AD ________________

Award Number: DAMD17-96-1-6164

TITLE: The Role of a Novel Protease NES1 in Breast Cancer

PRINCIPAL INVESTIGATOR: Vimla Band, Ph.D.

CONTRACTING ORGANIZATION: New England Medical Center
Boston, Massachusetts 02111

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20000710 145

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (19 Aug 98 -18 Aug 99)

4. TITLE AND SUBTITLE
The Role of a Novel Protease NES1 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6164

6. AUTHOR(S)
Vimla Band, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Medical Center
Boston, Massachusetts 02111

E-MAIL:
Vimla.Band@es.nemc.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

This report contains colored photographs

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The proposed studies are aimed to understand the biochemical mechanism of conversion of normal mammary epithelial cells (MECs) into tumor cells. Previously, we isolated a gene NES1 (Normal Epithelial Cell Specific-1), whose mRNA and protein expression was dramatically decreased in breast cancer cell lines. Homology comparison of this novel peptide showed that NES1 belongs to a family of serine proteases. Significantly, transfection-mediated reconstitution of NES1 expression in NES1-nonexpressing breast cancer cell lines suppressed the oncogenic phenotype and cell spreading on extracellular matrix. Purification of NES1 demonstrated that NES1 is synthesized as Pre-pro-NES1 and is secreted as Pro-NES1. Pro-NES1 can be activated using glandular kallikrein. Glandular kallikrein-activated NES1 cleaves IGFBP-3 as efficiently as other serine proteases. We have generated a large series of NES1 mutants that should determine the structural domains that are necessary for its protease activity as well as tumor suppressor function. Together, these studies should reveal the functional role of a novel serine protease whose expression is down-regulated in breast cancer cells. Thus, these studies should directly contribute to further our understanding of the biology of breast cancer.

14. SUBJECT TERMS
Breast Cancer Mammary epithelial cells Transformation Protease Radiation Tumor Suppressors Immortalization

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

N/A Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

Yes In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

Yes For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Vimla Band 9/17/99
PI - Signature Date

TABLE OF CONTENTS

CONTENTS	PAGE #
Front Cover	1
SF298	2
Foreword	3
Table of contents	4
Introduction	5
Proposed specific aims	5-6
Body of report	6-10
Key research accomplishments	10-11
Reportable outcomes	11
Conclusions	11
References	12
Figures	13-19
Appendix	

INTRODUCTION

Carcinomas, the malignant tumors arising from epithelial cells, constitute the majority of human cancers. Malignant transformation represents a complex multi-step process in which genetic changes and environmental factors, including radiation, viruses, carcinogens and dietary factors, are thought to impinge on common cellular pathways resulting in uncontrolled growth, a hallmark of tumorigenic process [1]. Understanding the nature of these cellular pathways is a central goal in cancer biology. A critical impediment to our understanding of human breast cancer has been a lack of *in vitro* models for biochemical and molecular analyses. We have developed methods and reagents that have allowed establishment of models for mammary tumorigenesis [as reviewed in reference 2-4]. Using these models, we previously isolated a novel serine protease NES1 (normal epithelial specific-1) gene whose mRNA was expressed in all normal MECs but was markedly decreased in breast tumor cells [5]. Genes whose alterations dys-regulate normal cellular functions including cell proliferation, DNA repair, cell-cell and cell-matrix interaction, angiogenesis, senescence and apoptosis form the broad spectrum of cancer genes. Individual genes may be altered in expression either by mutation or by changes in their regulation which provided the basis for grouping cancer genes into two classes: Class I genes are mutated or deleted, whereas Class II genes are not altered at the DNA level. Rather they affect the cellular phenotype through changes in the expression. The Class II gene products affects normal cellular functions, cell-cell or cell-matrix interactions, invasion and metastasis but are not mutated or deleted unlike *bona fide* Class I tumor supressors [as reviewed in reference 6]. Based on lack of major deletions or rearrangement of the gene in tumor cell lines tested, we hypothesize that NES1 is a Class II tumor suppressor gene [6]. Consistent with this classification, reconstitution of NES1 in a NES1-negative breast cancer cell line suppresses the tumorigenic phenotype [7]. Furthermore, NES1 transfectants showed inhibition of morphological branching on extracellular matrix (Fig. 6). We have purified NES1 protein and have shown directly that NES1 is a serine protease (Figs. 1-4). **It is our hypothesis that NES1 cleaves a growth promoting factor and inactivates it, thereby causing growth inhibition and suppression of tumorigenesis.**

PROPOSED SPECIFIC AIMS WERE:

I. CHARACTERIZE THE EXPRESSION OF NES1 IN NORMAL AND TUMOR BREAST CELLS IN CULTURE AND IN TISSUE SPECIMENS.

1. Generate anti-NES1 antibodies.
2. Examine the expression of NES1 mRNA and protein in normal and tumor mammary cells in tissue sections and in cultures.
3. Examine the mechanisms of inducible NES1 expression.
4. Examine the effect of DNA damage on NES1 mRNA and protein expression.

II. ASSESS THE INFLUENCE OF ALTERATIONS IN NES1 EXPRESSION ON CELL GROWTH AND ONCOGENICITY.

1. Transfection of NES1 into mammary cells.

2. Growth properties and oncogenic behavior of NES1 transfectants.
3. Influence of reduced NES1 expression on growth, immortalization and oncogenicity of MECs.

III. CHARACTERIZE THE BIOCHEMICAL FUNCTIONS OF NES1.

1. Characterize the potential protease activity of NES1.
2. Examine non-protease biochemical functions of NES1 protein.
3. Mutational analysis of NES1 protein.

BODY OF THE REPORT

This section describes work accomplished from August 1998 to August 1999.

1) Characterization of NES1 protein and identification of its protease activity (AIM III). As described in detail in last year's annual report, we had expressed NES1 protein in insect cells and purified it using ammonium sulfate fractionation, ion exchange chromatography and gel filtration chromatography. Homology comparison of NES1 with other serine proteases and a computer search using a neural network-based cleavage site prediction program (http://www.cbs.dtu.dk/services/signalP) revealed a putative signal peptide cleavage site between amino acid 33-34 of NES1 polypeptide sequence. The amino terminal sequencing of NES1 purified from insect cells was performed to confirm the cleavage of hydrophobic signal peptide of NES1 during secretion. The N-terminal sequencing of secreted NES1 revealed a sequence of ^{34}ALLPQ^{38}. These results demonstrated that, similar to other serine proteases, NES1 is translated as an inactive pre-pro-NES1 composed of 276 aa including a hydrophobic signal sequence of 33 aa. Upon entry into the secretory pathway, the signal peptide is cleaved yielding the pro form of the protein which includes a propeptide of 9 aa preceding the mature NES1 protein of 234 amino acids (Fig. 1, amino acids 1-52). In an attempt to obtain the activated form of NES1, we performed limited proteolysis (to remove propeptide amino acid 34-42) using low concentrations of trypsin or glandular kallikrein, which are known to have arginine-restricted specificity. Glandular kallikrein treatment clipped the propeptide from NES1, converting it to mature NES1 in a dose-dependent manner (Fig. 2). Proteolytic activity of activated NES1 was measured by the release of resorufin-labeled peptides of Casein Resorufin (Boehringer Mannheim), a universal protease substrate, into TCA soluble fraction (Fig. 2). These results demonstrate that NES1 is proteolytically competent upon activation with glandular kallikrein. In order to define specific substrates for activated NES1 protein, we used a number of para-nitro anilide synthetic substrates (Diapharma Group Inc.) that are known to be specifically cleaved by Plasmin (H-D-Val-Leu-Lys-pNA), Trypsin (H-D-Ile-Pro-Arg-pNA), Tissue plasminogen activator (H-D-Val-Phe-Lys-pNA), Glandular kallikrein (H-D-Val-Leu-Arg-pNA), Plasma kallikrein (H-D-Pro-Phe-Arg-pNA), Elastase(<Glu-Pro-Val-pNA), Chymotrypsin (MeO-Suc-Arg-Pro-Tyr-pNA) and Urokinase (pyro-Glu-Gly-Arg-pNA). NES1 protease failed to cleave any of these synthetic substrates, whereas each of these substrate was clearly cleaved by their respective protease (Fig. 3). In every experiment kallikrein-activated NES1 cleaved Casein Resorufin, indicating that experimental conditions are appropriate for NES1 protease

activity. These data clearly show the specificity of NES1 protease.

2) Protease activity of NES1 against IGFBP-3 (AIM III). As indicated in the original grant, NES1 is highly homologous with serine proteases of the trypsin-like family, kallikrein family and activators of kringle family. The chromosomal localization of NES1 gene places NES1 near the kallikrein family of genes which are clustered on chromosome 19q13.3 [7]. This family includes a well characterized protein, human prostate specific antigen.

It was shown that kallikreins, such as PSA cleave insulin-like growth factor-binding protein-3 (IGFBP-3) [8]. Based on these findings, we examined the potential protease activity of NES1 protein against IGFBP-3 protein. Last year we showed a limited proteolytic activity of NES1 against IGFBP-3. However, we show now that activated-NES1 exhibits high proteolytic activity towards IGFBP-3 (Fig. 4). These data clearly show that NES1 is a serine protease.

3) Expression of NES1 in a second NES1-negative breast cancer cell line MDA-MB-435 results in reduced anchorage-independent growth (AIM II). We have shown earlier [7] that reconstitution of NES1 in MDA-MB-231 cell line inhibits its ability to grow in an anchorage independent manner and its ability to form tumors when implanted in nude mouse [7]. We wanted to explore if this phenomenon is generalable and not specific to just one cell line. For this purpose, we used another breast cancer cell line, MDA-MB-435, that is known to grow in an anchorage independent manner. MDA-MB-435 cells were transfected with NES1, subjected G418 selection, and six independent stable clones each of the vector- and the NES1-transfected MDA-MB-435 cells were tested for the expression of NES1 mRNA and protein. Three clones expressing high levels of NES1 were examined for their ability to grow on soft agar. As shown in Fig. 5, all three NES1-expressing clones exhibited dramatic reduction in growth on soft agar. These data are similar to NES1-transfectants of MDA-MB-231 cells [7]. These clones will be examined for their capability to grow in nude mice assay.

4) NES1 inhibits branching morphogenesis of MDA-MB 231 cells (AIM II). Since NES1 is a serine protease and its overexpression inhibits soft agar growth as well as tumor formation [7 and Fig. 5], we hypothesized that NES1 may cleave an important protein(s) required for cell spreading and migration. To test this hypothesis, NES1 and vector transfected MDA-MB-231 cells were grown in reconstituted basement membrane, matrigel. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major components are laminin, collagen IV proteoglycans, TGFβ, fibroblast growth factor, tissue plasminogen activator and other growth factors [9-11]. Matrigel is commonly used to recreate the *in vivo*-like tissue architectural phenotypes and has proven useful for studying morphogenesis of a number of epithelial tissue types, the most dramatic example being the assembly of structures resembling the alveoli of lactating mammary glands and secretion of milk proteins by mouse mammary epithelial cells when maintained in matrigel [11-12]. Matrigel has also been used successfully to study invasive potential of cancer cells. Previous studies have correlated these morphological changes of cancer cells embedded in matrigel with their metastatic potential [12-14]. Previous studies have shown invasive cells such as MDA-MB 231, 436, 435, BT549 and HS578T cells

exhibit branching morphology in matrigel in contrast to non-invasive cells such as MCF-7 and T-47D that exhibit smooth spherical colonies on matrigel [12-14]. Our hypothesis was that re-expression of NES1 in invasive MDA-MB-231 cancer cells may cause non-invasive normal phenotypic changes. Even though no morphological differences were noticed between vector or NES1 transfected cells when cultured on plastic, we wished to examine if NES1 inhibits the growth of MDA-MB-231 cells when grown on matrigel. As shown in Fig. 6, under these conditions, the two cell types showed striking differences. Vector-transfected cells extended, spread and formed branching structures, whereas NES1-transfected cells aggregated into tight smooth and spherical colonies (Fig. 6 A & B). This distinct behavior was apparent after 24-48 h in culture. Furthermore, the NES1-transfectants did not proliferate readily on the matrix, whereas the vector-transfected cells reached high density and covered the dish over the course of a week. These data clearly demonstrate that NES1 inhibits the invasive phenotype of breast cells.

4) Construction of NES1 mutants using site directed mutagenesis (AIM III). The predicted NES1 polypeptide shows high homology with various families of serine proteases. Importantly, residues surrounding the active site serine are highly conserved (R-D-S-G-G in trypsin and S-D-S-G-G in NES1) [5]. The other two amino acids of the catalytic triad of serine proteases (equivalent to trypsin residues H57 and D102) are also strictly conserved; the corresponding residues in NES1 are H86 and D137 [5]. NES1 also possesses essentially all of the residues that form the specificity pocket of serine proteases, which is involved in substrate binding and specificity [5]. Interestingly, homology search with other proteases also revealed unique amino acid regions in NES1 polypeptide at positions 44-48, 123-130 and 148-151. These homology comparisons provided a clear rationale for mutational analysis of the role of protease activity in NES1 function. Full length NES1 cDNA in pCMV-neo plasmid (used for transfection in MDA-MB-231 cells) was used to perform site-directed mutagenesis using Quick Change site-directed mutagenesis kit (Stratagene). Mutagenic oligonucleotide primers were designed individually according to the desired alanine substitution or point deletion of critical amino acids. The primers were of 30-40 bases in length having desired mutation in the middle of the primer. The melting temperature (Tm) was designed to be greater than 78°C and the primers had a minimum of 40 % GC content. The following mutants were generated.

Mutants of catalytic triad: S229A, ΔS229, D137A, ΔD137, H86A, ΔH86.

Mutants of predicted cleavage site for activation : R42A, ΔR42

Mutants of Substrate binding domain: D223A, ΔD223, P224A, ΔP224, G241A, ΔG241, L243A, ΔL243, W245A, ΔW245

Mutants in conserved residues surrounding catalytic serine: G230A, ΔG230

Mutants in unique regions: Δ44-48, Δ123-130, Δ148-151

A total of twenty-three mutants were made and the entire cDNA sequence was determined. The

desired mutations were confirmed in all cases and no additional mutations were observed. These mutants will be examined for their ability to cleave IGFBP-3 and inhibit tumorigenic phenotypes.

5) Standardization of immunohistochemical technique to detect NES1 expression on in vitro NES1-expressing MDA-MB-231 cells (AIM I). Vector or NES1-transfected MDA-MB-231 cells were examined for NES1 expression using Vectastain ABC Kit protocol (Vector Laboratory). As shown in Fig. 7, NES1-transfected cells showed strong cytoplasmic particulate staining staining with anti-NES1 antibody (indicative of NES1 being a secreted protein) and no staining with control rabbit IgG (Fig. 7B and C). As expected, no staining was seen in vector-transfected cells (Fig. 7A). Importantly peptide competition showed complete inhibition of NES1 staining (Fig. 7C). These experiments show that the antibody that we have raised against a peptide is specific for NES1 and that it is suitable for immunohistochemistry. This antibody will be now used to examine the expression of NES1 protein in normal, primary, and metastatic breast tumor samples.

Methods utilized:

1) Transfection. NES1 cDNA was cloned into the pCMV-neo vector using standard molecular biology procedures. 8 μg of HindIII linearized plasmid was used for transfection into MDA-MB-435 cells using the calcium phosphate co-precipitation method, as described earlier [7]. After G418 (1 mg/ml) selection for 2 weeks, single colonies were isolated using cloning cylinders and subcultured at 1:3 split ratio.

2) Amino-terminal sequence determinations. Purified NES1 from Insect cell supernatant was run on a 10.5 % SDS-PAGE under reducing conditions. Proteins were blotted to PVDF membranes. The membrane was rinsed with 1 % acetic acid and was placed in 0.1 % Ponceau-S (Sigma) for 5 minutes, rinsed sequentially with 1% acetic acid and water. NES1 band at around 30 kDa was isolated and used for sequence determination. Automated amino-terminal sequence determinations were performed in Applied Biosystems 477A protein sequencer (Applied Biosystems, Foster City, CA).

3) Activation of pro-NES1 with recombinant glandular Kallikrein. Pro-NES1 (5 μg) was incubated with increasing doses of rhK2 (0.1-5.0 μg) in 100 μl of 0.1 M Tris-HCl, pH 7.5, 0.1M NaCl. The activation reaction was terminated by the addition of aprotinin (1.0-50 μg, respectively) at various times. Fifty microliters of 4 % casein resorufin and 50 μl of incubation buffer (0.2 M Tris-HCl pH 7.8, 0.02 M $CaCl_2$) was then added and reaction mixture was incubated at 37^0C overnight. Reaction was stopped by adding 480 μl of 5 % TCA, further incubated for 5 minutes, centrifuged for 5 min and 400 μl of reaction supernatant was mixed with 600 μl of assay buffer (0.5 M Tris-HCl, pH 8.8). Absorbance was measured at 574 nm against sample blank.

4) Measurements of enzymatic activity. Hydrolysis of the peptide substrates was measured at 405 nm in a DU series 70 spectrophotometer (Beckman). All reactions were performed at 37^0C

and initiated by the addition of 5 μg of activated NES1 to 1 ml of the appropriate substrate in their specified buffer (composition of buffers were as per Diapharma Group Inc. specifications).

5) IGFBP-3 proteolysis. Proteolytic assays were performed as described earlier [8] in 40 μl PBS with $CaCl_2$ (0.05 mM). Five microgram each of inactivated and activated NES1 was used for proteolytic reaction. 2.5 μl of normal serum was used as negative control whereas pregnancy serum (2.5 μl), NGF and PSA (100 μg/ml) were used as positive controls. It is known that pregnancy serum, NGF and PSA can efficiently cleave IGFBP-3 [8, 16]. ^{125}I IGFBP-3 (30,000 cpm) was added to each digestion mixture and incubated for 6 hours at 37^0C. At the completion of incubation, the digestion mixture was subjected to 12.5 % SDS-PAGE under non-reducing conditions. The gel was then dried and autoradiographed.

6) Matrigel growth experiments. For examining the growth of vector- and NES1-transfectants on matrigel, 1.5×10^4 cells were plated in 24 well plates on the top of 1:1 serum-free medium diluted matrigel substrate (7 mg/ml). Morphological changes were examined after 24-48 hrs using microscopy.

7) Immunohistochemical staining. 10^4 vector or NES1-transfected MDA-MB-231 cells were plated in 8 well chamber slide in α-MEM medium for 48 hours. Cells were washed with phosphate buffered saline, and fixed in 10 % buffered-formalin for 5 min at room temperature. Immunostaining was done using Vectastain ABC kit as recommended by manufacturer (Vector Laboratory). Briefly, cells were washed with PBS three times, treated with 3 % hydrogen peroxides (to quench the endogenous peroxidase activity), washed once with PBS, and incubated with either IgG or 0.2 μg/ml of affinity purified anti-peptide NES1 antibody (NES1 antibody was made in rabbits using amino acids sequences 38-52, reference 5). Peptide competition was performed using 30 fold excess of the peptide for overnight. Color was developed using peroxidase substrate kit (Vector Laboratory), and counter stained with hematoxylin. After dehydration, slides were mounted with Permount, and photographed.

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified a novel serine protease whose expression is down-regulated in breast cancer cell lines.

2. We have demonstrated that NES1 is synthesized as Pre-Pro-NES1, gets secreted as Pro-NES1, and gets activated as NES1 with glandular kallikreins.

3. Similar to other serine protease, activated-NES1 cleaves casein resorufin and IGFBP-3. These experiments demonstrate that NES1 is a serine protease.

4. Reconstitution of NES1 in a NES1-negative tumor cell line dramatically reduced its tumorigenic phenotype (anchorage independent growth and ability to grow in nude mice).

5. Reconstitution of NES1 in a NES1-negative tumor cell line dramatically reduced its ability to morphologically branch on extracellular matrix.

REPORTABLE OUTCOMES

-J Goyal, K M Smith, J M Cowan, D E Wazer, S W Lee and V Band. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res., 58:4782-4786, 1998.

-J Goyal, K M Smith, J M Cowan, D E Wazer, S W Lee and V Band. The role for NES1 serine protease as a novel tumor suppressor. Poster presented by Dr. Goyal at the American Association for Cancer Research, 90th annual meeting, Philadelphia, PA. Proceeding of Am. Asso. Cancer Res. volume 40, p35, 1999.

Summary and Conclusions.

Our efforts to isolate genes that were differentially expressed in a normal mammary epithelial cell strain 76N and its γ-irradiation-transformed derivative, led to isolation of a novel serine protease whose expression was down-regulated in a majority of breast tumor cell lines. Introduction of NES1 into a NES1-negative breast tumor cell lines suppressed the tumorigenic phenotype of these cells, as revealed by an essentially complete suppression of anchorage-independent growth in soft agar and growth as implanted tumors in nude mice. The potential tumor suppressor role of NES1 is further supported by our recent observation that NES1 reconstitution led to inhibition of cells branching on matrigel. NES1 protein characterization showed that NES1 protein is synthesized as Pre-pro-NES1 and then secreted as pre-NES1. Glandular kallikrein is required to fully activate NES1. Glandular kallikrein activated NES1 clearly exhibit serine protease activity against casein resourfin and IGFBP-3. Interestingly, however, NES1 showed no activity against many other synthetic substrates indicating that NES1 may have specific substrate(s). Based on our recent studies, we hypothesize that NES1 cleaves a growth promoting factor and inactivates it, thereby causes growth inhibition and suppression of tumorigenesis.

Altogether, our studies support a novel tumor suppressor function for the serine protease NES1 which is transcriptionally down-regulated during breast tumor progression. Given this information, it is likely that NES1 may also be involved in a critical aspect of regulating normal epithelial cell growth and/or differentiation. This year we plan i) to examine biological substrate for NES1 protein; ii) to examine various mutants of NES1 for protease activity and their ability to have tumor suppressor function; and iii) to examine if reduced NES1 expression in vivo in human breast cancer specimens correlates with tumor progression using immunohistochemical techniques. These studies are designed to examine the tumor suppressor role of NES1 in breast cancer.

LITERATURE CITED:

1. Farber, E. The multistep nature of cancer development. Cancer Res., 44: 4217-4223, 1984.
2. Band V. Preneoplastic transformation of human mammary epithelial cells. Seminars in Cancer Biol. 6:185-192, 1995.
3. Wazer DE and Band V. Molecular mechanisms of immortalization of human mammary epithelial cells. Radiation Oncology Investigations, 3:430-434, 1996.
4. Band V. The role of retinoblastoma and p53 tumor suppressor pathways in human mammary epithelial cell immortalization. Int. J. Oncol., 12:499-507, 1998.
5. Liu, X-L., Wazer, D. E., Watanabe, K., and Band, V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res., 56: 3371-3379, 1996.
6. Sager, R. Expression genetics in cancer:shifting the focus from DNA to RNA. Proc. Natl. Acad. Sci. 94: 952-955, 1997.
7. Goyal, J., Smith, K.M., Cowan, J.M., Wazer, D.E., Lee, S.W. and Band, V. The role of NES1 serine protease as a novel tumor suppressor. Cancer Res. 58: 4782-4786, 1998.
8. Cohen, P., Graves, H. C. B., Peehl, D. M., Kamarei, M., Giudice, L. C., and Rosenfeld, R. G. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. 75: 1046-1053, 1992.
9. Kleinman, H.K.,McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K., Martin, G.R. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21:6188-6193, 1982.
10. McGuire, P.G., Seeds, N.W. The interaction of plasminogen activator with a reconstituted basement membrane matrix and extracellular macromolecules produced by cultured epithelial cells. J. Cell. Biochem. 40:215-227,1989.
11. Lin, C.Q, and Bissell, M.J. Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J. 7:737-743, 1993.
12. Terranova, V.P., Hujanen, E.S., Martin, G.R. Basement membrane and the invasive activity of metastatic tumor cells. J. Natl. Cancer Inst. 77:311-316 1986.
13. Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M. and McEwan, R.N. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res.47: 3239-3245, 1987.
14. Kramer,R.H., Bensch,K.G., and Wong, J. Invasion of reconstituted basement membrane matrix by metastatic human tumor cells. Cancer Res. 46: 1980-1986, 1986.
15. Thompson, E.W., Paik, S., Brunner, N., Sommers, C.L., Zugmaier, G, Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., Martin, G.R., and Dickson, R.B. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J. Cellu. Phys. 150:534-544, 1992.
16. Rajah, R., Bhala, A., Nunn, S. E., Peehl, D. M. and Cohen, P. 7S nerve growth factor is an insulin-like growth factor-binding protein protease. Endocrinol. 137:2676-2682, 1996.

↱ Pre-pro-NES1(-42)

1	M	R	A	P	H	L	H	L	S	A	A	S	G
14	A	R	A	L	A	K	L	L	P	L	L	M	A

↱ Pro-NES1(-8)

27	Q	L	W	A	A	E	A	A	L	L	P	Q	N

↱ Mature NES1(+1)

40	D	T	R	L	D	P	E	A	Y	G	A	P	C

Fig. 1. Amino acid sequences of N-terminus of NES1. Pre-pro-NES1 contains a hydrophobic signal peptide (aa 1-33) that gets cleaved while secretion. Pro-NES1 contains eight amino acids (34-42) that is cleaved to activate NES1 as a protease.

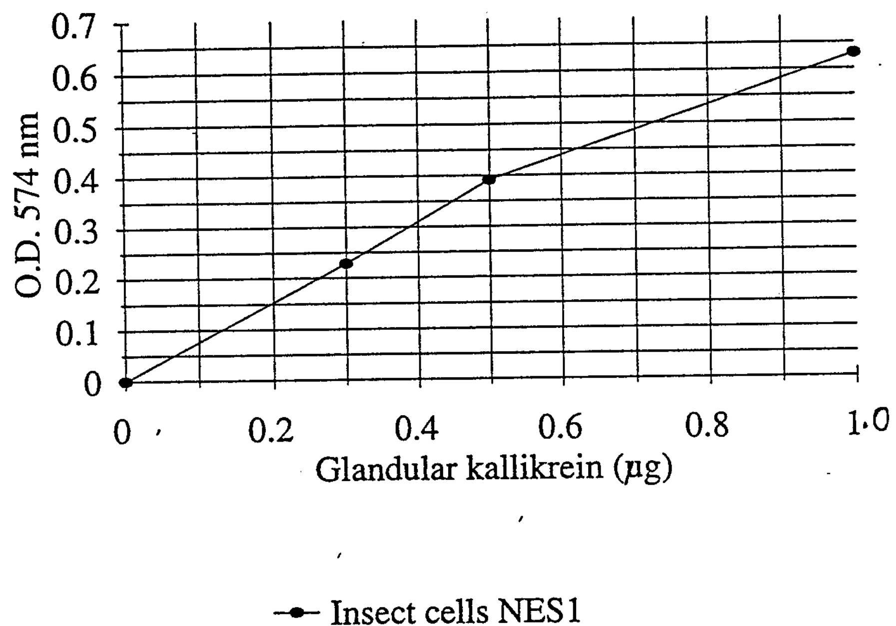
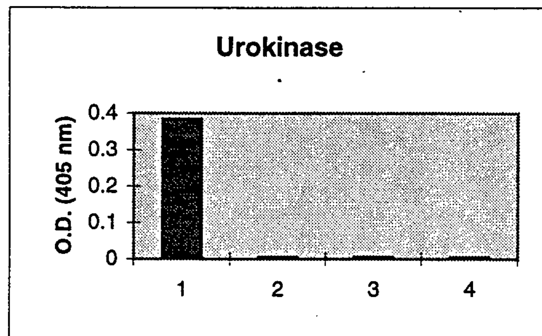
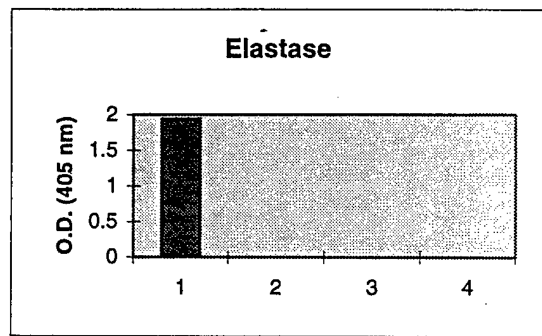
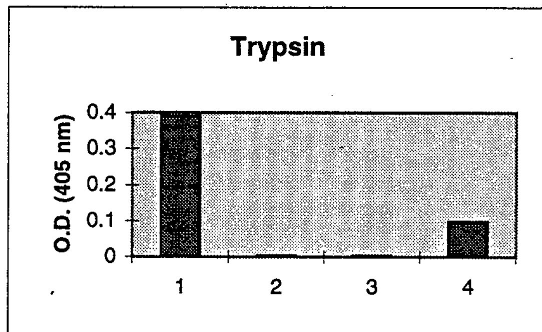
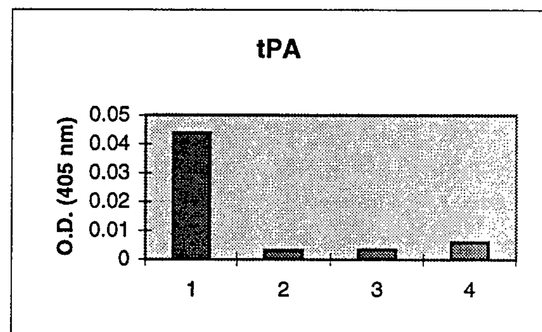


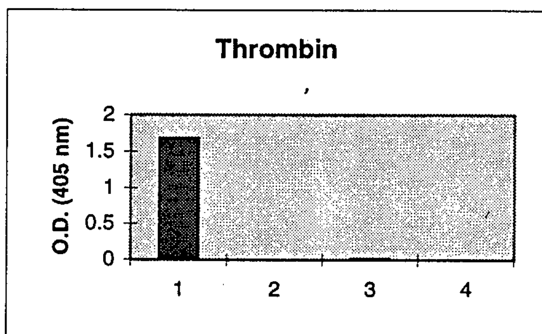
Fig.2. Protease activity of NES1 purified from insect cell supernatant. Increasing concentration of glandular kallikrein was used to activate NES1 (5µg). Activated NES1 in Tris buffer (0.2 M Tris-HCl, pH 7.8, 0.02 M CaCl2) was incubated with 50 µl of 0.4% Casein Resorufin substrate at 37°C. Reaction was stopped with 5% TCA and absorbance was read at 574 nm.

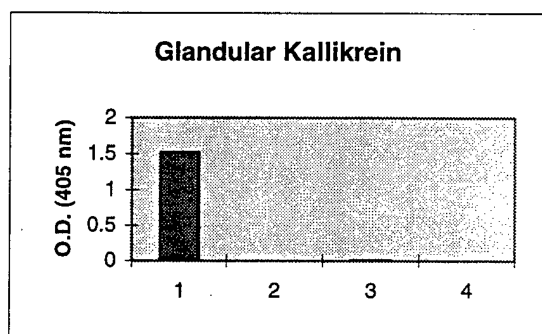


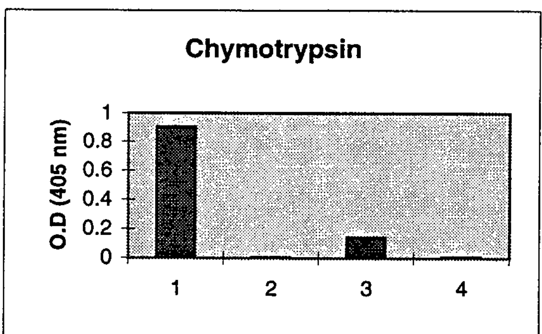












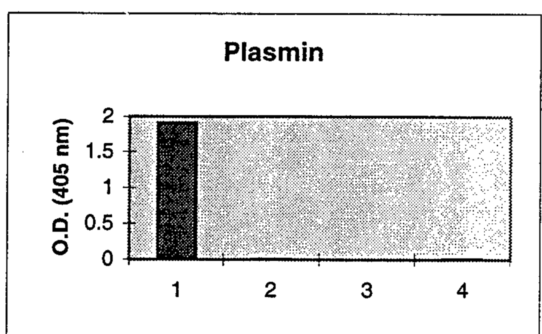


Fig. 3. Proteolytic activity of NES1 (5 μg) against the para-nitroanilide synthetic substrates against the indicated proteins (as described in Results). Reactions were performed as described in Methods. Increase in absorption was measured at 405 nm. 1. Positive Control 2. Inactive-NES1 3. Activated-NES1 4. Negative Control.

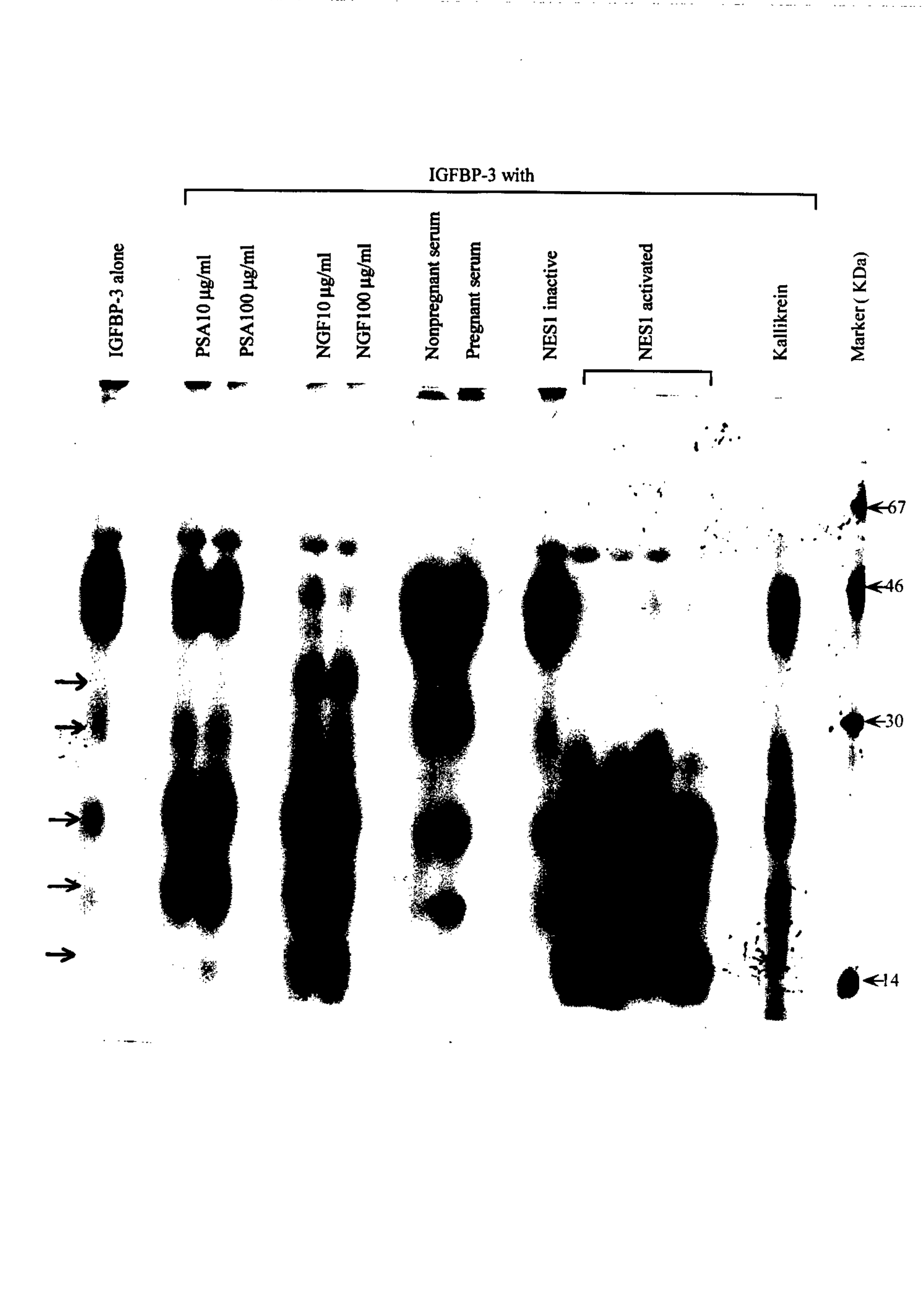
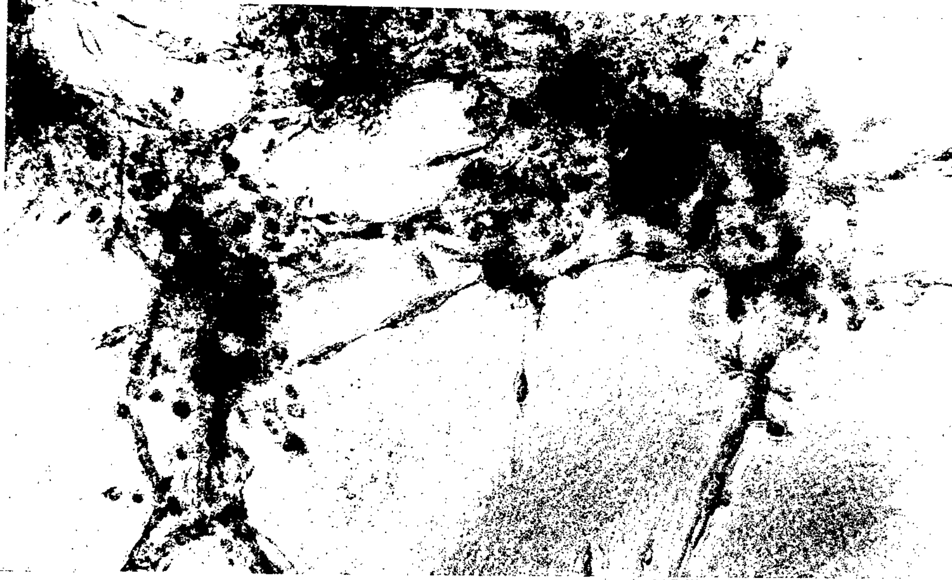


Fig. 4. Demonstration of proteolytic activity of NES1 against [^{125}I]IGFBP-3. 30,000 cpm of [^{125}I]IGFBP-3 was incubated for 6 hours at 37°C with either alone or with PSA (10 or 100 μg/ml), NGF (10 or 100 μg/ml), non-pregnant serum (2.5 μl), pregnant serum (2.5 μl), inactive NES1 (5 μg), glandular kallikrein (1 μg), or glandular kallikrein-activated NES1 protein (5 μg NES1 activated with 1 μg of kallikrein) as indicated. The digestion mixture was subjected to 12.5 % SDS-PAGE, and autoradiographed. Arrows indicate proteolytic products.

Fig. 5. Anchorage-independent growth of NES1-transfected clones. Three clones of vector-transfected (lower row) or NES1-transfected (upper row) MDA-MB-435 cells (1x 10^5/60-mm dish) were plated in soft agar, colonies were photographed after 2 weeks. All of the three vector-transfected clones formed colonies when grown in soft agar, whereas the clonogenicity of the three NES1-transfectants was markedly decreased.

A.



B.

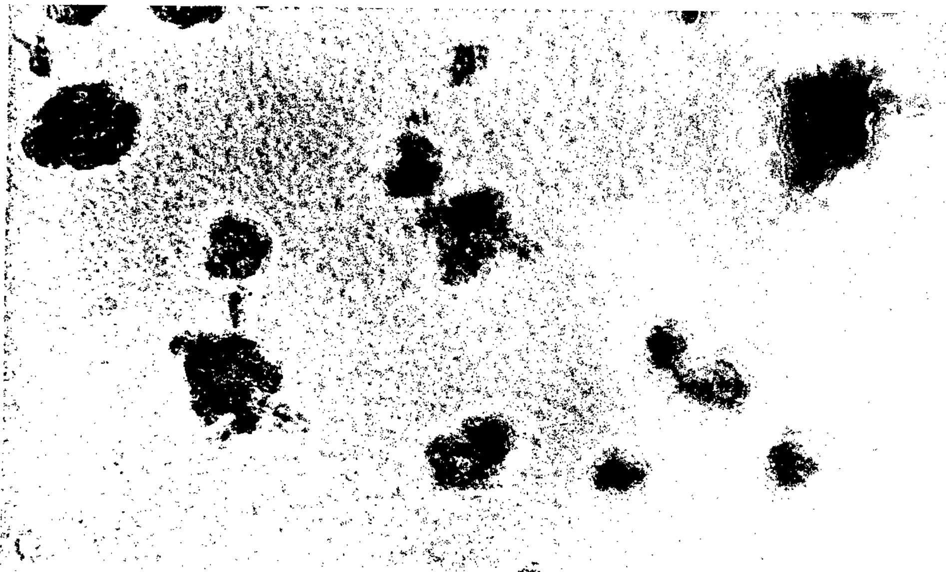


Fig 6A. &B. Branching morphogenesis of MDA-MB-231 cells on matrigel. $1.5x10^4$ Vector-transfected (A) and NES1-transfected (B) cells were cultured on matrigel (1:1 diluted with serum free medium) in 24 well plates. Cells were photographed after six days.

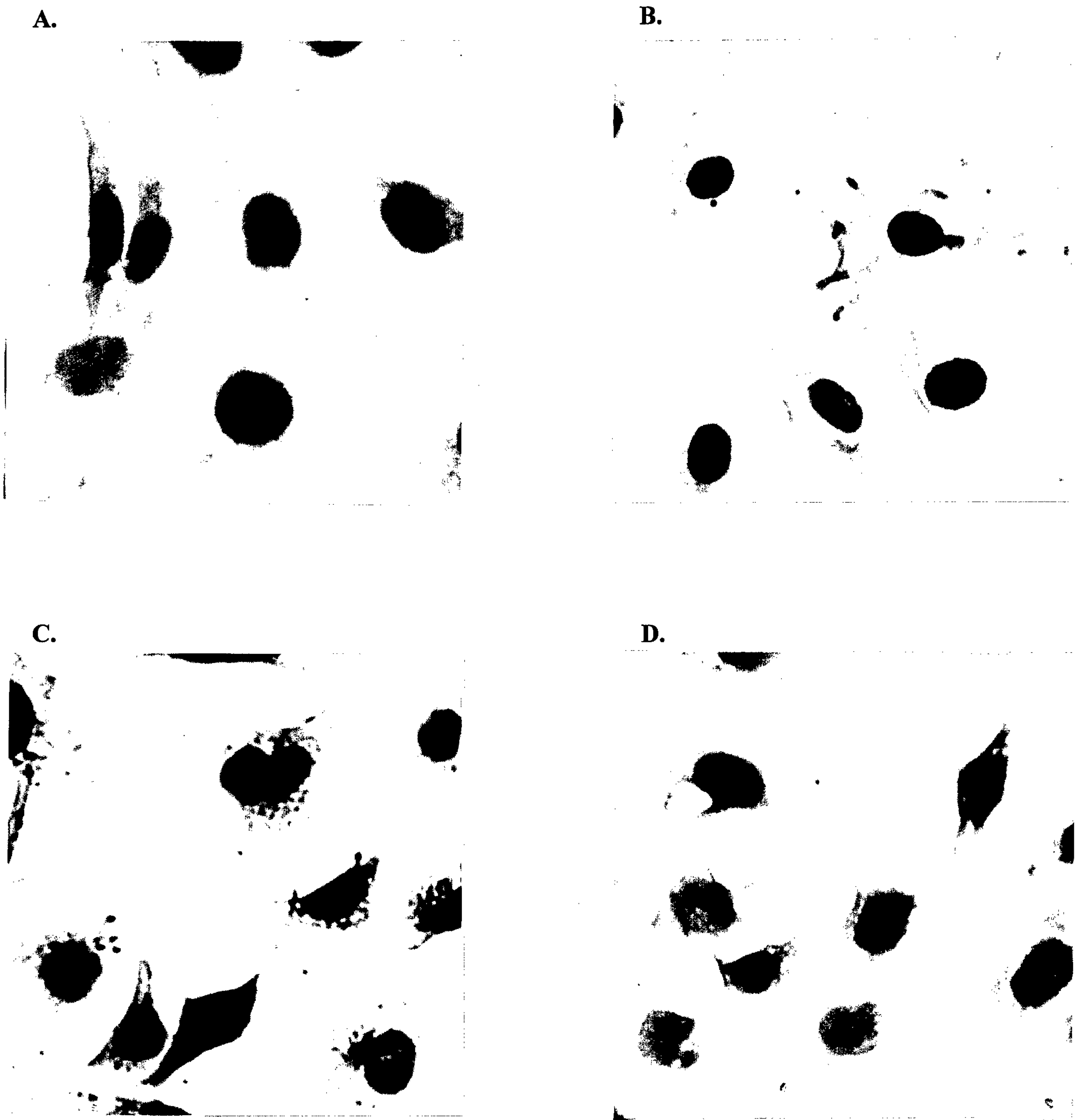


Fig.7. Analysis of expression of NES1 protein by immunohistochemistry. Vector (A), or NES1-transfected (B,C,D). MDA-MB-231 cells were grown on 8-well plates, fixed, treated with control rabbit IgG (B) or purified anti-NES1 peptide antibody (A,C,D) and stained using Vectastain kit. Panel D shows competition with 30 fold excess of NES1 peptide.

[CANCER RESEARCH 58, 4782–4786, November 1, 1998]

Advances in Brief

The Role for NES1 Serine Protease as a Novel Tumor Suppressor[1]

Jaya Goyal, Karen M. Smith, Janet M. Cowan, David E. Wazer, Sam W. Lee, and Vimla Band[2]

Departments of Radiation Oncology [J. G., D. E. W., V. B.], and Pediatrics [J. M. C.], New England Medical Center, and Department of Biochemistry [V. B.], and Genetics Program [K. M. S., V. B.], Tufts University School of Medicine, Boston, Massachusetts 02111, and Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115 [S. W. L.]

Abstract

Previously (Liu *et al.,* Cancer Res., *56:* 3371–3379, 1996), we isolated a novel serine protease-like gene—Normal Epithelial Cell Specific-1 (*NES1*)—that is expressed in normal mammary epithelial cells but is down-regulated in most breast cancer cell lines. Here, we demonstrate that stable expression of NES1 in the NES1-negative MDA-MB-231 breast cancer cell line suppressed the oncogenicity as revealed by inhibition of the anchorage-independent growth and tumor formation in nude mice. Fluorescence *in situ* hybridization localized the *NES1* gene to chromosome 19q13.3, a region that contains genes for related proteases (including the prostate-specific antigen) and is rearranged in human cancers. Similar to breast cancer cell lines, prostate cancer cell lines also lacked NES1 mRNA and protein expression. Together, these results strongly suggest a tumor-suppressor role for NES1 in breast and prostate cancer.

Introduction

Carcinomas, the malignant tumors arising from epithelial cells, constitute the majority of human cancers. In nearly all cases, the etiology of these cancers is unknown. Malignant transformation represents a complex multistep process in which genetic changes and environmental factors including radiation, viruses, carcinogens, and dietary components are considered to play a role (1).

To gain insight into biochemical pathways involved in epithelial cell oncogenesis, we and others have used *in vitro* models of epithelial cell transformation. In one such model, we exposed the normal mammary epithelial cell strain 76N to fractionated doses of γ-irradiation *in vitro,* which was similar to a therapeutic regimen used for cancer treatment and which resulted in the tumorigenic conversion of the cells (2). To isolate genes the products of which contributed toward oncogenic transformation in this system and to identify differentially expressed mRNAs, we carried out subtractive hybridization between the normal parental cell strain 76N and its radiation-transformed cell line 76R-30. This strategy resulted in the isolation of a novel putative serine protease, NES1,[3] the mRNA expression of which was observed in 76N cells but was down-regulated in 76R-30 cells (2). Using a panel of normal and tumor mammary epithelial cell lines, we showed that the expression of NES1 mRNA and protein was absent in a majority of breast tumor cell lines (2).

The predicted NES1 polypeptide showed high homology to a number of serine proteases (3), in particular the members of the trypsin family, the kallikrein family, and the family of proteases that activate the kringle domain-containing growth factors (4–6). The kallikrein family includes the PSA that is increased in the serum of prostate cancer patients and serves as a prognostic marker (7, 8). The kringle domain-containing growth factors include human tissue plasminogen activator and human hepatocyte growth factor activator, which have been linked to oncogenesis (5, 6, 9, 10). The former is increased during tumorigenic progression of cells, whereas the latter is a mitogenic growth factor for a known proto-oncogene, c-*met* (hepatocyte growth factor receptor; Ref. 11). The involvement of close homologues of NES1 in oncogenic transformation suggests a potential function for NES1 in cell growth.

Here, we show that the transfection-mediated reconstitution of NES1 expression in a NES1-negative breast tumor cell line, MDA-MB-231, results in suppression of the tumorigenic phenotype both *in vitro* and *in vivo.* The *NES1* gene localized to chromosome 19q13.3 within the same region that contains *PSA* (8). Similar to mammary epithelial cells, NES1 mRNA and protein were expressed in normal and immortal prostatic epithelial cells but not in tumorigenic prostate epithelial cell lines. Together, these data suggest that NES1 plays a tumor suppressor role in breast, prostate, and possibly other epithelial cells.

Materials and Methods

Tissue Sample and Cell lines. Normal prostate tissue was from a prostactomy specimen obtained from the Beth Israel Deaconess Medical Center (Boston). Normal prostate epithelial cells immortalized with the human papilloma virus type 18 (designated as HPV18-I cells) or SV40 large T antigen (SV40-I; obtained from Dr. J. S. Rhim, National Cancer Institute, Frederick, MD; Refs. 12, 13), the ND1 prostate cancer cell line (obtained from Dr. P. Narayan, University of Florida, Gainesville, FL; Ref. 14), and the MDA-MB-231 breast cancer cell line (obtained from Dr. Ruth Lupu, Lawrence Berkeley National Laboratory, Berkeley, CA) have been described (15). PC3, DU-145, and LNCaP prostate cancer cell lines were obtained from American Tissue Type Collection (Manassas, VA). All of the cell lines used in this study were grown in α-MEM with 10% FCS (16).

Transfection. *NES1* cDNA was cloned into pCMV-neo vector and 8 μg of *Hin*dIII linearized plasmid was used for transfection into MDA-MB-231 cells using the calcium phosphate coprecipitation method as described earlier (16). After G418 (1 mg/ml) selection for 2 weeks, single colonies were isolated and subcultured at 1:3 split ratio.

Northern Blotting. Total cellular RNA was isolated from subconfluent monolayer cells using the guanidinium-isothiocynate method. Ten μg of each RNA was resolved on formaldehyde-1.2% agarose gel and was transferred onto a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Hybridization was carried out with ^{32}P-labeled *NES1* cDNA probe (nucleotide 651-1072) as described previously (2).

Western Blotting. Culture supernatants from vector- and *NES1*-transfected cells were collected for 24 h after serum deprivation. Fifty μg (breast cells) or 300 μg (prostate cells) of each supernatant [protein quantitation done using a Bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL)] were separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P Millipore, Marlborough, MA). The membranes were blocked with TBST (25 mM Tris, 0.15 M NaCl, and 0.1% Tween 20) containing 5% each of nonfat dry milk and BSA, incubated with either rabbit anti-NES1 antibody or monoclonal antibody against PSA (Ab-2, Neomarkers, Fremont, CA), followed by either goat antirabbit IgG or goat anti-

Received 7/23/98; accepted 9/17/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Department of Defense Breast Cancer Program.

[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, Box 824, New England Medical Center, 750 Washington Street, Boston, MA 02111. Phone: (617) 636-4776; Fax: (617) 636-6205; E-mail: VBAND@opal.Tufts.Edu.

[3] The abbreviations used are: NES1, normal epithelial specific-1; PSA, prostate-specific antigen; FISH, fluorescence *in situ* hybridization; IGF, insulin-like growth factor; IGFBP-3, insulin-like growth factor-binding protein 3; DAPI, 4′,6-diamidino-2-phenylindole; DAPI-II, DAPI with *P*-phenylenediamine in PBS and glycerol.

mouse IgG-horseradish peroxidase conjugates, respectively. Enhanced chemiluminescence detection was performed according to manufacturer's instructions (Amersham, Arlington Heights, IL).

Anchorage-independent Growth. MDA-MB-231 cells transfected with either vector or *NES1* were plated at 1×10^5/60-mm dish in a top layer of 0.3% agar (Bacto Agar, Difco, Detroit, MI) with a bottom layer of 0.5% agar (both in 1× complete MEM medium). Two ml of complete medium were added on top on every 4th day; plates were examined for clonal growth under a microscope every other day and photographed after 2 weeks.

Tumor Formation in Nude Mice. *Balb/C* nude mice (ages 4–6 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to adjust to the institutional animal facility for 1 week before the experiment was initiated. Subconfluent cultures of vector or *NES1*-transfected MDA-MB-231 cells were released with trypsin/EDTA (0.025% trypsin and 0.01% EDTA) and resuspended in normal saline. Five $\times 10^5$ cells in a volume of 0.2 ml were injected s.c. into the mammary fat pad area (below the nipple) of five mice per group. Mice were examined on alternate days for the presence of any palpable tumor growth, and tumor sizes were measured. Tumors were allowed to grow for 4 weeks, at which time mice were photographed and killed.

FISH. FISH was performed using a commercial kit following the procedures recommended by the supplier (Oncor, Inc., Gaithersburg, MD). A 140-kb human genomic DNA from bacterial artificial chromosome (Bac) library (obtained through Research Genetics Inc, Huntsville, AL., by screening the library with a *NES1* cDNA probe) was used as a probe for FISH analysis. 300 ng of genomic *NES1* DNA probe was labeled with biotin-14-dCTP (Oncor, Inc) using the Bioprime DNA labeling system (Life Technologies, Grand Island, NY). The time and concentration of DNA were adjusted to produce biotinylated products of 100–500 bp in length as judged by agarose gel electrophoresis. The labeled DNA was purified through a microspin S-200 HR column (Pharmacia Biotechnologies, Piscataway, NJ) and precipitated with 3 M sodium acetate-ethanol in the presence of human COT-1 DNA (human placental DNA from Life Technologies) and salmon sperm DNA to block nonspecific hybridization due to any repetitive human sequences in the probe. The probe was resuspended in Hybrizol VI hybridization solution (Oncor, Inc). Before hybridization, the probe was denatured at 72°C for 10 min., preannealed for 30 min at 37°C, and chilled on ice.

Cytogenetic metaphase chromosome preparations were prepared from human peripheral blood lymphocytes stimulated with phytohemagglutin (17). Before denaturation, the slides were pretreated in 2× SSC [1× = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)] for 30 min at 37°C followed by dehydration in an ethanol series (70–95%). Slides were denatured in 70% formamide, 2× SSC (pH 7.0) at 70°C for 2 min, dehydrated in an ice-cold ethanol series (70–95%), and air-dried. The denatured probe was added to the slide, covered with a glass coverslip, and sealed with rubber cement. The probe was hybridized in a moist chamber at 37°C overnight. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and antiavidin repetitive amplification steps (Oncor, Inc.). The slides were mounted in antifade medium containing DAPI-II (Vysis, Inc., Downers Grove, IL) for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC, DAPI, and propidium iodide.

Results

***NES1*-transfected Cells Lack Anchorage Independence as well as Ability To Grow in Nude Mouse.** The pattern of NES1 expression—with high levels in normal cells, reduced levels in radiation-transformed 76R-30, and an essentially complete lack of expression in most mammary tumor cell lines (2)—suggested a potential role of NES1 in tumor suppression. To directly assess whether NES1 can function as a tumor-suppressor protein, we introduced either pCMV-neo vector or *NES1*-pCMVneo plasmids into a breast cancer cell line, MDA-MB-231. The choice of the MDA-MB-231 cell line was based on its lack of NES1 expression and its known ability to grow in an anchorage-independent manner as well as its ability to form tumors when implanted in nude mouse (2, 15). After G418 selection, six independent stable clones each of the vector and the *NES1*-transfected MDA-MB-231 cells were tested for the expression of NES1 mRNA and protein. As shown in Fig. 1*A*, 4 (clones 2, 4, 5, and 6) of 6 *NES1*-transfected MDA-MB-231 clones expressed high levels of NES1 mRNA whereas the remaining two (clones 1 and 3) showed very little or no mRNA expression. When analyzed for NES1 protein secretion, four clones (clones 2, 4, 5, and 6) showed considerably high levels of protein as compared to two other clones (clones 1 and 3; Fig. 1*B*). As expected, none of the vector-transfectants showed any NES1 protein (*Lane 1* and data not shown). On the basis of the protein expression, the three strong positive clones (clones 2, 4, and 5) were used for further analyses to examine the effect of NES1 on tumorigenicity.

Previous studies (15) have demonstrated a direct correlation between the tumorigenic phenotype of cancer cell lines such as MDA-MB-231 and the ability to grow in an anchorage-independent manner and to form tumors when implanted into nude mice. As shown in Fig. 2*A*, all of the three vector-transfected clones formed colonies when grown in soft agar, whereas the clonogenicity of the three *NES1*-transfectants was markedly decreased. The experiment was repeated three times, each in triplicate, with similar results. Thus, overexpression of NES1 in a NES1-negative breast cancer cell line abolished the ability of cells to grow in an anchorage-independent manner.

When 5×10^5 cells of *NES1*-transfectant clone 4 or a vector-transfectant were injected s.c. into the mammary gland area of mice, 5 of 5 vector-transfected mice showed palpable tumors within 8–10 days of injection, and these grew progressively, reaching a 2.0×2.0 cm^2 size by 4 weeks (Fig. 2*B*). The tumor from one vector transfectant-implanted mouse was excised to examine for histopathology and to assess the ability of tumor cells to grow *in vitro*. These experiments demonstrated that the tumor was an adenocarcinoma, as expected

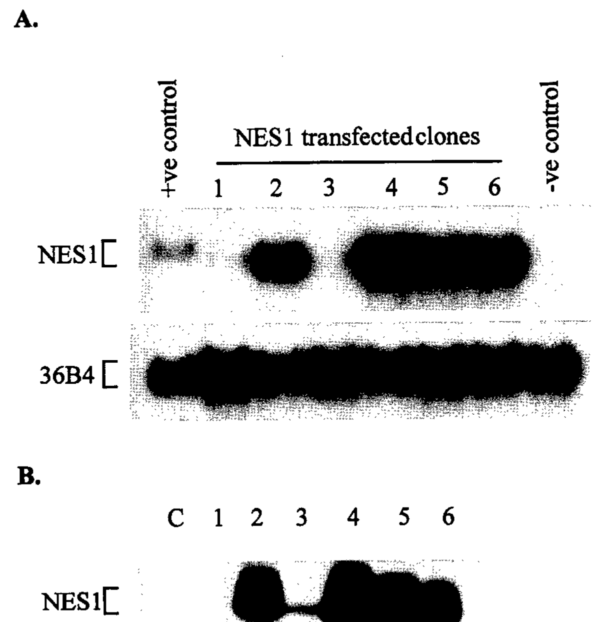


Fig. 1. *A*, Northern blot analysis of NES1 mRNA expression in *NES1*-transfected MDA-MB-231 cells. Nylon membranes with 10 μg of total RNA from different *NES1*-transfected clones (clones 1–6) of MDA-MB-231 cells were hybridized with a ^{32}P-labeled NES1 probe and visualized by autoradiography. Positive (+*ve*) control, normal mammary epithelial 76N cells; negative (-*ve*) control, vector-transfected MDA-MB-231 cells. 36B4 probe was used as a loading control. *B*, Western blot analysis of NES1 protein in *NES1*-transfected MDA-MB-231 cells. Aliquots of culture supernatant derived from different *NES1*-transfected clones (clones 1–6) of MDA-MB-231 cells containing 50 μg of protein were resolved by a SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were immunoblotted with an anti-NES1 antiserum followed by goat antirabbit IgG conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence. Control (*C*), supernatant from vector-transfected MDA-MB-231 cells.

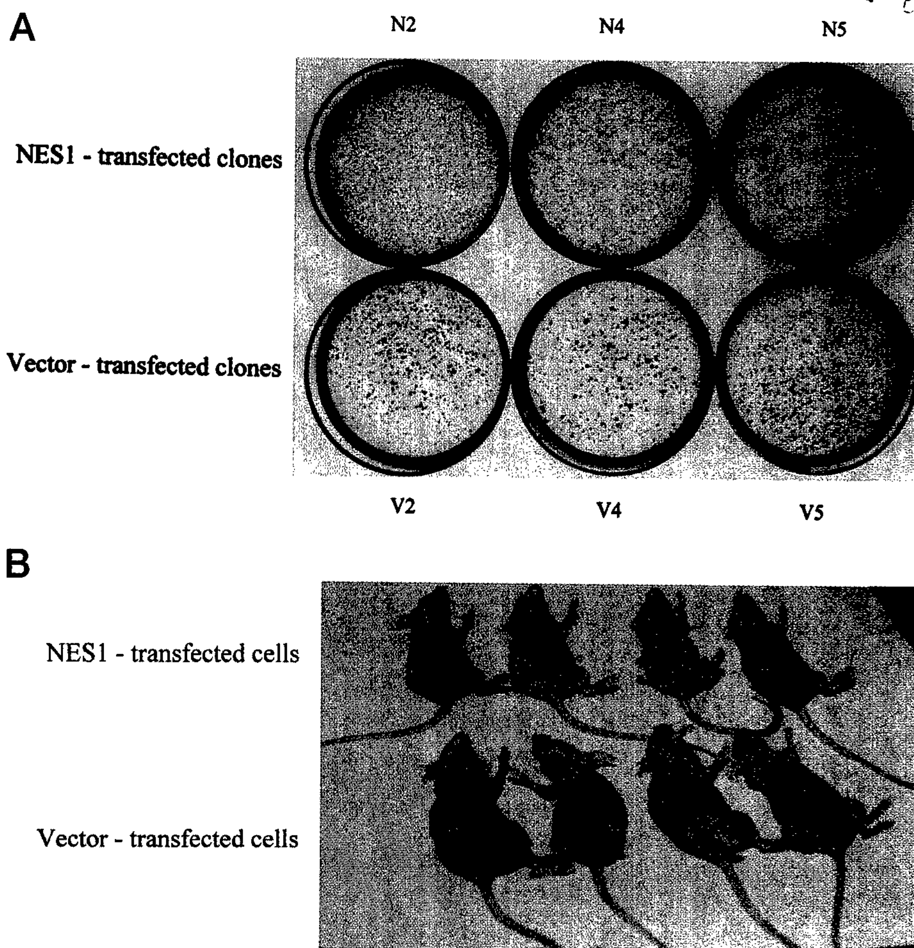


Fig. 2. *A*, anchorage-independent growth of *NES1*-transfected clones. Various clones of vector-transfected (*V2, V4, V5*) or *NES1*-transfected (*N2, N4, N5*) MDA-MB-231 cells (1×10^5/60-mm dish) were plated in soft agar, and colonies were photographed after 2 weeks. *B*, growth of vector- or *NES1*-transfected MDA-MB-231 cells as tumors upon implantation in nude mice. Five $\times 10^5$ cells of vector or *NES1*-transfected MDA-MB-231 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 4 weeks at which time the mice were photographed and killed.

(15), and these cells proliferated in cell culture (data not shown). In contrast to vector-transfected MDA-MB-231 cells, none of the mice implanted with *NES1*-transfected MDA-MB-231 showed any palpable or visible tumors by 4 weeks. The mice were then killed, and the injected area was examined thoroughly for any nonpalpable tumor growth. However, no tumor growth was observed. Taken together, these data clearly demonstrate the ability of NES1 to suppress tumorigenesis in MDA-MB-231 cells.

Localization of the *NES1* Gene to Chromosome 19q13.3 by FISH Analysis. The potential role of NES1 as a tumor suppressor suggested by the above experiments prompted us to examine the chromosomal localization of the *NES1* gene. For this purpose, we performed pulsed-field gel electrophoresis of *Not*I-digested Bac clone and found that it contained a 140-kb *NES1* DNA insert. Southern blotting of an *Eco*RI-digest of DNA isolated from several single colonies of the Bac clone compared with genomic DNA of 76N normal mammary epithelial cell strain using a *NES1* cDNA probe indicated that the 140-kb genomic clone contained most of the *NES1* gene (data not shown). This 140-kb probe was used for FISH analysis. Thirty-one metaphase spreads were analyzed for chromosomal localization of NES1. In all metaphase spreads, hybridization signals were found on both copies of the 19q (Fig. 3, as indicated by *arrows*). Longer metaphase spreads allowed localization of the *NES1* gene to 19q13.3, and 90% of these spreads had both chromatids stained. Propidium iodide counterstaining to evaluate chromosomal banding confirmed the presence of NES1 on 19q13.3 (data not shown). This analysis localized *NES1* gene to the same region where the gene for prostate cancer-associated serine protease PSA is localized (8). Furthermore, this region is known to undergo loss of heterozygosity in solid tumors including pancreatic carcinomas, astrocytoma, ovarian cancer, and thyroid tumors (17), consistent with a potential tumor-suppressor role of NES1.

NES1 mRNA and Protein Expression Is Down-Regulated in Prostate Cancer Cell Lines. The localization of *NES1* gene to chromosome 19q13.3, where PSA is localized, prompted us to analyze the status of NES1 expression in normal, immortal, and tumor cells derived from the prostate gland. Northern blot analysis revealed that, although NES1 was expressed at high levels in normal tissue from a prostactomy specimen and two HPV-18 and SV40-immortalized normal prostate cell lines, four of four prostate tumor cell lines completely lacked the NES1 mRNA expression (Fig. 4*A*). Importantly, the analysis of NES1 protein in the supernatants of the immortalized prostate cells secreted significant levels of NES1 protein, whereas no protein was detected in the supernatants of prostate tumor cell lines (Fig. 4*B*). Importantly, when these supernatants were tested for PSA levels, a significant level of PSA protein was detected in one of the four (LNCaP) prostate cancer cell lines. These data are consistent with the published reports on the secreted levels of PSA in LNCaP cells (13). Thus, similar to our previous results in breast cells, NES1 mRNA expression is down-regulated during tumorigenic progression in prostate cancer. Taken together, these experiments suggest that NES1 may play a tumor-suppressor role in breast as well as prostate cancer.

Discussion

Our efforts to isolate genes that were differentially expressed in a normal mammary epithelial cell strain 76N and its γ-irradiation-

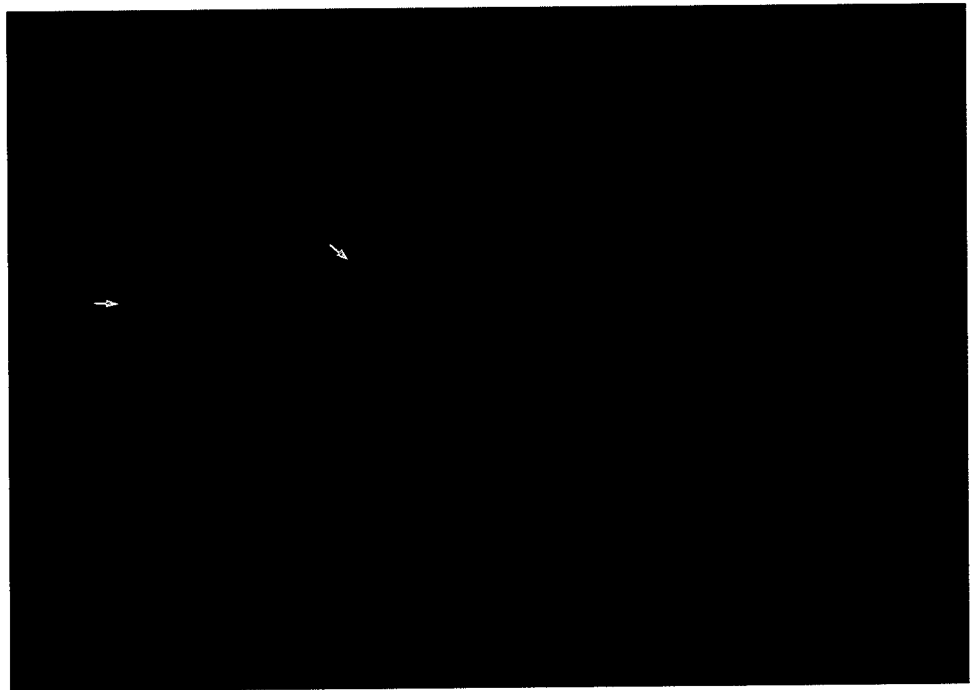


Fig. 3. Localization of *NES1* gene by FISH analysis. FISH analysis was performed using a commercial kit following the procedures recommended by the supplier (Oncor, Inc.) using a biotin-14-dCTP labeled-140-kb *NES1* genomic DNA probe. Cytogenetic metaphase chromosome preparations were from human peripheral blood lymphocytes stimulated with phytohemagglutin. Immunocytochemical detection of the hybridized probe was achieved using avidin-FITC and antiavidin repetitive amplification steps. The slides were mounted in antifade medium containing 4,6-diamino-2-phenylindole DAPI-II for counterstaining of the chromosomes. Chromosomes were evaluated under a Zeiss fluorescence microscope equipped with appropriate filters for the visualization of FITC and DAPI. *Arrows* point to *NES1*-specific hybridization signals in the telomeric region of the long arm of both chromosomes 19 at band q13.3.

transformed derivative, led to the isolation of a novel serine protease whose expression was down-regulated in a majority of breast tumor cell lines (2). Here, we demonstrate that, similar to breast cancers, NES1 expression is also down-regulated at the mRNA level in prostate cancer-derived cell lines, whereas both the mRNA and NES1 proteins are abundantly expressed in immortal prostate epithelial cell lines. Together, the down-regulation of NES1 expression during tumorigenic progression in two different epithelial tumors further supports the likelihood that NES1 functions in regulating cell proliferation, differentiation, or other traits that are deregulated during oncogenesis.

On the basis of its lack of expression in breast cancer cells, we examined the consequence of *NES1* transfection into a NES1-negative breast tumor cell line MDA-MB-231, which allowed an assessment of several aspects of tumorigenic phenotype (2). We demonstrate that *NES1* gene indeed suppressed the tumorigenic phenotype of these cells, as revealed by an essentially complete suppression of anchorage-independent growth in soft agar and growth as implanted tumors in nude mice. This effect was not due to an overall suppression of cell proliferation, inasmuch as vector-transfected cells and *NES1*-transfected cells grow comparably under regular culture conditions.

The potential tumor suppressor role of NES1 is further supported and expanded by our studies using FISH analysis, which localized *NES1* gene on chromosome 19 region *q13.3*. As we have reported earlier, NES1 is highly homologous with serine proteases of the trypsin-like family, kallikrein family, and activators of kringle family. The chromosomal localization of *NES1* gene places NES1 in the kallikrein family of genes that are clustered on chromosome 19q13.3. These results suggest that NES1 may be derived by gene duplication from a common ancestor of the kallikrein family of serine proteases. In humans, this family includes four members: (*a*) human renal/pancreatic kallikrein; (*b*) human glandular kallikrein; (*c*) human PSA; and (*d*) the recently isolated protease M (18). Notably, except for protease M, all of the other members of this family are secreted proteins (2, 3, 18). Interestingly, similar to NES1, protease M also exhibits a reduced expression in breast and prostate tumor cell lines compared with normal cells (18). Although PSA levels are elevated in the serum of prostate cancer patients and their high levels represent a

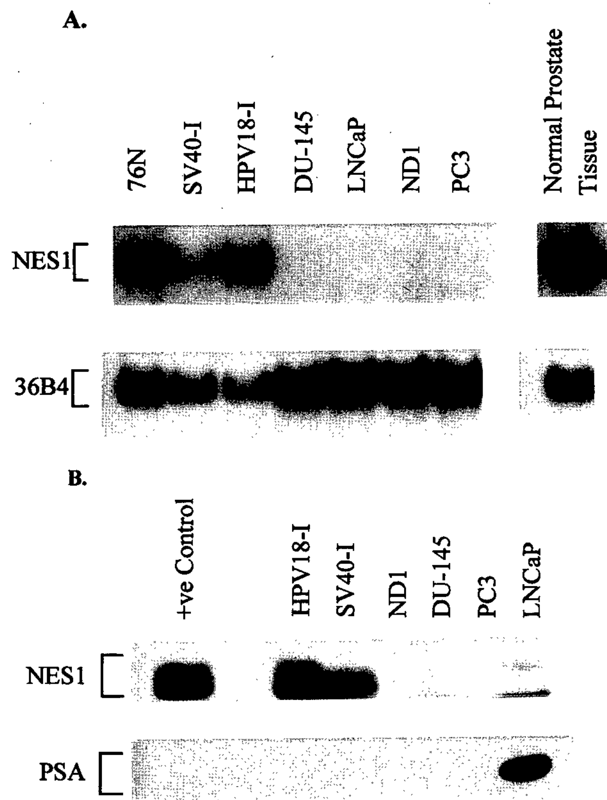


Fig. 4. *A*, Northern blot analysis of NES1 mRNA expression in prostate-derived normal, immortalized, and tumor epithelial cells. Nylon membranes with 10 μg of total RNA from normal prostate tissue, immortalized prostate cell lines SV40-I and HPV18-I, and prostate tumor cell lines DU-145, LNCaP, ND1, and PC3, were hybridized with a ^{32}P-labeled NES1 probe and visualized by autoradiography. 76N normal mammary epithelial cell strain was used as a positive control. 36B4 probe was used as a loading control. *B*, Western blot analysis of NES1 protein in prostate-derived immortalized and tumor epithelial cell lines. Aliquots of culture supernatants derived from different cells (as indicated above) containing 300 μg of protein were resolved by a SDS-10% PAGE and transferred to polyvinylidene difluoride membrane. Membranes were immunoblotted either with an anti-NES1 antiserum (*above panel*) or with an anti-PSA monoclonal antibody followed by goat antirabbit IgG or goat antimouse IgG horseradish peroxidase conjugates, respectively. Detection was by enhanced chemiluminescence. Positive (*+ve*) control, supernatant from *NES1*-transfected MDA-MB-231 cells.

marker of poorer prognosis (7, 8), PSA was reported as marker of better prognosis in breast cancer (19). Thus, two additional members of the kallikrein family that colocalize on 19q13 with NES1 also appear to be closely related to the process of human oncogenesis. Finally, 19q13 is known to be rearranged in a variety of human solid tumors including pancreatic carcinomas, astrocytomas, ovarian cancers, and thyroid tumors (17). Although any direct involvement of NES1 or other kallikrein family member in these rearrangements remains to be analyzed, the chromosomal location of NES1 is fully consistent with its role as a tumor suppressor.

The mechanism of how NES1 induces suppression of the tumorigenic phenotype is currently unknown. Given that NES1 is a secreted serine protease, it is likely that its targets are also extracellular, either components of the extracellular matrix, extracellular growth regulatory molecules or cell surface receptors. It is well documented that normal cellular behavior is regulated by both positive and negative factors. NES1 could mediate its tumor-suppressor role either by generating an inhibitory factor(s) or by terminating the action of an activating factor(s).

Recent studies have shown a role for kallikreins, such as PSA in cleaving IGFBP-3, whereas IGFBP-1,-2,-4, and -6 were not cleaved significantly (as reviewed in Ref. 20). IGFBP-3 has been shown to have IGF1-dependent and IGF1-independent inhibitory effects on cell growth and potential cleavage of IGFBP-3 by PSA may be expected to enhance cell growth, consistent with the correlation of the high levels of PSA and low levels of IGFBP-3 with tumor burden in prostate cancer (20, 21). However, a similar mechanism for NES1 would not account for its tumor suppressor function. Interestingly, whereas a decrease in the levels of IGFBP-3 was observed in the sera of patients with prostate cancer, a significant elevation of IGFBP-2 was noticed (22). Furthermore, patients with high serum levels of PSA also showed elevated levels of IGFBP-2 (22). Interestingly, breast carcinoma cells synthesize a number of IGFBPs, with estrogen receptor-positive and -negative cells secreting different types of IGFBPs (23). Recently, it has been shown that IGFBP-3 predisposes breast cancer cells to program cell death in a non-IGF-dependent manner (24). It is, therefore, possible that NES1 may target one or more IGFBP family members. Additional studies will need to address whether this is the case and whether other targets of NES1 relevant to its tumor-suppressor function exist.

Altogether, our studies support a novel tumor-suppressor function for the serine protease NES1 that is transcriptionally down-regulated during breast and prostate tumor progression. Given this information, it is likely that NES1 may also be involved in a critical aspect of regulating normal epithelial cell growth and/or differentiation.

References

1. Farber, E. The multistep nature of cancer development. Cancer Res., *44:* 4217–4223, 1984.
2. Liu, X-L., Wazer, D. E., Watanabe, K., and Band, V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res., *56:* 3371–3379, 1996.
3. Biochemistry. L. Stryer (ed.), 3rd ed., pp 226–259. New York: W. H. Freeman and Company, 1988.
4. Wang, M-H., Gonias, S. L., Skeel, A., Wolf, B. B., Yoshimura, T., and Leonard, E. J. Proteolytic activation of single-chain precursor macrophage-stimulating protein by nerve growth factor-γ and epidermal growth factor-binding protein, members of the kallikrein family. J. Biol. Chem., *269:* 13806–13810, 1994.
5. Degen, S. J. F., Rajput, B., and Reich, E. The human tissue plasminogen activator gene. J. Biol. Chem., *261:* 6972–6980, 1986.
6. Naka, D., Ishii, T., Yoshiyama, Y., Miyazawa, K., Hara, H., Hishida, T., and Kitamura, N. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J. Biol. Chem., *267:* 20114–20119, 1992.
7. Watt, K. W., Lee, P. J., M'Timkulu, T., Chan, W. P., and Loor, R. Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc. Natl. Acad. Sci. USA, *83:* 3166–3170, 1986.
8. Becker, C., and Lilja, H. Individual prostate-specific antigen (PSA) forms as prostate tumor markers. Clin. Chim. Acta, *257:* 117–132, 1997.
9. Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P. I., Nielsen, L. S., and Skriver, L. Plasminogen activators, tissue degradation, and cancer. Adv. Cancer Res., *44:* 139–266, 1985.
10. Lamszus, K., Jin, L., Fuchs, A., Shi, E., Chowdhury, S., Yao, Y., Polverini, P. J., Laterra, J., Goldberg, I. D., and Rosen, E. M. Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice. Lab. Invest., *76:* 339–353, 1997.
11. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int. J. Cancer, *74:* 301–309, 1997.
12. Bright, R. K., Vocke, C. D., Emmert-Buck, M. R., Duray, P. H., Solomon, D., Fetsch, P., Rhim, J. S., Linehan, W. M., and Topalian, S. L. Generation and genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens. Cancer Res., *57:* 995–1002, 1997.
13. Webber, M. M., Bello, D., Kleinman, H. K., Wartinger, D. D., Williams, D. E., and Rhim, J. S. Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis (Lond.), *17:* 1641–1646, 1996.
14. Narayan, P., and Dahiya, R. Establishment and characterization of a human primary prostate adenocarcinoma cell line (ND-1). J. Urol., *148:* 1600–1604, 1992.
15. Zugmaier, G., Paik, S., Wilding, G., Knabbe, C., Bano, M., Lupu, R., Deschauer, B., Simpson, S., Dickson, R. B., and Lippman, M. Transforming growth factor $\beta 1$ induces cachexia and systemic fibrosis without an antitumor effect in nude mice. Cancer Res., *51:* 3590–3594, 1991.
16. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc. Natl. Acad. Sci. USA, *87:* 463–467, 1990.
17. Heim, S., and Mitelman, F. (eds.). Cancer Cytogenetics: Chromosomal and Molecular Genetic Aberrations of Tumor Cells. New York: John Wily and Sons, Inc., 1995.
18. Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S. C., and Sager, R. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med., *2:* 624–636, 1996.
19. Yu, H., Diamandis, E. P., and Sutherland, D. J. Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin. Biochem. *27:* 75–79, 1994.
20. Cohen, P., Graves, H. C. B., Peehl, D. M., Kamarei, M., Giudice, L. C., and Rosenfeld, R. G. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. *75:* 1046–1053, 1992.
21. Rajah, R., Katz, L., Nunn, S., Solberg, P., Beers, T., Cohen, P. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. Prog. Growth Factor Res., *6:* 273–284, 1995.
22. Ho, P. J., and Baxter, R. C. Insulin-like growth factor-binding protein-2 in patients with prostate carcinoma and benign prostate hyperplasia. Clin. Endocrinol., *46:* 145–154, 1997.
23. Clemmons, D. R., Camacho-Hubner, C., Coronada, E., and Osborne, C. K. Insulin-like growth factor binding protein secretion by breast carcinoma cell lines; correlation with estrogen status. Endocrinology, *127:* 2679–2686, 1990.
24. Gill, Z. P., Perks, C. M., Newcomb, P. V., and Holly, J. M. P. Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. J. Biol. Chem., *272:* 25602–25607, 1997.