breast cancer cells and that the lack or loss of ER expression, which is characteristic of more advanced and aggressive disease, leads to the dramatic and specific up-regulation of eN and increased generation of extracellular adenosine.

## MATERIALS AND METHODS

**Reagents.** All common chemicals were purchased from Mallinckrodt or Sigma Chemicals (St. Louis, MO) and were of highest quality available. Antibodies against Thy-1 (sc-9163), cyclin D1 (sc-8396), and CD24 (sc-11406) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-urokinase-type plasminogen activator receptor (399R) from Ameri-

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can Diagnostica, Inc. (Greenwich, CT), and anti-β-actin from Oncogene (Boston, MA).

**Cell Lines and Culture Conditions.** All original cell lines used in this work were obtained from the American Type Culture Collection through the Tissue Culture Facility, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill. Cells were maintained in MEM supplemented with Eagle salts, sodium pyruvate, nonessential amino acids, and 10% FBS (most cell lines) in McCoy's supplemented with 15% FBS (SK-BR-3 cells), in Leibowitz L-15 supplemented with 10% FBS (MDA-MB-468 cells), and in mammary epithelial cell growth medium (MEGM) supplemented with bovine pituitary extract, human epidermal growth factor (hEGF), insulin, hydrocortisone, and 10% FBS (BioWhittaker medium for MCF-10A cells) in $CO_2/O_2$ atmosphere at 37°C. The origin of Adr2 and AdrR MCF-7 Adriamycin-resistant cell sublines was from Dr. Youcef M. Rustum (Roswell Park Cancer Institute, Buffalo, NY). ERα-expressing and vector-transfected MDA-MB-231 cells were obtained from Dr. Craig Jordan at the University of Chicago (18) and were maintained in estrogen-free conditions. Normal human mammary epithelial cells were obtained from Bio-Whittaker (Cambrex Bio Science, Walkersville, MD) and maintained in supplied complete medium for human mammary epithelial cells.

**Incubation in Estrogen-Free Conditions and Preparation of Lysates.** Switching to estrogen-free conditions required meticulous washing of attached cells in PBS (three times) and preincubation in phenol red-free and 10% double charcoal-treated FBS for 60 min to remove intracellular stores of estrogens, trypsinization, and plating on 55-$cm^2$ plates at 30% confluency. Typically, cells were incubated for 48 h in estrogen-free conditions before DMSO (vehicle, 1:10,000 dilution), estradiol, or antiestrogens were added, as indicated in figure legends. All compounds were freshly prepared in DMSO to prevent loss of potency. At the time of harvesting, cells were washed in cold PBS containing 1 mM sodium vanadate, 2 mM NaF, 1.0 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, leupeptin, and Pefabloc and phosphatase inhibitors mix (Sigma Chemicals). Cells were scraped, pelleted, and lysed in the same buffer containing 1% Triton X-100. Lysates were kept at −80°C until used. Each experiment was repeated at least two times.

**Western Blot Analysis.** Generation of rabbit polyclonal antibodies and Western blotting procedure were described previously (19). Briefly, protein samples obtained by scraping adherent cells with a mixture of protease inhibitors (30 μg/lane) were separated by 10% SDS-PAGE. After transfer to Immobilon-P membrane and blocking with PBS-containing 5% fat-free dry milk and 0.2% Tween 20, eN was detected using purified rabbit antibodies (0.05 and 0.15 μg/ml for cell panel and MCF-7 lysates, respectively), followed by incubation with horseradish peroxidase-labeled antirabbit secondary antibodies.

**Northern Blot Analysis.** Total cellular RNA was extracted from trypsinized cells using TRI reagent (Molecular Research Center, Cincinnati, OH), and 20 μg were loaded in each lane and separated in 1% agarose, 100 mM 4-morpholinepropanesulfonic acid buffer, and 2.0 M formaldehyde. Fractionated RNA was transferred onto Nytran nylon membranes (Schleicher-Shuell, Keene, NH), hybridized to labeled eN and ERα cDNA probes, as previously described (20), and exposed for 6 h to 6 days at −80°C. Double-labeled eN cDNA probe ($\alpha P^{32}$ dCTP and dATP) was used to detect eN mRNA in MCF-7 cells.

**Measurements of Extracellular Adenosine Generation from AMP and ATP and Adenosine Deaminase (ADA) Assay.** Cells were plated at $1–4 \times 10^3$ cells/well in 48-well flat-bottomed plates. Twenty-four h after plating, medium was replaced with 80 μl of serum-free Opti-MEM, and cells were preincubated for an additional 30 min. The reaction was initiated by the addition of (8-$^{14}$C) AMP (final concentration 20 μM, $3 \times 10^5$ cpm/well), cold adenosine at a final concentration 10 μM, 1 μM deoxycoformycin to inhibit ADA activity, and 10 μM dipyridamol to inhibit adenosine uptake in a total volume of 200 μl. Samples (25 μl each) were withdrawn at 0, 30, and 60 min and analyzed for radiolabeled adenosine by thin layer chromatography on Kodachrome microcrystalline cellulose plates with fluorescent indicator. Rates of adenosine generation were linear within the times specified, and the maximum amount of dephosphorylated AMP did not exceed 10%.

To determine extracellular ATP degradation products, $4 \times 10^4$ cells/well (24-well plate) were incubated in a 300-μl of total volume in the presence of 10 μM ($C^{14}$) ATP in RPMI 1640 without phenol red and FBS. Samples of 100 μl were withdrawn at 0, 1, 2, and 4 h and analyzed by HPLC linked to a radioactivity detector.

The ADA activity assay in cell lysates of MCF-7 and MDA-MB-231 cells was performed as described before (20).

**Plasmid Constructs and Transient Transfection Assays.** The full-length eN promoter (969 bp) was cloned into pCAT-Basic (Promega, Madison, WI) vector as described previously (21). Human ERα cDNA in a pCDNA3 vector was obtained from Dr. Kenneth Korach at National Institute of Environmental Health Sciences in Research Triangle Park in North Carolina. MCF-7 parental and MCF-7/Adr2 drug-resistant cells were transfected using FuGene6 reagent from Roche (Indianapolis, IN) at 3 μg DNA/9 μl of the reagent. Cells were incubated in regular medium, harvested after 48 h, and processed as described previously (21). pCDNA-β-galactosidase control vector was cotransfected to determine transfection efficiency (3–6% for MCF-7/Adr2 cells and 12–19% for MCF-7 parental cells).

## RESULTS

Our preliminary survey of several normal and cancer cell lines showed a variable expression of eN (2). A more focused analysis of eN expression in MCF-10A and nine breast cancer cell lines revealed a high eN expression at the mRNA and protein levels in five of six ER-negative cells, whereas three ER+ lines had very low or undetectable expression (Fig. 1, *A* and *B*). eN expression was detected in MCF-7 extracts only after a 20-fold increase in exposure time. An examination of two prostate cancer cell lines, LNCaP and PC-3, showed a similarly inverse correlation with the androgen receptor status (data not shown). MCF-7 cells that were selected for doxorubicin resistance lost ER expression and simultaneously increased eN expression (Fig. 1*C*), an observation previously reported by Ujhazy *et al.* (11). Comparison of eN expression in hyperplastic (nontumorigenic) MCF-10A, cancer MDA-MB-231 and

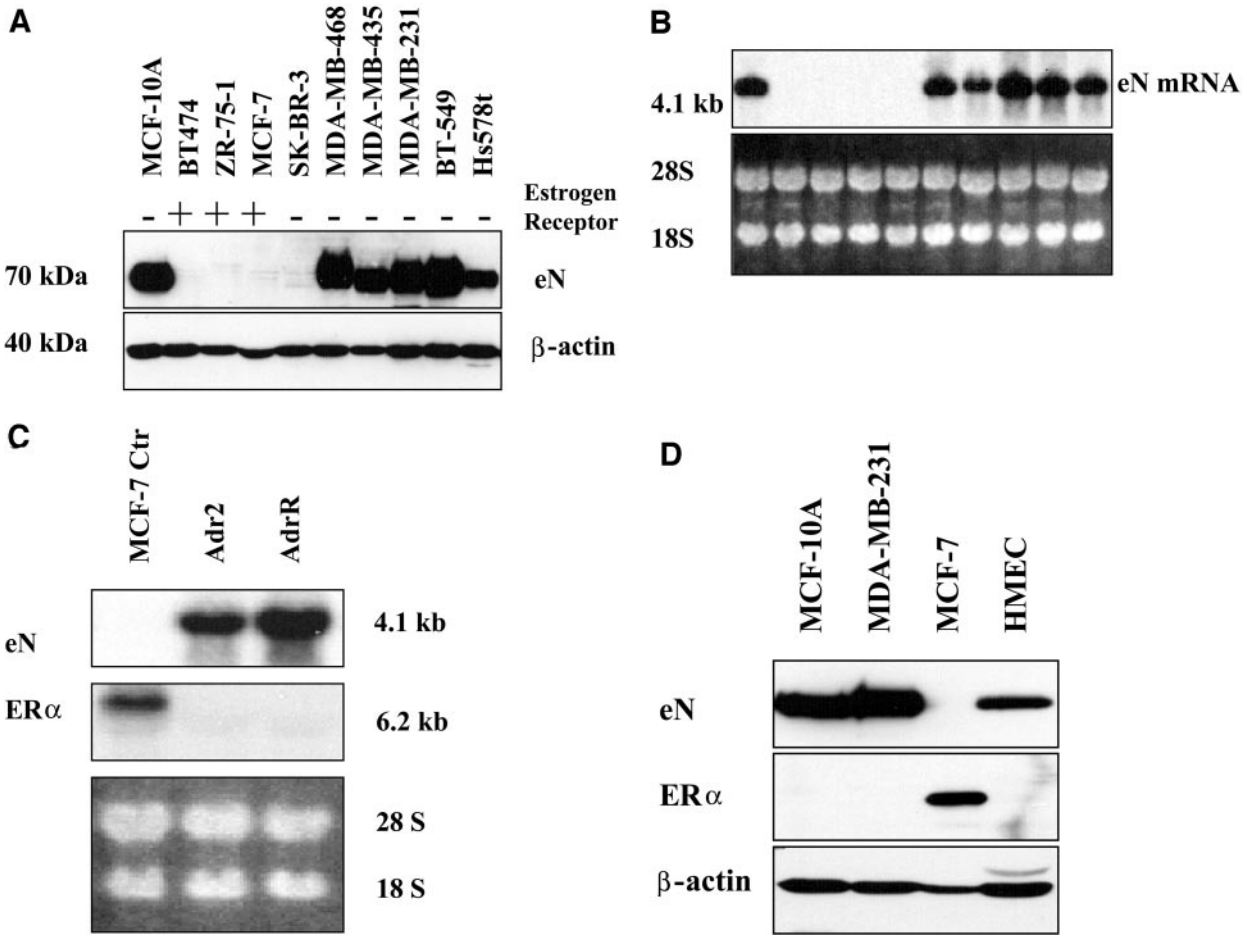


*Fig. 1* Relationship between estrogen receptor (ER) and ecto-5′-nucleotidase (eN) expression in 12 breast cancer cell lines. The panel includes ER-negative hyperplastic MCF-10A cells and ER-positive and -negative tumor-derived cell lines. *A*, Western blot analysis of eN protein levels. *B*, Northern blot analysis of eN mRNA levels. *C*, correlation of loss of ER expression after development of drug resistance in MCF-7/Adr2 and MCF-7/R cells with increased expression of eN mRNA. *D*, comparison of eN and ERα protein expression in malignant (MDA-MB-231 and MCF-7), hyperplastic (MCF-10A), and normal human mammary epithelial cells (HMECs). Cells were maintained in appropriate medium and total RNA and/or protein isolated and processed as described in "Materials and Methods."

MCF-7, and normal human mammary epithelial cells revealed that eN expression was high in MDA-MB-231 and MCF-10A and intermediate in human mammary epithelial cells, all of which were ER− (Fig. 1*D*).

To determine whether protein levels correlate with the enzymatic activity of membrane eN in intact cells, we incubated cells with 10 μM ($C^{14}$) AMP. ER-negative breast cancer cell lines MDA-MB-231 and BT-549 produced 32–104-fold more adenosine from added AMP, as compared with two ER-positive lines (Table 1). Adenosine generation was markedly reduced in the presence of 20 μM adenylymethylenediphosphate (AMPCP), an eN inhibitor. Because ATP is the major nucleotide released from cells, we investigated whether breast cancer cells differed in rates of adenosine generation from extracellular ATP. Data in Fig. 2 demonstrate that MDA-MB-231 (ER−) cells, in contrast to MCF-7 cells, generate adenosine and inosine from extracellular ATP. These results indicate that ATP degradation leads to extracellular adenosine formation and that eN is essential for this process. Similar differential results were obtained with ZR-75-1 (ER+) and BT-549 (ER−) cells (data not shown). To better approximate the *in vivo* situation, ATP degradation, as opposed to the rate of adenosine generation from AMP, was analyzed in the absence of inhibitors of ADA and nucleoside transport. Under these conditions, there was accumulation of inosine and/or hypoxanthine in MDA-MB-231 cells, which most likely resulted from the extracellular or intracellular metabolism of generated adenosine (Fig. 2). The extracellular adenosine levels may also be modulated by adenosine deaminase, although ADA activity differed by only 2-fold (2.03 ± 0.36 nmol/min/mg protein in MCF-7 cells and 1.03 ± 0.09 nmol/min/mg protein in MDA-MB-231 cells, ±SD, $n = 4$). Given the 32-fold difference in eN activity, we conclude that eN is the enzymatic activity responsible for extracellular adenosine formation by ER-negative cells.

Because these results suggested that the ER might directly regulate the expression of eN, we analyzed the levels of eN mRNA in MCF-7 cells treated with either estradiol as an agonist or tamoxifen as an antagonist. As shown in Fig. 3*A*, tamoxifen

*Table 1* Generation of adenosine from extracellular AMP in breast cancer cell lines

| Cell line | Estrogen receptor status | Rates of adenosine generation (nmol/h/$10^6$ cells) | |
|---|---|---|---|
| | | +AMPCP | −AMPCP |
| ZR-75-1 | + | 3.5 ± 0.3 | 12.1 ± 5.3 |
| MCF-7 | + | 14.1 ± 1.1 | 37.7 ± 6.5 |
| MDA-MB-231 | − | 146.2 ± 10.6 | 1212 ± 417 |
| BT-549 | − | 162.7 ± 7.3 | 1253 ± 489 |

Data represent the means ± SD of three independent experiments. Adenylymethylenediphosphate (AMPCP), an inhibitor of ecto-5′-nucleotidase, was used at 20 μM.

increases eN mRNA in cells cultured in regular medium, whereas estradiol decreases eN mRNA in estrogen-stripped medium. Note that Northern blots for eN in MCF-7 cells were hybridized with double-labeled probe and exposed 10–20-times longer to detect eN-specific signals. Similar effects were seen with eN protein levels: addition of estradiol caused a concentration-dependent decrease of eN expression and incubation in estrogen-free medium increased the eN expression in a time-dependent manner (Fig. 3*B–D*). The decrease in eN expression is apparent at 0.1–1.0 nM estradiol, the physiological range of estradiol concentrations in premenopausal women. The ER antagonists, tamoxifen at 1 μM and ICI-182,780 at 0.1 μM, reversed the effect of estradiol on eN expression (Fig. 3*D*). In contrast to the effects on eN, there was no effect of estradiol or antiestrogens on the expression of other glycosylphosphatidyl inositol-linked membrane proteins, urokinase-type plasminogen activator receptor or CD24 (two proposed markers for breast carcinoma) or Thy-1 (22–24). The lack of effect of estradiol on urokinase-type plasminogen activator receptor expression has also been reported in MDA-MB-231 cells stably expressing ERα (25). Estradiol increased and estrogen antagonists decreased the expression of cyclin D1 (Fig. 3*D*), a known target of ER signaling (26–28). These data show that in the context of several other breast cancer markers, the down-regulation of eN expression by estradiol is specific for eN. Interestingly, a similar pattern of expression, and negative regulation by ER, has been recently reported for a novel breast cancer marker EphA2 (29), as well as for a few other genes (30–32). ERα protein levels were also decreased in the presence of estradiol, increased after treatment with tamoxifen, and almost completely down-regulated in the presence of the pure antiestrogen ICI-182,780. Expectedly, adenosine generation increased in estrogen-deprived conditions ~3-fold and decreased 2-fold in the presence of estradiol (Table 2). It is of note that AMPCP-inhibitable and eN-specific nucleotidase activity constitute ~64% of total activity in MCF-7 cells (Table 1); therefore, the actual amount of eN-dependent adenosine generation decreased substantially more in the presence or absence of estradiol then a direct comparison of rates would indicate. The identity of the nonspecific AMP hydrolase that may have contributed to adenosine generation at 10 μM AMP is unknown but most likely represents one or several of nonspecific phosphatase(s) that are expressed in epithelial cells. These enzymes, however, have much higher $K_m$s than eN and therefore are not likely to participate in adenosine generation at the lower physiological AMP concentrations of <1 μM (33, 34).

To demonstrate that estradiol-mediated regulation of eN could be achieved in ER-negative cells by restoration of ER expression, we used MDA-MB-231 cells stably transfected with an ERα (S30 variant). The ERα expression partially restores the sensitivity of these cells to estradiol (18). Incubation of S30 cells with 1 nM estradiol for 48 h resulted in decreased expression of eN protein and mRNA (Fig. 4, *A* and *B*). Because the ERα transfection alone is not sufficient to down-regulate eN expression, these data also demonstrate that estradiol is required for ERα-mediated decrease of eN expression. To investigate whether the suppression of eN expression by ER is mediated at the transcriptional level, we analyzed the effect of ERα cotransfection on the eN promoter activity in transient transfection assays using the wild-type 969 bp eN promoter fragment (21). For this purpose, we used parental MCF-7 cells and the drug-resistant variant MCF-7/Adr2 that has lost ER expression (Fig. 1*C*). Data shown in Fig. 5 indicate that eN promoter activity measured in cells incubated in regular medium,

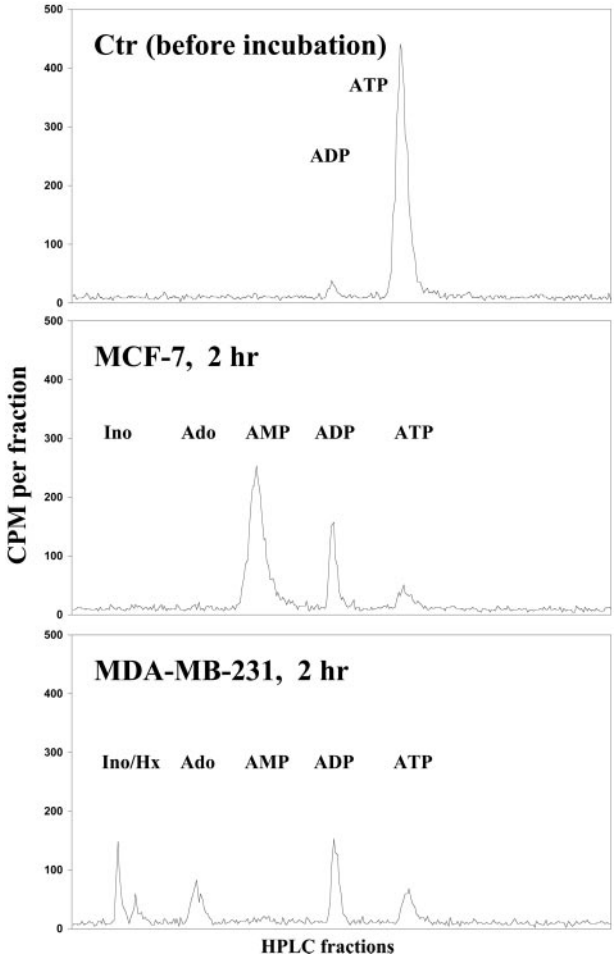


*Fig. 2* Analysis of extracellular ATP degradation products in the medium incubated with MCF-7 and MDA-MB-231 cells. Peaks represent the amount of radioactivity (cpm/ml) corresponding to authentic nucleotides and nucleosides. The data shown represent results obtained in two independent experiments.

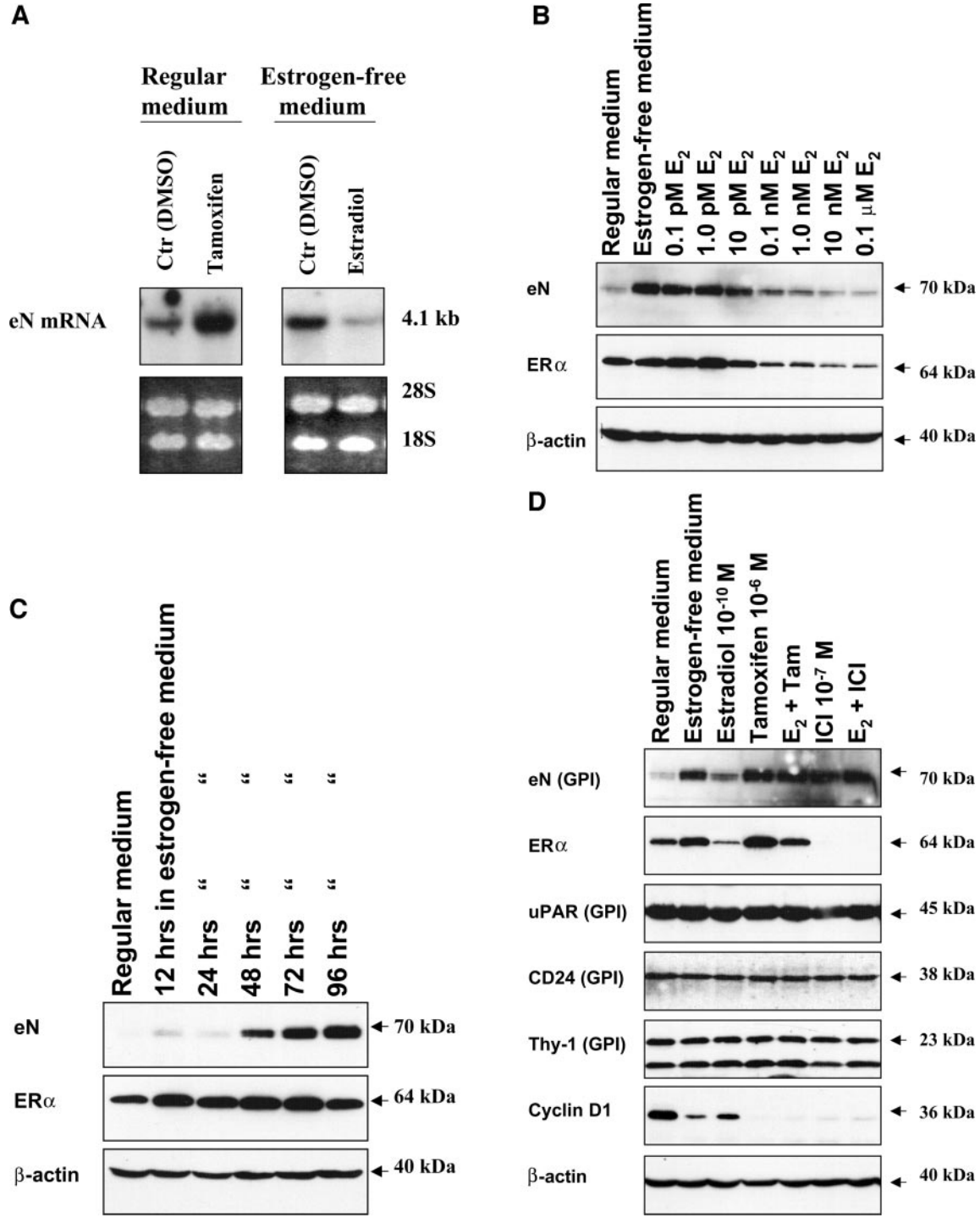


*Fig. 3* The effect of estrogen-free medium, estradiol ($E_2$), ICI-182,780 and tamoxifen on the expression of ecto-5′-nucleotidase (eN) mRNA and protein in MCF-7 cells. *A*, estradiol decreases and tamoxifen increases the expression of eN mRNA in MCF-7 cells. Cells were incubated in regular medium supplemented with 10% FBS (tamoxifen treatment, 48 h) or preincubated for 48 h in phenol red-free medium supplemented with 10% double charcoal-treated FBS followed by the addition of estradiol for 48 h. *B*, dose response effect of estradiol on MCF-7 cells preincubated for 48 h in phenol red-free and double charcoal-treated FBS. Cells were treated with estradiol for additional 48 h. *C*, effect of time of incubation of MCF-7 cells in phenol red-free and double charcoal-treated FBS on eN protein expression. *D*, effect of estradiol, ICI-182,780, and tamoxifen on the expression of eN, other glycosylphosphatidyl inositol (GPI)-linked membrane proteins, and cyclin D1 expression in MCF-7 cells. Cells were preincubated for 48 h in phenol red-free medium supplemented with 10% double charcoal-treated FBS before treatment with indicated compounds for an additional 48 h. Detection of eN mRNA and protein in MCF-7 cells required 10–20-fold longer exposures of blots and also required double-labeled eN cDNA probe or higher concentration of eN antibodies when compared with blots shown in Fig. 1. This longer exposure is responsible for the background on some eN Western blots. The identity of the lower Thy-1-immunoreactive band is unknown. Other experimental conditions are described in "Materials and Methods."

*Table 2* The effect of estradiol on adenosine generation from extracellular AMP in MCF-7 cells[a]

| Treatment | Rates of adenosine generation (nmol/h/$10^6$ cells) |
|---|---|
| Regular medium | 17.9 ± 5.3 |
| Estrogen-stripped medium | 67.8 ± 4.1 |
| Estrogen-stripped medium + 0.1 nM estradiol | 38.7 ± 1.4 |

[a] Cells were preincubated in estrogen-free medium for 48 h before estradiol or vehicle (DMSO) was added and then incubated for an additional 48 h. Generation of adenosine was measured as described in "Materials and Methods." A representative experiment in triplicate is shown. In three independent experiments, adenosine generation increased by 2.7-fold (±1.0, SD) in estrogen-free medium *versus* regular medium and decreased 2.0-fold (±0.3, SD) in estradiol-treated cells *versus* estrogen-free medium.

when adjusted for transfection efficiency and compared with promoterless pCAT-Basic, is ~8-fold higher in MCF-7/Adr2 cells. Cotransfection with the ERα significantly decreases eN promoter activity in both cell lines. These data suggest that transcriptional regulation is an important component in the repression of eN expression by ER. Because a strong negative effect is seen in MDA-MB-231 S30 and MCF-7/Adr2 cells that express a high level of endogenous eN, results shown in Figs. 4 and 5 also indicate that ERα activity has a dominant effect. However, the reverse scenario, the decrease of ERα activity found in estrogen-free conditions and in the presence of antiestrogens in cells with low endogenous eN, does not lead to the high eN levels found in ER-negative cells (compare Tables 1 and 2 for quantitative difference). This difference suggests that additional factors are necessary for additional induction of eN expression in ER-negative cells.

Protein kinase C (PKC) activation by phorbol esters (phorbol 12-myristate 13-acetate) has been known to strongly induce eN expression (20). To test whether PKC-mediated eN expression may be modulated by ER activity, we cultured MCF-7 cells in estrogen-stripped medium and induced eN expression by phorbol ester in the absence and presence of estradiol. Data shown in Fig. 6 suggest that although phorbol 12-myristate 13-acetate strongly up-regulates eN expression, they do not attain the levels seen in ER-negative cells (Fig. 6*B*). However, consistent with the dominant suppressive activity of ERα, the addition of estradiol dramatically reverses the increased expression induced by phorbol 12-myristate 13-acetate alone (Fig. 6). The difference in incubation times between the experiments presented in Figs. 3 and 6 may account for the different basal expression of eN in estrogen-stripped medium. It took at least 48–72 h in estrogen-free medium to observe increase in eN expression (Fig. 3*C*). This may be due to trace levels of estrogen present in cells after transfer to estrogen-free conditions. Addition of ICI 182,780 was able to compete out this remaining estrogen effect and increase eN expression as early as 24 h (Fig. 6*A*, *Lane 7*). Addition of both the ICI compound and 100 ng/ml phorbol 12-myristate 13-acetate lead to a toxic effect on MCF-7 cells that may account for the reduced eN expression under these conditions (Fig. 6*A*, *Lane 9*).

Elevated activity of eN was previously found in primary breast carcinoma (10). In a recent study, Martin *et al.* (35) used a differential display strategy to identify 170 genes that were differentially expressed in a breast cancer cell line MDA-MB-435 when compared with normal mammary epithelium. The identified genes were then used to survey 18 clinical specimens using cDNA arrays. The list of genes altered in the highly metastatic MDA-MB-435 cells included eN; although, this information was not in the published version. We have accessed the unpublished data and found that eN expression was absent in all seven clinical samples with the level of ER expression of ≥20% and was elevated in four of six ER-negative samples

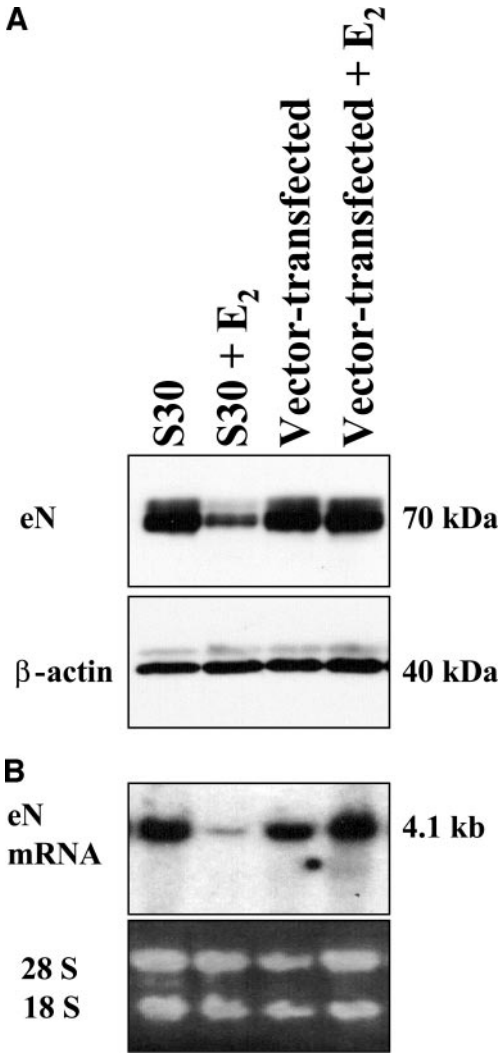


*Fig. 4* Effect of estrogen receptor (ER)α expression on estradiol ($E_2$)-dependent regulation of eN. MDA-MB-231 ER− cells were stably transfected with ERα cDNA expression vector (S30) or with vector alone (16). Cells were incubated in phenol red-free medium supplemented with 5% double charcoal-stripped FBS and treated with estradiol (1 nM) or DMSO for 48 h. eN protein expression (*A*) and mRNA expression (*B*). Occasionally, we observed two closely migrating eN bands on Western blots that may be also seen in Figs. 3 and 6. It is possible that glycosylation differences account for these bands. Other experimental conditions as described in "Materials and Methods." These experiments are representative of two, with similar results.

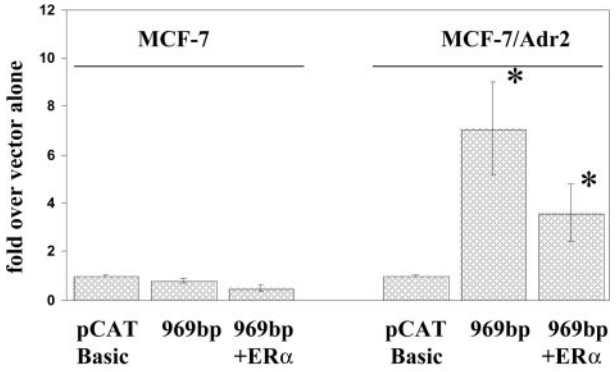


*Fig. 5* The effect of cotransfected estrogen receptor (ER)α on the 969-bp ecto-5′-nucleotidase promoter activity in MCF-7 and MCF-7/Adr2 cells. Cells were incubated in regular medium. Data show mean values from three independent experiments ± SD, *, $P < 0.05$ (student unpaired *t* test). Experimental details in "Materials and Methods."

(Table 3).[4] Using a differential ER expression threshold of 20% (*i.e.*, low < 20%; high > 20%), there was a statistically significant correlation between eN expression and low or absent ER status (Spearman correlation coefficient, −0.58; $P < 0.014$). Thus, this finding in primary breast tumors supports our *in vitro* observations that ER may be an important factor in the negative regulation of eN expression in breast carcinoma.

## DISCUSSION

ERs play an important role in maintaining physiological processes of breast tissue. However, they are also critical factors in the etiology and progression of human breast and gynecological cancers and consequently are important pharmacological targets of anticancer therapy (36). ER-negative breast cancers that are associated with a more aggressive course have been correlated with a specific gene expression profiles (35, 37–43). Although several of the directly ER-responsive genes have been identified (44, 45), the loss or lack of ER expression may also contribute to a broad range of phenotypic changes that occur during the transition to a more aggressive and metastatic breast cancer. We have found that estradiol, acting through the ER, strongly down-regulates the expression of eN. The major function of eN is to produce extracellular adenosine, and given the well-defined angiogenic, cytoprotective, and anti-inflammatory functions of this regulatory molecule, we propose that increased generation of adenosine in ER-negative breast carcinoma could have direct tumor-promoting consequences.

The mechanism of ERα-mediated regulation of target genes may be direct, *i.e.*, by binding to the ERα-responsive element, or indirect, *i.e.*, either by binding to transcription factors or proteins that form multiprotein complexes within the transcription machinery or by affecting the expression of other transcription factors (29–32). Because we have not identified the ERα consensus binding site within the 969-bp proximal promoter fragment, we conclude that the down-regulation of eN expression by ER likely involves an indirect mechanism. A number of reports documented that ER may specifically regulate the activity of activator protein-1 and Sp1 transcription factors (46–49), and we have previously shown that these sites are important for the eN promoter activity (21).

The estradiol and/or ERα-mediated down-regulation of eN promoter activity and eN expression in MCF-7/Adr and MDA-MB-231 ERα-transfected cells suggest that ERα has a dominant

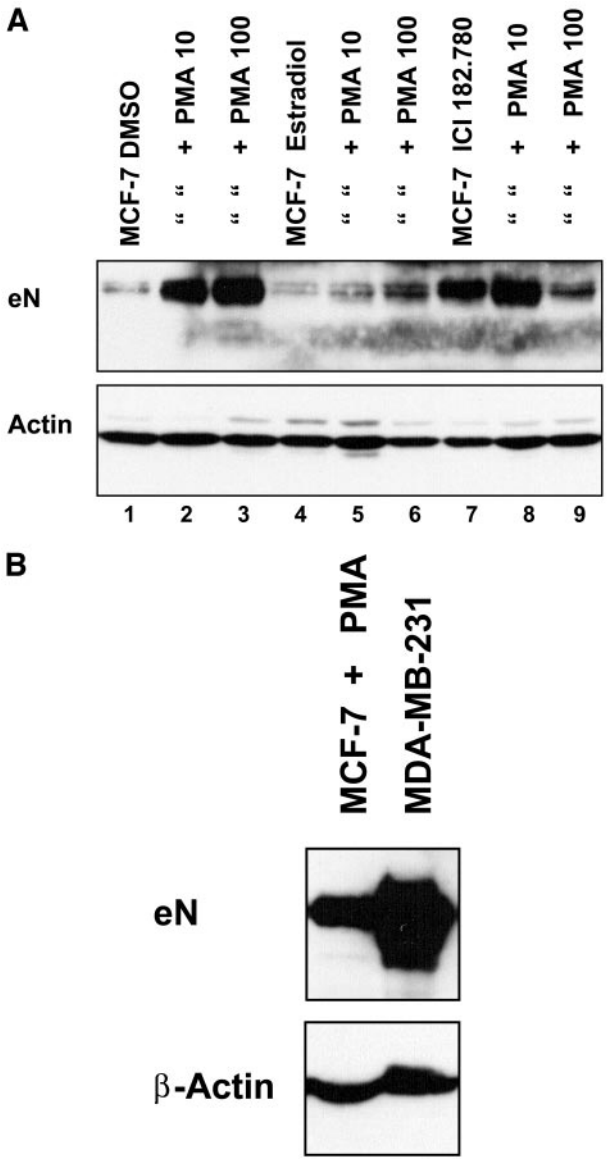


*Fig. 6* The effect of estradiol on phorbol ester-induced eN expression in MCF-7 cells (*A*). Comparison of expression of eN protein in PMA-stimulated (100 ng/ml) MCF-7 and MDA-MB-231 cells (*B*). Cells were incubated in phenol red-free medium supplemented with 5% double charcoal-stripped FBS and treated either with estradiol (1 nM) and phorbol esters (10 or 100 ng/ml) or simultaneously with both for 24 h. MDA-MB-231 cells were incubated in regular medium. Other experimental conditions as described in "Materials and Methods." Representative experiment of three, with similar results, is shown.

[4] Internet address: http://mbcf.dfci.harvard.edu/labs/pardee/expression_patterns.html.

*Table 3* Correlation of ER and eN expression in breast carcinoma[a]

| | Tumor type | Estrogen receptor status | Ecto-5′-nucleotidase |
|---|---|---|---|
| 1. H24 | Ductal invasive | 90% | 0.1[b] |
| 2. PT12 | Ductal invasive | 90% | 0.1 |
| 3. H34 | Metastatic | 86% | 0.1 |
| 4. H16 | Metastatic | 70% | 0.1 |
| 5. PT6 | Ductal invasive | 67% | 0.1 |
| 6. H35 | Ductal invasive | 64% | 0.1 |
| 7. H17 | Ductal invasive/ lobular invasive | 31% | 0.1 |
| 8. PT1 | Ductal invasive | 19% | 7.2 |
| 9. H19 | Ductal invasive | 18% | 0.1 |
| 10. PT9 | Ductal invasive | 15% | n/a[c] |
| 11. PT10 | Ductal invasive | 13% | 4.4 |
| 12. PT8 | Ductal invasive | 10% | 1.1 |
| 13. H31 | Ductal invasive | 0% | 9.8 |
| 14. H4 | Lobular invasive | 0% | 0.1 |
| 15. H43 | Metastatic | 0% | 2.8 |
| 16. PT4 | Ductal invasive | 0% | 16.7 |
| 17. H10 | Metastatic | 0% | 2.1 |
| 18. H33 | Ductal invasive | 0% | 0.1 |

[a] Data from Martin *et al* (35).
[b] The value of 0.1 (arbitrary units) is equivalent to background.
[c] n/a, value not available from the original data.

effect and the loss of its expression or inhibition of its activity is a prerequisite for the increased eN expression. However, mere removal of ER activity in estradiol-stripped medium, although causing an increase in eN expression in MCF-7 cells, is not sufficient to bring the expression to levels seen in ER-negative cells, and likely, other factors are necessary to further induce eN expression. Examination of eN expression in cell lines and clinical samples (Fig. 1 and Table 3) appears to support this conclusion, whereas high ER expression invariably correlates with low eN levels, one cell line (SK-Br-3), and three clinical samples with low or absent ER that do not express eN. Although the identity of factor(s) that may be important for additional induction of eN expression is unclear, PKC-dependent signaling is a likely candidate. PKC activation has been shown to increase the expression of eN (20, 21, 50–52), and the inverse relationship between ER activity and PKC activity (53–55) indicates that PKC could provide the necessary stimuli for increased eN expression in ER-negative breast cancer cells. Our data show that PKC and ER have opposing effects on eN expression. Furthermore, we have shown that estradiol dramatically reverses eN expression induced by phorbol esters, indicating that ER activity has a dominant role over PKC pathway activation.

One previous study on the expression of eN (CD73) in breast carcinoma did not reveal a significant correlation between eN expression and ER status (56). This could be due to the low number of eN-positive samples (4%) among these tumors. A second possibility is that a certain level of ER expression has to be reached to decrease eN expression. Analysis of clinical samples from Martin *et al.* (35) reveals that eN is uniformly absent only in samples that express ER in ≥20% of cancer cells, suggesting that a minimal level of ER expression is required for this effect.

Although general effects of adenosine on vasodilation, angiogenesis, cytoprotection, and immune functions are well documented, *in vitro* effects on cell growth seem to be more complex. A number of studies have shown that activation of adenosine receptors may both stimulate and inhibit cell proliferation (reviewed in Refs. 2, 56–63). This could reflect variability in the expression profile of adenosine receptors that may signal either through growth stimulatory or growth inhibitory intracellular signaling pathways. The adenosine-mediated stimulation of proliferation of endothelial cells (8, 59, 60) may suggest a direct involvement of this molecule in angiogenesis. In addition to stimulation of cell proliferation, potential mechanisms for tumor promotion include adenosine-stimulated increased secretion of either vascular endothelial growth factor or interleukin 8 (61, 62). The role of adenosine in solid tumor physiology is of increasing interest, and additional studies will determine whether either the expression of eN itself or adenosine generation mediated by eN could contribute to tumor progression. An important point of this study is that increased adenosine generation in tumors may not merely result from ischemia and necrosis but is a part of an active process that includes induction of expression of eN. In the absence of eN expression, extracellular nucleotides would likely wash out into circulation. Additional identification of specific oncogenic mechanisms that govern expression of eN during cancer progression will be important to determine the significance of this protein for tumor growth. It is important to emphasize in this regard that the eN expressing cancer cell lines described in this study are more invasive *in vitro* and tumorigenic in nude mice than are the eN-negative lines (13–17, 64–66), supporting to some extent the growth-promoting properties of eN. If this relationship can be confirmed on larger number of clinical samples, especially in conjunction with outcome data, eN may emerge as a useful marker for a subset of more aggressive breast carcinomas.

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