

The Normal Epithelial Cell-Specific 1 (NES1) Gene is Up-Regulated by Steroid Hormones in the Breast Carcinoma Cell Line BT-474

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Abstract. The normal epithelial cell-specific 1 (NES1) gene encodes a serine protease which was found to be down-regulated in breast cancer. There is evidence that NES1 acts as a tumor suppressor gene in breast cancer cells. To further understand its role in breast tumorigenesis, we investigated the effect of estrogens, androgens, and progestins on NES1 gene expression, in the breast cancer cell line BT-474, at the transcription level. The reverse transcriptase polymerase chain reaction method was used to monitor changes in the NES1 mRNA. Our experiments showed that NES1 gene expression is up-regulated promptly in response to 17 β -estradiol, 5 α -dihydrotestosterone (DHT) and norgestrel stimulation. NES1 gene mRNA started to increase 2 hours after estradiol stimulation and 8 hours after DHT stimulation. The stimulation of NES1 by estradiol can be dramatically blocked by the estrogen antagonists ICI 182,780 and 4-hydroxytamoxifen. Mifepristone (a synthetic antiprogesterin) can partially block the up-regulation of the NES1 gene by norgestrel. Dose-response experiments indicated that the lowest stimulatory concentration of 17 β -estradiol, DHT, and norgestrel is 10⁻¹¹ M, 10⁻¹⁰ M, and 10⁻¹⁰ M, respectively. The production of NES1 mRNA increased coordinately with increasing concentration of the stimulants. These results suggest that the NES1 gene is primarily regulated by estrogen, but also by androgen and progestin in the breast cancer cell line BT-474. It appears that NES1 may be involved in a pathway that counterbalances the action of estrogens and androgens in steroid hormone responsive tissues.

The normal epithelial cell-specific 1 (NES1) gene was identified by subtractive hybridization by virtue of its down-regulation in breast cancer cell lines. Its cDNA has been cloned and found to be 1.4 kb in length. This gene is

predicted to encode a secreted serine protease. It has 50-63% sequence identity with three distinct serine protease families, including the trypsin family, the kringle family, and the human kallikrein family (1). Our previous work has shown that the NES1 gene spans about 5.5 kb of genomic DNA, which contains five coding exons and one non-coding exon and it resides on chromosome 19q13.3-4, in the same region where the human kallikrein gene family is localised (2). These data suggest that the NES1 gene and the human kallikrein family have likely originated from the same ancestor gene and remain closely linked.

The NES1 gene has been found to be down-regulated in established breast cancer cell lines, but its exact role in breast tumorigenesis is not known. An in-vitro study showed that by over-expressing the NES1 gene, the growth of the breast cancer cell line MDA-MB-231 was suppressed, and when this cell line was transplanted into nude mice, tumor formation was reduced (3). These findings indicate that the NES1 gene can negatively regulate cell growth and that it may act as a tumor suppressor gene.

Estrogens play an essential role in breast cancer development (4-6). Their involvement with breast cancer tumorigenesis lies in the fact that they can increase breast epithelial cell proliferation, thus facilitating malignant transformation (7).

To gain insight into the role of NES1 in breast cancer tumorigenesis, in this study, we investigated the effect of steroid hormones including estrogens, androgens, and progestins on the expression of the NES1 gene in the breast cancer cell line BT-474. The possible regulatory effects of steroid hormones on the expression of this potentially important gene have not as yet been reported.

Materials and Methods

Chemicals. 17 β -estradiol, 5 α -dihydrotestosterone, cyproterone acetate, and norgestrel were from Sigma Chemical Co, St Louis, MO. 4-hydroxytamoxifen was purchased from Research Biochemicals International, Boston, MA. ICI 182,780 was kindly donated by Zeneca, Mississauga, Canada. Mifepristone and nilutamide were kindly donated by Roussel-UCLAF, Romainville, France.

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Breast cancer cell lines. The breast cancer cell lines BT-474 and MDA-MB-453 were purchased from the American Type Culture Collection (ATCC), Rockville, MD.

Stimulation experiments. The BT-474 and MDA-MB-453 breast cancer cell lines were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200mmol/L), bovine insulin (10mg/L), fetal bovine serum (10%), antibiotics and antimyotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

Dose-response experiments. The BT-474 cells were cultured and stimulated with various steroid hormones as described above, except that the steroid hormone concentration varied from 10^{-8} to 10^{-13} M. Cells were collected 24 hours later.

Blocking experiments. The BT-474 cells were cultured as described in the stimulation experiments. To block the steroid hormone receptors, blockers for different steroid hormones were added for 1 hour into the culture media at a final concentration of 10^{-7} M, which was 100 times higher than the concentration of the stimulants. Then, different steroid hormones were added into the culture media at a final concentration of 10^{-9} M. Cells were collected 8 hours later.

Time course experiments. BT-474 cells were cultured and stimulated as described in stimulation experiments. Cells were harvested for mRNA extraction at 1, 2, 4, 8, 24, 36, and 48 hours.

Reverse transcriptase polymerase chain reaction. Total RNA was extracted from the breast cancer cells using Trizol reagent (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2µg of total RNA was reverse transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20µl. PCR was carried out in a reaction mixture containing 1µl of cDNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl₂, 200 (M dNTPs (deoxynucleoside triphosphates), 150ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Inc. Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 12 minutes to activate the Taq Gold DNA polymerase, followed by 30 cycles of 94°C for 30 s, 62°C for 1 min and a final extension at 62°C for 10 min. Equal amounts of PCR products were electrophoresed on 2.5% agarose gels and visualized by ethidium bromide staining. The sequences of the amplification primers are described in Table I.

Results

Stimulation experiments. The experiments described in this study were all done independently at least three times and we obtained similar results. Representative data are shown here. The BT-474 breast cancer cell line has been found to be estrogen, androgen, and progesterone receptor positive (our data not shown). Therefore, we used this cell line to examine whether the expression of the NES1 gene can be affected by estrogens, androgens, and progestins. When RT-PCR was performed on mRNA from the BT-474 cells without any stimulation, the expected size of PCR product was detected

Table I. Primers used for RT-PCR.

Gene	Direction	Primer sequence	Expected PCR product size
NES1	forward	5'-CACTCTGGCAAGGGTCCTG-3'	383bp
	reverse	5'-GATCACCTGCTGCTTCTTC-3'	
pS2	forward	5'-GGTGATCTGCGCCCTGGTCCT-3'	328bp
	reverse	5'-AGGTGTCCGGTGGAGGTGGCA-3'	
PSA	forward	5'-TGCGCAAGTTCACCCTCA-3'	754bp
	reverse	5'-CCCTCTCCTTACTTCATCC-3'	
Actin	forward	5'-ACAATGAGCTGCGTGTGGCT-3'	372bp
	reverse	5'-TCTCCTTAATGTCACGCACGA	

(383 base pairs) but its intensity was very faint, indicating that the basal expression level of the NES1 gene in this cell line is very low. When RT-PCR was repeated with cells stimulated with ethanol, the intensity of the PCR product was similar to the one obtained with non-stimulated cells. This finding suggested that ethanol does not have any stimulatory effect on the expression of the NES1 gene in the BT-474 cell line (Figure 1A). Since the steroid hormones used in this study were dissolved in ethanol, the cells treated with ethanol were used as controls, representing the basal NES1 expression level.

pS2 is a classical estrogen regulated gene. It is known that this gene is up-regulated by estrogens, but not androgens or progestins in breast cancer cell lines (8, 9). PSA has been found to be up-regulated by androgens and progestins, but minimally or not at all by estrogens in breast cancer cell lines (10). In this study, the expression of the PSA and pS2 genes after steroid hormone stimulation was investigated. These genes serve as controls to ensure that the cell culture system and the RT-PCR method used were able to correctly reflect the mRNA level change after steroid hormone stimulation. Actin is another control which indicates that an equal amount of cDNA from the cells stimulated with different amount of steroid hormones was used for RT-PCR. Actin mRNA is not altered by steroid hormones.

In the stimulation experiments, the breast cancer cell line BT-474 was stimulated with 17β-estradiol, 5α-dihydrotestosterone (DHT), and norgestrel at a concentration of 10^{-8} M for 24 hours. When RT-PCR was performed on these cells, we found, as expected, that the mRNA level of the pS2 gene was increased by estradiol, but not DHT or norgestrel and the PSA gene was up-regulated by DHT and norgestrel, but not estrogen (Figure 1B). These results indicated that in this cell

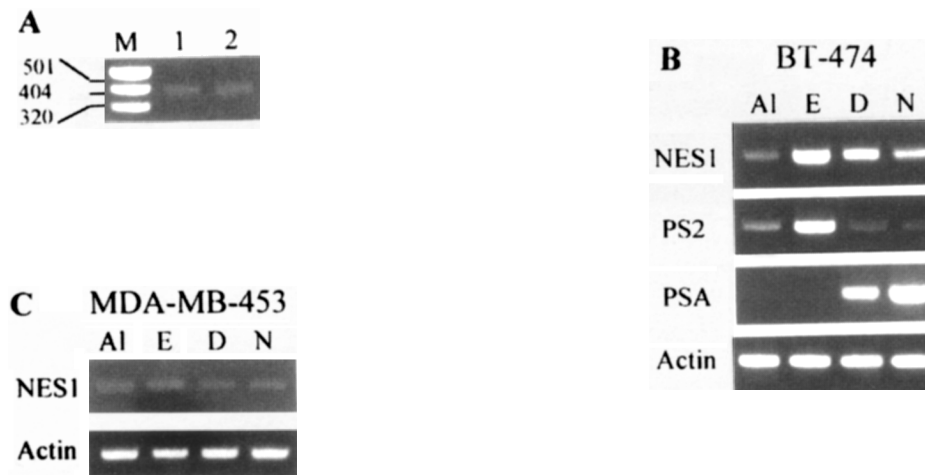


Figure 1. Representative RT-PCR reactions on mRNA from breast cancer cells stimulated with various steroid hormones. The same cDNAs were used for PSA, pS2, actin, and NES1 gene amplification. A. effect of ethanol on the expression of the NES1 gene. Lane M, molecular weight markers; Lane 1, non-stimulated cells; Lane 2, cells stimulated with ethanol. B. Stimulatory effect of ethanol (Al), 17 β -estradiol (E), DHT (D) and norgestrel (N) on the expression of the PSA, pS2, actin, and NES1 genes in the breast cancer cell line BT-474. C. Stimulatory effect of ethanol (Al), 17 β -estradiol (E), DHT (D) and norgestrel (N) on the expression of the NES1 gene in the breast cancer cell line MDA-MB-453. The size of the PCR products is shown in Table I. For discussion, see text.

culture system, estradiol, DHT and norgestrel could activate their own receptors and regulate target gene expression. The RT-PCR method used is able to clearly differentiate the mRNA level change after steroid hormone stimulation. To investigate the effect of estradiol, DHT, and norgestrel on the NES1 gene expression, RT-PCR was performed on the same cDNA samples. We found that the NES1 mRNA increased significantly in the cells stimulated with estradiol, DHT, and norgestrel (Figure 1B). The NES1 mRNA level is highest in the cells stimulated by estradiol, and lowest in the cells stimulated by norgestrel. These data suggest that estradiol and DHT can up-regulate NES1 mRNA strongly, while norgestrel's effect is relatively weak. The rank of potency is estrogen > androgen > progestin.

In order to determine whether the stimulatory effect of estradiol, DHT, and norgestrel on the NES1 gene expression was mediated by steroid hormone receptors, the same experiments were repeated in the breast cancer cell line MDA-MB-453, which is negative for estrogen and progesterone receptors, but has functional androgen receptor (11). By RT-PCR, the basal expression of NES1 was very low in this cell line. After stimulation by estradiol, DHT, and norgestrel, NES1 gene mRNA was not increased (Figure 1C), indicating that the estrogen and progesterone receptors are required for the up-regulation of the NES1 gene by estradiol

and norgestrel. Although the androgen receptor in this cell line has been reported to be functional, it failed to up-regulate both PSA (data not shown) and NES1 genes (Figure 1C).

Blocking experiments To further examine if estradiol, DHT, and norgestrel up-regulate the NES1 gene through hormone receptor action, we performed blocking experiments. Antagonists of estrogen, androgen and progesterone receptors were used to block their receptors prior to adding the steroid hormones. ICI 182,780 and 4-OH tamoxifen are established estrogen receptor antagonists and mifepristone is a strong progesterone receptor blocker. Nilutamide and cyproterone acetate are known androgen receptor antagonists. In Figure 2A., we demonstrate that the stimulatory effect of estradiol on the NES1 gene expression can be significantly suppressed by ICI 182,780 and 4-OH tamoxifen, but not by androgen or progesterone receptor blockers, suggesting that estrogen up-regulates the NES1 gene through its own receptor. On the other hand, only mifepristone can partially block the up-regulation of the NES1 gene by norgestrel (Figure 2B), indicating that the action of norgestrel is mediated at least partially through the progesterone receptor. Although cyproterone acetate and nilutamide inhibited DHT up-regulation of the PSA gene, they failed to suppress the stimulatory effect of DHT on the NES1 gene. Additionally,

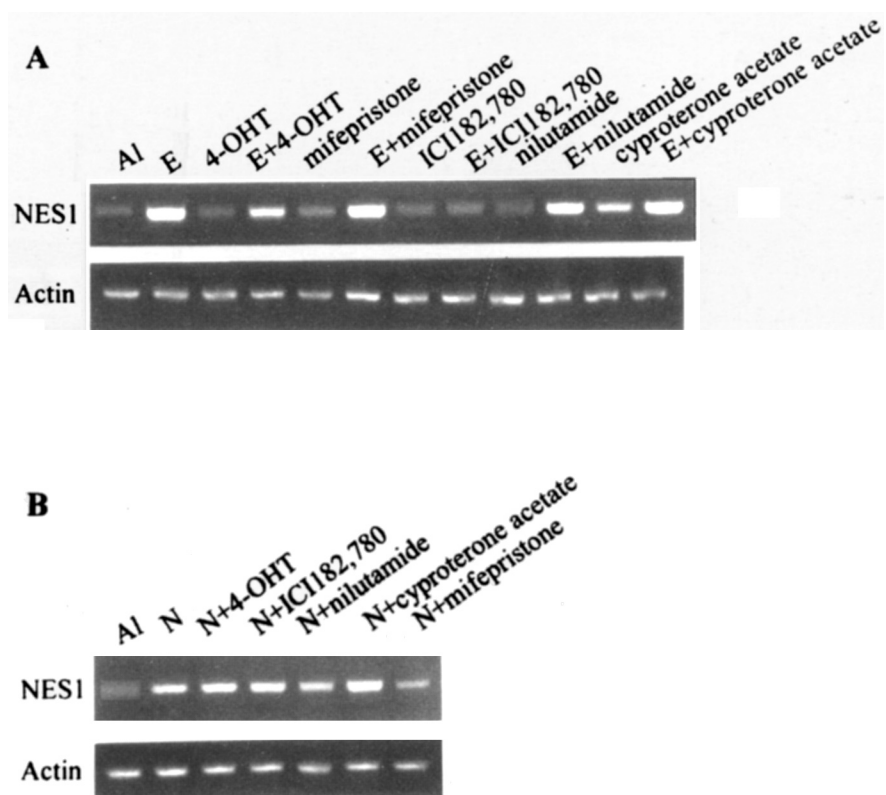


Figure 2. Representative RT-PCR reactions of the NES1 gene in the breast cancer cell line BT-474 stimulated with (A) 17β-estradiol (E) and (B) norgestrel (N) in the presence or absence of different steroid hormone antagonists. The compounds are indicated on the top of each lane. For discussion, see text.

the up-regulation on the NES1 gene by DHT was not blocked by estrogen and progesterone receptor blockers (data not shown).

Dose response experiments. To further investigate the stimulatory effect of the steroid hormones on the NES1 gene expression, we performed dose-response experiments. BT-474 cells were stimulated by different concentrations of estradiol, DHT, and norgestrel ranging from 10^{-13} to 10^{-8} M. Representative RT-PCR data are shown in Figure 3. NES1 mRNA increases proportionally with increasing concentration of the steroid hormones. The lowest stimulatory concentration of estradiol, DHT, and norgestrel is 10^{-11} M, 10^{-10} M, and 10^{-10} M, respectively.

Time course experiments. To study the kinetics of the NES1 mRNA change after steroid hormone stimulation, time course experiments were conducted. BT-474 cells were stimulated with estradiol and DHT at a concentration of 10^{-8} M and cells were harvested at different time points. We found that the PSA gene mRNA become detectable 8 hours after DHT stimulation, whereas, pS2 mRNA level started to increase 2 hours after estradiol stimulation. NES1 mRNA increased 2 hours after estradiol stimulation and 8 hours after

DHT stimulation. The increased expression of these three genes persisted up to at least 48 hours. These results show that the kinetics of NES1 gene expression are similar to those of pS2 and PSA genes (Figure 4).

Discussion

In this study, we found that the NES1 gene is up-regulated in response to estrogen, androgen, and progestin stimulation in the breast cancer cell line BT-474. Our data indicate that these steroid hormones regulate the NES1 gene expression, most likely through their own receptors. Our inability to block effectively androgen receptor action by anti-androgens in the BT-474 cells and the failure of DHT to up-regulate the PSA and NES1 genes in the MDA-MB-453 cells may reflect a more complicated mechanism. It has been suggested that the action of steroid hormones or steroid hormone receptor antagonists relies on the sophisticated interaction among hormone or antagonist, receptor, steroid hormone receptor co-activators or co-repressors, the basal transcription machinery, and gene promoter context (12, 13). We postulate that DHT and androgen receptor antagonists fail to interact

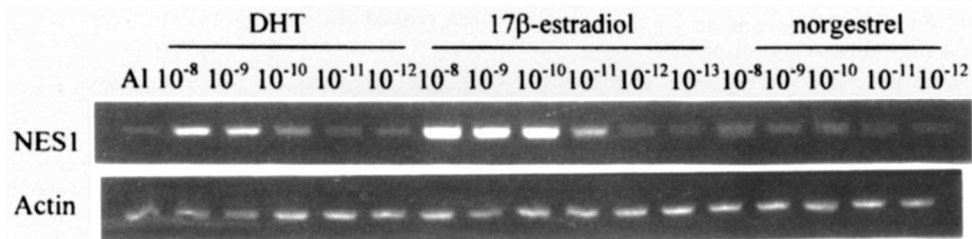


Figure 3. Representative RT-PCR reactions indicating the effect of increasing concentrations of 17β-estradiol (E), DHT (D) and norgestrel (N) on the NES1 gene expression in BT-474 cells. The numbers on the top of each lane denote the molar concentration of each steroid hormone.

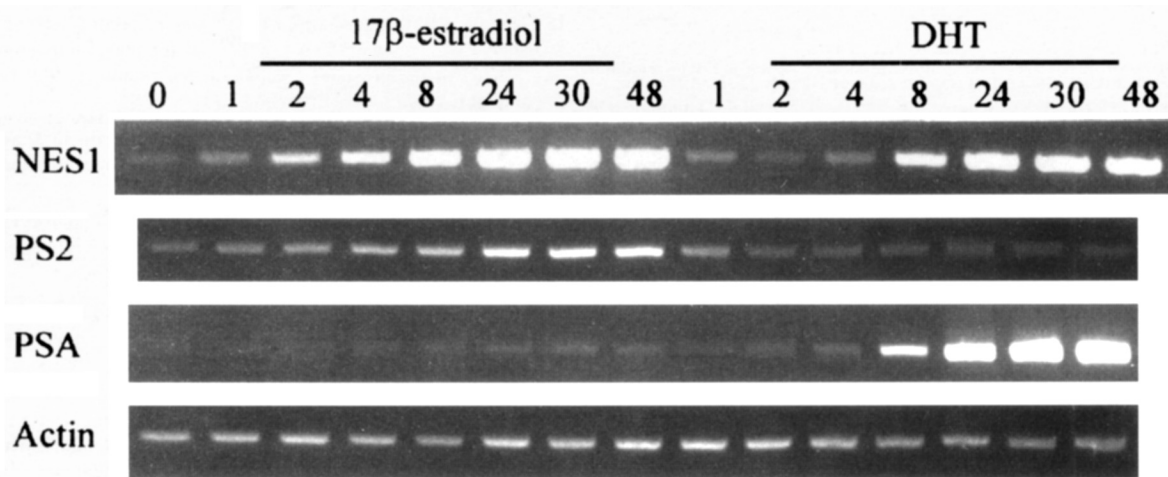


Figure 4. Kinetic changes of the PSA, pS2, and NES1 gene expression after 17β-estradiol (E) or DHT (D) stimulation in BT-474 cells. The time points in hours at which the cells were harvested, are indicated on the top of each lane.

successfully with other molecules, in the MDA-MB-453 and BT-474 cells.

pS2 is a classical estrogen regulated gene, and an estrogen response element has been found in its promoter region (8, 14). The PSA gene is well known to be specifically regulated by androgens and progestins (10). The 5'-end of this gene has androgen response elements to which the activated androgen and progesterone receptors can bind (15-17). Therefore, these two genes are considered to be directly regulated by estrogens and androgens at the transcription level. The rapid increase of NES1 mRNA and its similar kinetic behavior to pS2 and PSA, after estradiol and DHT stimulation, indicates that the NES1 gene may also be directly regulated by steroid hormones. Final proof must await functional analysis of the NES1 gene promoter.

The NES1 gene is homologous to the human kallikrein family, including PSA, KLK1 (pancreatic/renal kallikrein), and KLK2 (glandular kallikrein) (1). Although both PSA and

NES1 are down-regulated in breast cancer (1, 18), the regulation of their expression by steroid hormones in breast cancer cell lines is quite different. PSA is up-regulated by androgens and progestins, but not estrogens (10), while NES1 is up-regulated primarily by estrogens, and also by androgens and progestins. In addition, NES1 over-expression can inhibit tumor formation (3), whereas, this tumor suppressor function has not been studied with the PSA gene. These observations suggest that NES1 may function in a different way than PSA in normal or cancerous breast tissue.

Estrogen plays an essential role in stimulating breast epithelial cell proliferation. Given that by over-expressing the NES1 gene breast cancer cell growth is inhibited (3), the up-regulation of NES1 gene in response to estrogen stimulation may indicate that NES1 is involved in a pathway that counter balances the mitogenic effect of estrogen. Thus, loss of NES1 may lead to uncontrolled cell growth. In estrogen receptor negative breast cancer, the more aggressive phenotype may

partially be due to the loss of NES1 expression and its tumor suppressor function. Similarly, androgens also have potent stimulatory effects on the proliferation of prostate epithelial cells (19) and the NES1 gene is down-regulated in prostate cancer cell lines (3). These findings indicate that NES1 may function in a similar way (cell growth inhibitor) in both breast and prostate tumorigenesis, as already described for other genes (20).

In summary, our study indicates that the NES1 gene is up-regulated in response to stimulation by estrogens, androgens, and progestins. We suggest that NES1 may be involved in a pathway that counteracts the mitogenic activity of estrogen and androgen.

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