

NGEP, a Prostate-Specific Plasma Membrane Protein that Promotes the Association of LNCaP Cells

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Abstract

NGEP is a prostate-specific gene identified by analysis of expressed sequence tag databases. RNA analysis revealed two spliced forms of NGEP mRNA: a short form encoding a soluble protein (NGEP-S) and a long form encoding a polytopic membrane protein (NGEP-L). Transient expression of myc epitope-tagged NGEP-L showed that it was localized to the plasma membrane. We have now produced a specific antibody to the COOH terminus of NGEP-L and showed that it detects an ~100-kDa protein in extracts of normal prostate and prostate cancers that contain high levels of NGEP mRNA. The antibody detects a protein that is highly expressed on the apical and the lateral surfaces of normal prostate and prostate cancer cells by immunohistochemistry. The antibody does not detect a protein in the prostate cancer cell line LNCaP, which has very low NGEP mRNA levels. To study NGEP function, two stable LNCaP cell lines were prepared by transfection with NGEP-L and shown to contain similar amounts of NGEP-L protein as human prostate. Confocal immunofluorescence showed that NGEP-L is present on the plasma membrane of the transfected LNCaP cells and is highly concentrated at cell:cell contact regions. Furthermore, as the cell density increased, the cells formed large aggregates. A specific RNA interference that lowered NGEP-L levels prevented formation of cell aggregates. Our results suggest that NGEP-L has a role in promoting cell contact-dependent interactions of LNCaP prostate cancer cells and also that NGEP is a promising immunotherapy target for prostate cancer. [Cancer Res 2007;67(4):1594–601]

Introduction

Adenocarcinoma of prostate is the second leading malignancy in men in United States. It was estimated that in the year 2006 in the United States, 234,460 men would be diagnosed with prostate cancer and 28,000 would die of the disease (1).³ Despite recent advances in diagnosis and treatment, current therapies are unable to completely eliminate the androgen-independent prostate cancer cells that remain after androgen ablation (2–4). To develop improved treatments for prostate cancer, it is important to identify and characterize new molecular targets. Our laboratory has used a computer-based strategy of searching for expressed sequence tags (EST) that are expressed in prostate tissues to identify new genes

that are expressed in prostate cancer and not in essential normal tissues (5). One of the genes discovered by this approach is *NGEP*.

The *NGEP* gene, also now known as *TMEM16G*, is located on chromosome 2 at 2q37.3. There are two spliced forms of NGEP mRNA. The smaller transcript encodes a 179-amino acid cytoplasmic protein (NGEP-S) and the larger transcript encodes a 933-amino acid polytopic membrane protein (NGEP-L). RNA analysis has shown that NGEP is only detected in prostate samples [normal, benign prostate hyperplasia (BPH), and cancer], indicating it is a differentiation antigen made in normal prostate that continues to be expressed in cancers (1). In an initial attempt to localize the NGEP-L protein, we transfected 293T cells with a myc-tagged NGEP-L cDNA and showed that the protein was localized to the plasma membrane (6). The current study was undertaken to address the question of the location and possible function of NGEP-L in a prostate cancer cell line. Our study shows that in LNCaP cells expressing high levels of NGEP-L, the protein is concentrated at cell:cell contact regions where it seems to promote the association of cells into aggregates and that this aggregation is specifically prevented by RNA interference (RNAi), which dramatically lowers NGEP-L protein levels. This is the first study showing the presence of a prostate-specific protein in the cell:cell contact regions of prostate cancer cells and raises the possibility that NGEP-L may be responsible for cell contact-dependent interactions in the epithelial cells of the prostate.

Materials and Methods

Materials. DMEM and LipofectAMINE were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). Fatty acid-free bovine serum albumin (BSA), Triton X-100, octyl glucoside, and protease inhibitors were from Sigma (St. Louis, MO). Tissues were obtained from Cooperative Human Tissue Network (Charlottesville, VA).

Identification of NGEP-L related proteins in the human genome. The sequences of NGEP-L related proteins were collected by a BLAST search of a nonredundant human protein database or a BLAT search of the human genome assembly using the NGEP-L protein sequence as a query. Multiple sequence alignment of NGEP-L and related proteins were prepared using T-Coffee (7) and manually adjusted and visualized using CHROMA (8).

Generation of the Rab-Fc-NGEP-L (875–933) fusion protein. The COOH terminus of the NGEP-L (residues 875–933) was expressed in transfected human kidney 293T cells as a fusion protein with a rabbit immunoglobulin (IgG) 1 Fc fragment. The expression vector for the Rab-Fc-NGEP-L (875–933) was produced by subcloning the NGEP-L fragment 875 to 933 in the pSec-Tag Rab-Fc vector using plaque-forming units (Pfu)

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³ National Center for Health Statistics, Division of Vital Statistics, Centers for Disease Control [Accessed 2006 January]. Available from: <http://www.cdc.gov/nchs/nvss.htm>.

Polymerase (Stratagene, La Jolla, CA). The PCR products were purified using QIAquick Purification kit (Qiagen, Valencia, CA), digested with *SacII* and *XhoI*, and cloned in pSec Tag-Rab-Fc digested with *SacII* and *XhoI*. The plasmids were sequenced in the regions that underwent genetic manipulations. The Rab-Fc-NGEP-L (875–933) plasmid was transfected in 293T cells using LipofectAMINE Plus (Invitrogen) and the manufacturer's protocol. Supernatants from the transfected cells were collected for 5 days beginning 48 h post-transfection and purified on a protein A column (Immunopure Plus protein A, Pierce, Rockford, IL). Protein concentrations were measured by a Coomassie protein assay reagent (Pierce) according to the manufacturer's protocol and checked on SDS-PAGE gels.

Generation of NGEP-L (875–933) 6×-His fusion protein. Expression vectors for the NGEP-L (875–933) 6×-His was constructed by subcloning the NGEP-L (875–933) sequences of human NGEP-L into the pET28a vector (Novagen, Madison, WI) between *NdeI* and *XhoI* sites by overlap extension PCR using *Pfu* polymerase. The vector is designed to introduce a COOH-terminal His₆ tag between the *XhoI* site and the stop codon for affinity purification of expressed proteins.

Escherichia coli strain BL21 (ΔDE3; Novagen) was used as a host for protein expression. Five hundred milliliters of Luria broth supplemented with 50 μg/mL kanamycin were inoculated with 1 mL of overnight culture grown at 37°C. Cells were grown at 37°C until their absorbance at 600 nm reached ~0.6, and the protein expression was then induced with 0.5 mmol/L isopropyl-1-thio- β -galactopyranoside (Research Products, Mount Prospect, IL). After 4 h, cells were harvested by centrifugation at 5,000 × *g* and 4°C for 10 min. Cells were resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.4% (v/v) Triton X-100, 0.4% (w/v) sodium deoxycholate, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). After the suspension was sonicated, the inclusion body pellet was obtained by centrifugation at 50,000 × *g* for 15 min at 4°C. The pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.8% (v/v) Triton X-100, and 0.8% (w/v) sodium deoxycholate. After centrifugation at 50,000 × *g* for 15 min at 4°C, the pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl and 8 mol/L urea. Protein was purified using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. Purity of protein samples determined electrophoretically was >90%. Aliquots of purified proteins were stored at 20°C.

Polyclonal antibody production and purification. One hundred micrograms of Rab-Fc-NGEP-L (875–933) were injected into rabbits with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent immunizations. Sera were collected after the fourth and sixth immunizations and analyzed by ELISA against the *E. coli*-purified NGEP-L (875–933) 6×-His. An unrelated protein POTE-His, also made in *E. coli*, was used as a control to estimate the nonspecific signal coming from the antisera. Further, to see the cross-reactivity of the antisera, a competition assay was done using Rab-Fc-NGEP-L (875–933). The antibody was further purified by using a Superdex 200 column from which the IgG fraction was collected and concentrated.

Western blotting analysis. Human prostate tissue lysate was prepared by pulverization of the frozen prostate samples in lysis buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8), 0.8% Triton X-100, 0.2% NP40, 1% sodium deoxycholate, 1 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin]. Tissue and cell debris were removed by centrifugation of the lysate at 14,000 rpm for 10 min. Protein concentration was determined by Coomassie protein assay reagent according to the manufacturer's protocol.

Fifty micrograms of protein extract from different prostate tissues were separated on a 4% to 20% Tris-glycine gel (Invitrogen) and transferred to a 0.2-μm immunoblot polyvinylidene difluoride membrane (Invitrogen) in transfer buffer [25 mmol/L Tris/192 mmol/L glycine/20% (v/v) methanol (pH 8.3)] at 4°C for 2 h at 50 V. Filters were probed with 1:1,000 rabbit antiserum or preimmune serum. Primary antibodies were detected with the donkey antirabbit secondary antibody conjugated with horseradish peroxidase (HRP; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and their respective signals were detected by using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences).

Generation of stable cell lines and RNAi. LNCaP cells were grown in RPMI 1640 containing 10% FBS, 1 mmol/L pyruvate, 2 mmol/L glutamine, and 100 μg/mL penicillin and 100 μg/mL streptomycin in a 5% CO₂/95% air humidified incubator at 37°C. For stable cell line generation, the cells at 80% confluency in six-well plates were incubated in serum-free and antibiotic-free RPMI 1640 containing 4 μL LipofectAMINE and 6 μL Plus reagent with 6 μg DNA. Cells were incubated for 4 h at 37°C, after which complete medium was added to bring the final concentration to 10% FBS. Twenty-four hours after the start of the transfection, cells were passaged and split into 10 different plates at different dilutions. At 48 h, the medium was replaced with complete medium containing 750 μg/mL G418 for the selection of the stable clones. Multiple G418 colonies were picked and the clones expressing NGEP-L were selected using Western blot analysis with the polyclonal antibody against NGEP-L.

For NGEP-L RNAi experiments, LNCaP-CL-2 cells were transfected with 200 pmol NGEP-L-specific (D-023184-01) or control small interfering RNAs (siRNA; GL2-Luc; Dharmacon, Chicago, IL) using LipofectAMINE 2000 transfection reagent (Invitrogen) 48 h after plating of the LNCaP-CL-2 cells. Western analysis was done on the cells transfected with the siRNA and the extent of the inhibition of NGEP-L expression was studied. Each transfection was repeated twice in duplicate.

Immunocytochemistry. Cells were plated onto a LabTek chamber slide and grown for 2 days. After 48 h, cells were fixed for 20 min in 4% formaldehyde, treated for 10 min with 0.1% Triton X-100 in PBS, blocked for 30 min with 10% normal goat globulin in PBS, and then incubated at room temperature for 1 h with 1:100 diluted polyclonal antibody for NGEP-L. Subsequently, the cells were incubated at room temperature for 1 h with tetramethyl rhodamine-conjugated secondary antibodies (Invitrogen) at the concentration of 2 μg/mL and then mounted in antifade solution with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Labeled cells were analyzed by laser confocal microscopy using a ×63 oil immersion objective lens. For labeling of the endoplasmic reticulum (ER) and *cis*-golgi, 1: 100 anti-protein disulfide isomerase (PDI) monoclonal antibodies (mAb; Abcam, Cambridge, MA) and 1 μg/mL anti-human golgin-97, mouse monoclonal CDF4 (Invitrogen) were used, respectively, as the primary antibody. The secondary antibody used for the detection of ER and golgi was Alexa 488-labeled goat anti-mouse antibody (Invitrogen).

Immunohistochemistry. Paraffin-embedded prostate tissues were cut into 5-micron sections and mounted onto polylysine slides (Histoserv, Germantown, MD). The sections were deparaffinized in xylene followed by graded ethanol hydration into water. The sections were treated with antigen-retrieval solution (DAKO Genpoint, Carpinteria, CA) at 85°C for 20 min. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxidase for 10 min followed by washing in water twice for 5 min. Nonspecific labeling was blocked with 5% BSA in TBS for 20 min. After washing the sections in TBST for 5 min, tissues were incubated with polyclonal antibody against NGEP-L (dilution 1:1,500) in blocking buffer overnight at room temperature. After incubation, the sections were washed twice with TBST for 5 min. Sections were then treated with biotinylated goat anti-rabbit IgG (dilution 1:500; DAKO Genpoint) for 20 min at room temperature, washed once with TBST, and incubated with streptavidin HRP (dilution 1:400) for 30 min. The sections were washed once with TBST for 5 min and the secondary antibody was detected with 3',3'-diaminobenzidine peroxidase substrate (Sigma). Slides were counterstained with hematoxylin (Histoserv, Germantown, MD), dehydrated in graded ethanol and cleared in xylene, and mounted using Permount (Fisher Scientific, Pittsburgh, PA).

Results

To study the cellular location and function of NGEP-L protein, we generated a specific polyclonal antibody to NGEP-L. Because NGEP-L is a member of the TMEM16 protein family, we used the data of Galindo and Vacquier (9) and two additional members identified in this study to choose a region of NGEP-L that is different from other family members. Our sequence comparison of the TMEM16 family members showed that among the paralogs, the

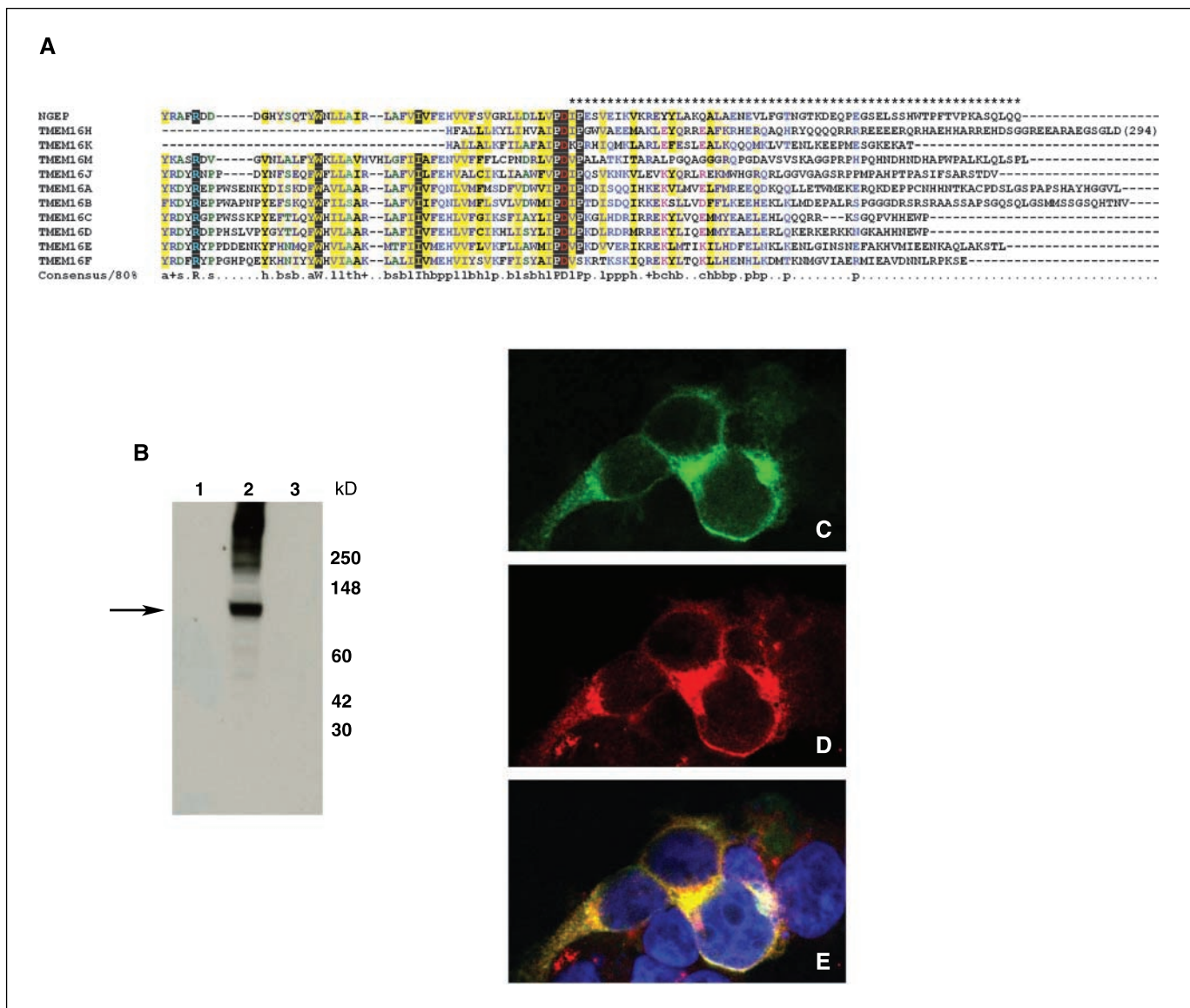


Figure 1. Generation of antibodies against NGEPL. **A**, multiple sequence alignment of COOH termini of 11 human NGEPL-related proteins (TMEM16 family). Amino acid residues encoded by exons 22 to 25 of NGEPL-L (amino acids 829–933) and its homologous regions in NGEPL-L related proteins are shown. TMEM16H and TMEM16K lack the exon equivalent to NGEPL-L exon 22. *, peptide sequence used for antibody production. The unaligned residues of TMEM16H are omitted (parenthesis). Markings in the consensus line are as follows: *capital letters*, amino acid residue identical in 80% or more sequences; *l*, aliphatic (ILV); *+*, positive (HKR); *t*, tiny (AGS); *a*, aromatic (FHWR); *c*, charged (DEHKR); *s*, small (ACDGNPSTV); *p*, polar (CDEHKNGRST); *b*, big (EFHIKLMQRWY); *h*, hydrophobic (ACFGHILMTVWY). The GenBank gi number and amino acids shown here are as follows: TMEM16A, 40354210:838–960; TMEM16B, 9966845:876–999; TMEM16C, 13899227:887–981; TMEM16D, 30520318:823–920; TMEM16E, 47106048:806–913; TMEM16F, 70778895:808–910; NGEPL-L (TMEM16G), 49533623:829–933; TMEM16H, 55741655:851–1232; TMEM16J, 59858803:680–782; TMEM16K, 27545336:332–392; and TMEM16M, 51459226:477–584. **B**, Western blot analysis of NGEPL-transfected cell extracts with an NGEPL-L antibody. The expected 100-kDa protein is detected in extracts transfected with pcDNA3.1-NGEPL-L myc (*lane 2*). A cell extract from vector only-transfected cells or pcDNA3.1-POTE21-transfected cell produced no signal (*lanes 1 and 3*, respectively). Forty micrograms of protein lysate were loaded onto a 4% to 20% gel and Western blot analysis was done using NGEPL polyclonal antibody at a dilution of 1:1,000. HRP-labeled donkey anti-rabbit secondary antibody at a dilution of 1:2,000 was used for detection. **C** to **E**, 293T cells were transiently transfected with a plasmid encoding EGFP-NGEPL. After 24 h, the cells were stained with anti-NGEPL antibodies. The fluorescence of EGFP-NGEPL in the 293T cells expressing the fusion protein (**C**; *green*) completely colocalized with the anti-NGEPL (**D**; *red*) as shown in (**E**; *yellow*). Nuclei were stained with DAPI (*blue*).

COOH termini have high sequence diversity (Fig. 1A). Based on this information, we chose residues 875 to 933 to generate NGEPL-L antibodies.

Preparation of Rab-Fc-NGEPL-L (875–933) protein and generation of the polyclonal antibodies. Rabbits were immunized with a Rab-Fc-NGEPL-L (875–933) fusion protein composed of the Fc fragment of rabbit IgG1 and the COOH-terminal amino acids (875–933) of NGEPL-L as described in Materials and Methods. Blood

was collected from the rabbits after the fourth and sixth immunizations and the antibody titer and specificity were determined by ELISA on plates coated with NGEPL-L (785–933) 6×-His protein made in *E. coli* (data not shown). The antibody was purified on a Superdex 200 column to remove albumin and other proteins and the IgG fraction was collected and concentrated.

The minimum amount of recombinant NGEPL-L (785–933) that could be detected on a Western blot by the purified polyclonal

antibody was determined using different dilutions of polyclonal antibody and different concentrations of NGEP-L (785–933) 6×-His. Using Western blot analysis, the NGEP-L antisera at a dilution of 1:1,000 could detect 1 ng of recombinant NGEP-L (785–933; data not shown). To determine if the polyclonal NGEP-L antibody could detect NGEP-L protein in mammalian cells, 293T cells that do not express NGEP-L were transfected with a pcDNA3.1/NGEP-L-myc plasmid. Total lysates were subjected to SDS-PAGE. As shown in Fig. 1B, the polyclonal antibody specifically detected a strong band of ~100 kDa in 293T cells transfected with pcDNA3.1 NGEP-L-myc. It also detected higher molecular bands very likely representing aggregated protein (Fig. 1B, lane 2). (Aggregation of polytopic proteins is often observed when these proteins are produced at high levels by transfection.) No signal was detected in the untransfected cells (Fig. 1B, lane 1) or in 293T cells expressing a cDNA encoding a control protein POTE21 (Fig. 1B, lane 3; ref. 10). These data establish that the polyclonal antibody against NGEP is specific and can detect NGEP-L expressed in mammalian cells.

We showed previously that NGEP-L tagged with myc localizes in the plasma membrane of transfected 293T cells (6). To determine if the polyclonal antibody detects the same protein, we chose to immunologically colocalize transfected NGEP-L by transfecting 293T cells with enhanced green fluorescent protein (EGFP)-NGEP-L and determining if the EGFP signal (green) colocalizes with one produced by an antibody to NGEP-L detected with a tetramethyl rhodamine goat anti-rabbit antibody (red). The images in Fig. 1C–E show that the two signals overlap, indicating the NGEP-L antisera detects NGEP-L in transfected cells (Fig. 1E). No signal was obtained from the untransfected 293T cells or when prebleed sera was used for the immunofluorescence on NGEP-L-transfected cells

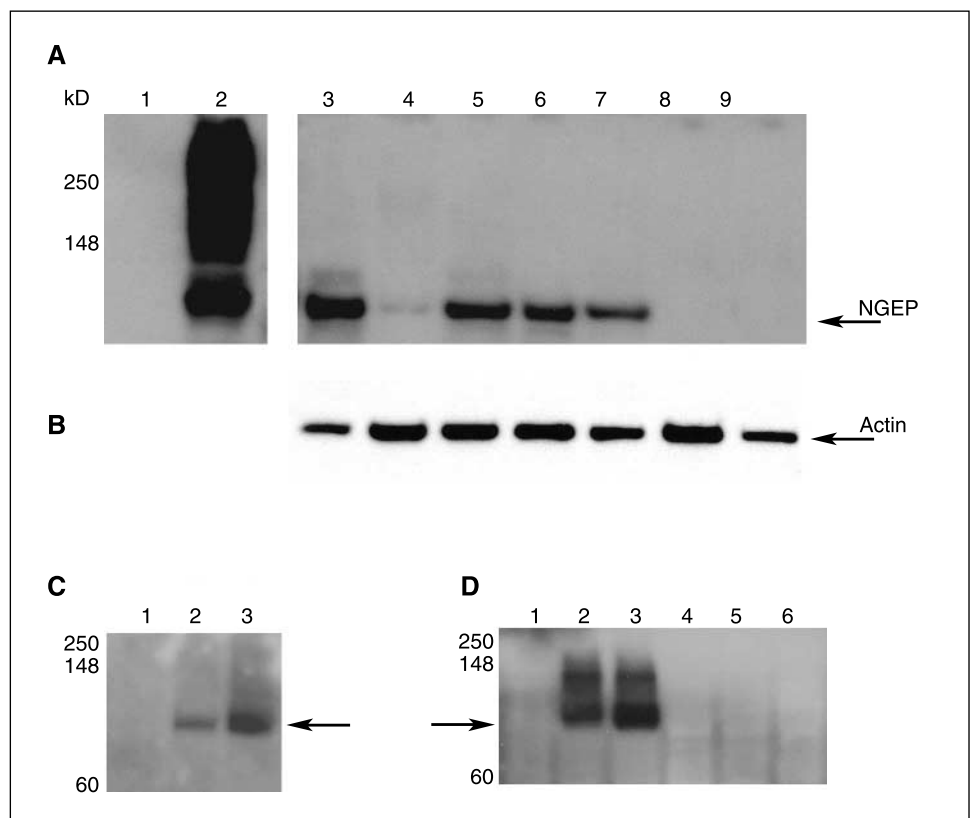
(data not shown), showing the antibody is specific to NGEP-L and can detect NGEP-L in formalin fixed cells.

Expression of NGEP in prostate tissue. Because RNA studies have shown that *NGEP-L* is a prostate-specific gene (6), we used the antisera to test for the presence of NGEP-L in protein extracts of normal prostate and prostate cancer and a few normal tissues. Fifty micrograms each of tissue lysates were resolved on a 10% SDS gel, blotted, and then reacted with the polyclonal NGEP-L antibody. As shown in Fig. 2A, a band of ~100 kDa was detected in protein extracts of 293T cells transfected with pcDNA3.1 NGEP-L-myc (Fig. 2A, lane 2) as well as bands in the high molecular weight regions due to aggregated protein. A specific band at the expected M_r of ~100 kDa was detected in tissue extracts from normal prostate (Fig. 2A, lane 3), BPH (Fig. 2A, lanes 4 and 5), and prostate cancers (Fig. 2A, lanes 6 and 7). The expression of NGEP-L in one of the BPH samples was low (Fig. 2A, lane 4). No signal was detected in liver and brain tissue lysates. To evaluate the quality of the lysates, we did a Western blot with a β -actin antibody as shown in Fig. 2B; a similar amount of actin was detected in all the tissue lysates. We conclude the antisera can specifically detect NGEP-L in prostate tissue lysates.

Generation of NGEP-L-expressing prostate cell line. Previously, we found that NGEP mRNA is present at extremely low levels in LNCaP cells using reverse transcription-PCR. This level is much less than the levels present in tissue and tumor samples from patients, suggesting NGEP-L expression was lost when the LNCaP cell line developed. As expected, attempts to detect NGEP-L protein in LNCaP cells by Western blot were unsuccessful (data not shown).

To study the location and possible function of NGEP-L in prostate cancer cells, we established two stable LNCaP cell lines expressing NGEP-L by transfecting NGEP-L into these cells using a

Figure 2. Western analysis of NGEP-L in prostate tissue and prostate cancer cell lines. **A**, Western blot analysis of NGEP-L expression: 50 μ g of lysate from 293T nontransfected 293T cells (lane 1), 30 μ g of cell lysate from 293T transfected with cDNA encoding NGEP-L (lane 2), and 50 μ g of tissue lysate from normal prostate (lane 3), BPH (lanes 4 and 5), prostate cancer (lanes 6 and 7), brain (lane 8), and liver (lane 9) were analyzed by Western blotting using rabbit anti-NGEP-L antibody (1:1,000). **B**, the blot was probed with anti- β -actin (1:250; Abcam) as a loading control. **C**, Western analysis of NGEP-L expression in the stable cell lines. The samples (40 μ g) loaded are the following: 1, LNCaP-V; 2, LNCaP-CL-1; and 3, LNCaP-CL-2. A protein band ~100 kDa was observed for NGEP-L-transfected cells. **D**, subcellular fractionation was done using differential centrifugation in LNCaP-CL-2 and LNCaP-V. The samples (40 μ g cells extracts) loaded are the following: 1, LNCaP-CL-2 cytosol fraction; 2, LNCaP-CL-2 nuclear fraction; 3, LNCaP-CL-2 membrane fraction; 4, LNCaP-V cytosol fraction; 5, LNCaP-V nuclear fraction; and 6, LNCaP-V membrane fraction. NGEP-L is present in the crude membrane and nuclear fractions but not in the cytosolic fraction.



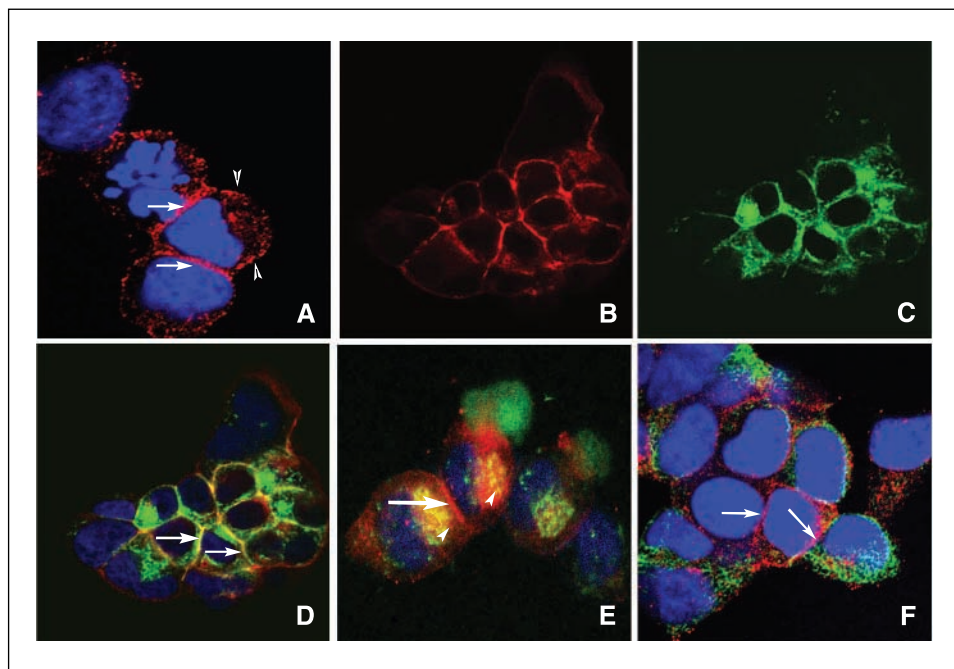


Figure 3. NGEP-L is present in the cell:cell contact regions in the LNCaP-CL-2 cells. *A*, immunofluorescence of NGEP-L was done on LNCaP-CL-2 using the rabbit NGEP-L antibody and the signal was detected using tetramethylrhodamine goat antirabbit. *Arrows*, NGEP-L (red) was predominantly localized in the cell:cell contact regions. There was also presence of NGEP-L in the plasma membrane (*arrowheads*) and also intracellular organelles. *B* to *D*, the LNCaP-CL-2 cells were stained with tetramethylrhodamine B isothiocyanate-labeled phalloidin (*B*; red) and the immunofluorescence of NGEP-L was done using rabbit NGEP-L antibody followed by Alexa 488-labeled goat antirabbit antibody (*C*; green). Phalloidin labels the F-actin beneath the plasma membrane and NGEP-L is present in the membrane of the cells. *Arrows*, NGEP-L is predominant in the cell:cell contact regions (*D*; yellow). *E*, LNCaP-CL-2 cells were stained with golgin-97 antibody, a *cis*-golgi protein (green), and costained with anti-NGEP-L antibody (red). *Arrowhead*, colocalization (yellow) of golgin-97 and NGEP; *arrow*, localization of NGEP in the cell-cell contact regions. *F*, LNCaP-CL-2 cells were stained with PDI antibody, an ER protein (green), and costained with anti-NGEP antibody (red). The nuclei were stained with DAPI.

pCDNA3.1-based vector as described in Materials and Methods. As shown in Fig. 2C, NGEP-L expression in CL-2 (Fig. 2C, lane 3) is higher than expression in CL-1 (Fig. 2C, lane 2). In addition, there is no detectable expression of NGEP-L in a vector-transfected LNCaP-V cell (Fig. 2C, lane 1). These cell lines were used for further experiments.

NGEP-L is a plasma membrane protein. To verify that NGEP-L was tightly associated with the membrane fraction of cells, LNCaP-CL-2 cells were disrupted by homogenization and subjected to low- and high-speed centrifugation, and the samples were analyzed by Western blots. As shown in Fig. 2D, NGEP-L was detected in both the low (Fig. 2D, lane 2) speed ($1,000 \times g$) and the high (Fig. 2D, lane 3) speed membrane fractions ($100,000 \times g$) but not in the soluble fraction of the cells (Fig. 2D, lane 1). In both membrane fractions (Fig. 2D, lanes 2 and 3), we observed a major band of the expected size (~ 100 kDa) for NGEP-L and a higher molecular weight band (probably an aggregate), which was not present in any of the fractions of the vector-transfected cells (Fig. 2D, lanes 4–6).

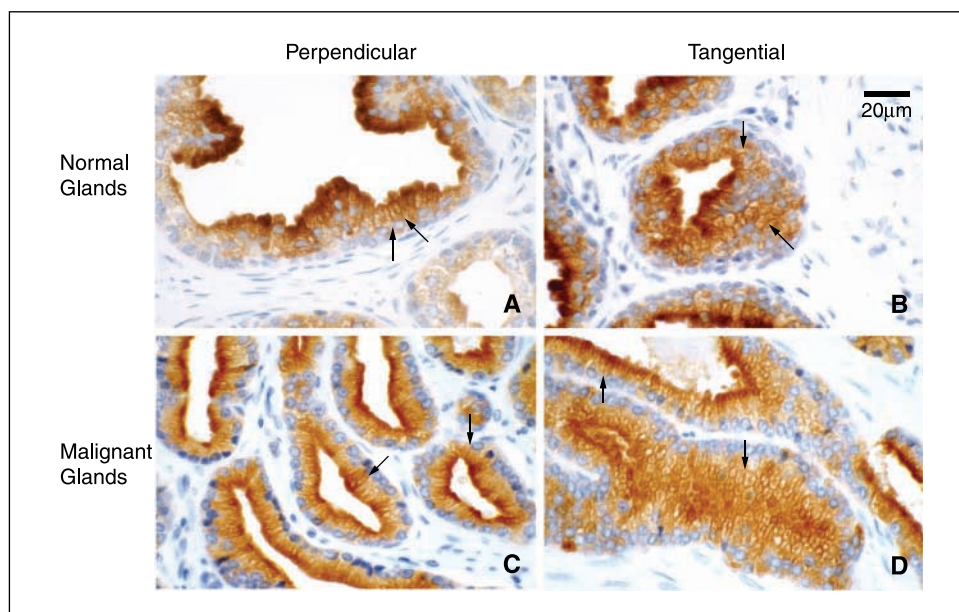
We next did immunofluorescence studies with LNCaP-CL-2 cells. As shown in Fig. 3A, there is strong red fluorescence signal (tetramethylrhodamine) in the cell:cell contact regions and a weaker signal in the regions of the plasma membrane where the cells are not touching other cells. In addition, there is some punctate staining in the interior portion of the cell. To confirm the plasma membrane location of NGEP-L, we double stained cells using Alexa 488 (green) to stain NGEP-L and tetramethylrhodamine B isothiocyanate-labeled phalloidin to stain the F-actin network that lies very close to the plasma membrane. As shown in

Fig. 3B, phalloidin stains the actin present adjacent to the plasma membrane region. Figure 3C shows the green NGEP-L signal that is very strong in cell:cell contact regions but also present inside the cell. Figure 3D shows that there is very good colocalization of NGEP-L and actin in the region of the cell membrane as would be expected at this level of resolution. The bright yellow signal at cell junction regions is consistent with an increase in NGEP-L in that region. There was no NGEP-L signal detected when we analyzed LNCaP-V cells or in LNCaP-CL-2 cells using prebleed sera establishing the specificity of the signal observed (data not shown).

To determine the location of NGEP-L protein within the cell, we did double labeling experiments with a *cis*-golgi marker (golgin-97) and with an ER marker (PDI) in LNCaP-CL-2 cell. As shown in Fig. 3E, there is colocalization of some of the intracellular NGEP-L signal with the *cis*-golgi marker but not with the ER marker (see Fig. 3F). It is likely that this signal represents NGEP-L being processed in the Golgi compartment before delivery to the cell membrane.

To determine the location of NGEP in normal prostate and prostate cancer, we did immunohistochemistry using four different formalin-fixed, paraffin-mounted prostate cancer specimens obtained from radical prostatectomy. Figure 4 shows the staining of the NGEP in both normal and malignant glands from one of the prostate cancer specimens. In the normal glands, NGEP is present at the apical and the lateral intercellular region between acinar epithelial cells, when sectioned perpendicular to the basement membrane (Fig. 4A). Figure 4B shows the same expression pattern, when sectioned tangential to the basement membrane. Figure 4C and D shows the perpendicular and the tangential sections from

Figure 4. Location of NGEP-L in prostate. Slides were prepared from formalin fixed, paraffin-embedded human prostate cancer samples. NGEP-L was detected as described in Materials and Methods using a polyclonal antibody to NGEP-L at a dilution of 1:1,500. Arrows, NGEP-L signal on the lateral surface of the cells. A and B, normal prostate. C and D, prostate cancer. The signal is visible in both perpendicular and tangential sections of normal and malignant prostate glandular epithelia with respect to the basement membrane on which the epithelial cells sit. Perpendicular sections (A and C). Tangential sections (B and D).



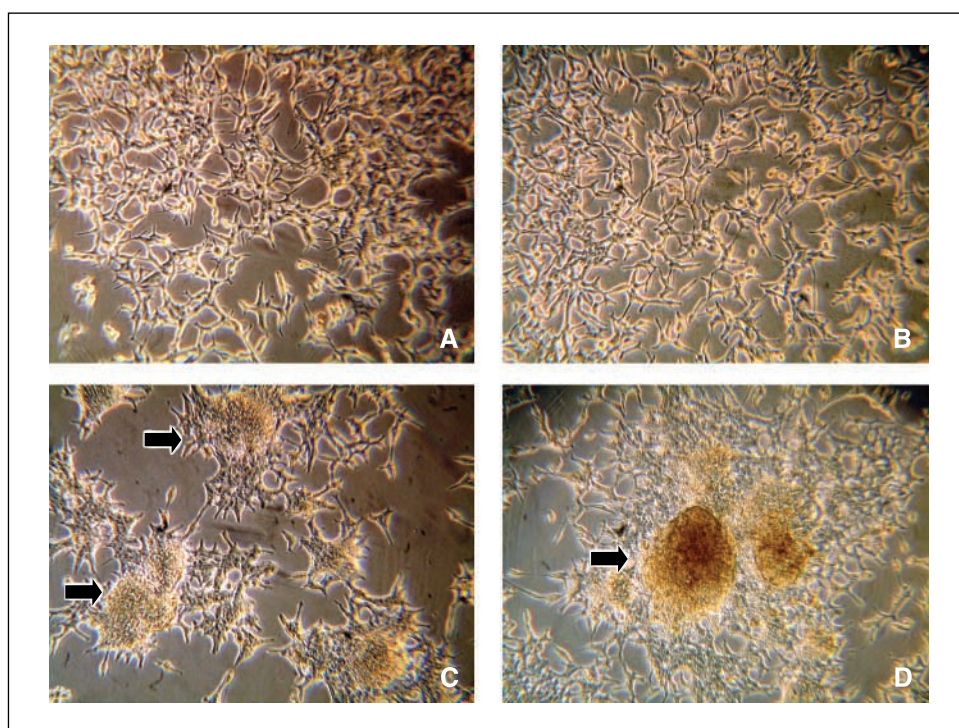
malignant prostate adenocarcinoma glands, respectively. In the malignant prostate glands, NGEP was expressed on the lateral and the apical surface of the epithelial cells, similar to the distribution in normal prostate glands.

Effect of NGEP-L on cell morphology. To examine if expression of NGEP-L in the cell:cell contact regions can alter the behavior or shape of the LNCaP cells, we plated 4.5×10^4 cells from each of the two clones in a six-well plate and observed the cells for several days. Figure 5 shows phase-contrast micrographs of living cells taken on day 7. Two striking differences were observed when compared with untransfected LNCaP cells (Fig. 5A) or

LNCaP-V cells (Fig. 5B). Cells expressing NGEP-L protein formed large aggregates and the cells not in aggregates were rounder and less spread out than control cells (Fig. 5C and D). This behavior was not due to clonal variation because we made the same observation in two different cell lines.

RNAi-mediated inhibition of NGEP-L expression. To determine if the aggregation and morphologic changes of the NGEP-L-expressing cells were due to expression of NGEP-L, we carried out siRNA experiments to lower the NGEP-L levels. We reasoned that if the change in the morphology of the cells is due to NGEP expression, then down-regulation of NGEP-L would revert the cell

Figure 5. Expression of NGEP-L in the LNCaP cells alters the morphology of the cells. Phase-contrast photographs of growing LNCaP cells. A, parental LNCaP cells. B, LNCaP-V cells. C, LNCaP-CL-1. D, LNCaP-CL-2. NGEP expression shows a circular morphology, more cell:cell adhesion morphology compared with the control cells. Arrows, aggregation of the cells due to NGEP-L.



shape to that of the parental LNCaP cells and eliminate the aggregates. Four siRNA oligonucleotide sets were tested for their ability to lower NGEP-L levels measured on a Western blot before deciding on the best construct for the efficient knockdown. siRNA (D-023184-01) specific to NGEP-L was chosen and was used for the studies shown here. To show down-regulation of the NGEP-L protein level in the LNCaP CL-2 cells, Western blot analysis was carried out. As shown in Fig. 6A, NGEP-L siRNA repressed the expression of NGEP-L protein in the LNCaP-CL-2 cells (Fig. 6A, lanes 1–3), but the control siRNA did not (Fig. 6A, lane 4). The maximum reduction in protein levels was observed 4 days after transfection.

To determine if the down-regulation of NGEP-L protein has any effect on the aggregation or morphology of the LNCaP-CL-2 cells, we monitored the cells after transfection with the siRNAs. Figure 6B to G shows the phase-contrast micrographs of the LNCaP-CL-2 cells transfected with NGEP-L-specific siRNA, control siRNA, and the nontransfected cells at days 4 and 6 after transfection. The LNCaP-CL-2 cells transfected with siRNA-NGEP-L show a morphology that is similar to that of the parental untransfected LNCaP cells (Fig. 6B and E and compare it with Fig. 5A and B). The cells are more elongated compared with the LNCaP cells expressing NGEP-L. The LNCaP-CL-2 cells (Fig. 6D and G) and the LNCaP-CL-2 cells transfected with nonspecific siRNA (Fig. 6C and F) have a similar morphology. They form clumps of cells as shown in Fig. 6C, D, F, and G, arrows.

To determine if the NGEP-L interaction responsible for the cell:cell adhesion requires calcium, we did Ca^{2+} chelation with EGTA. We reasoned if the cell:cell adhesion due to NGEP-L requires calcium, depletion of the extracellular calcium will cause the aggregates to come apart. We treated the LNCaP-CL-2 cells with 2 mmol/L EGTA for 1 h and monitored the cells. We found that EGTA treatment caused the LNCaP-CL-2 cells to detach from the dish. However, the few cells that remained on the plate were still in clumps (data not shown).

Discussion

The *NGEP* gene was originally identified by analyzing the EST database and searching for genes expressed in prostate and prostate cancer and not other tissues. Proteins encoded by such genes could be useful therapeutic targets for cancer therapy. An analysis of NGEP mRNA showed that there are two spliced variants (6). One of these encodes a short cytoplasmic protein (NGEP-S) and the other encodes a long polytopic membrane protein (NGEP-L), which is a member of the TMEM16 family of proteins (9, 11). The antibody used in the current study was generated against the COOH terminus of NGEP-L because that region is the most diverse among the family members. The antibody specifically detected a ~100-kDa protein in samples of normal prostate, prostate cancer, and BPH but not in other tissues examined, such as liver and brain. This finding indicates that the antibody is specific for NGEP-L. We then examined two prostate cancer cell lines, PC3 and LNCaP, for NGEP-L protein expression and it was not detected in either. This result was not surprising because NGEP mRNA is not detected in PC3 cells and is expressed at a very low level in LNCaP cells (6). We then used LNCaP cells to evaluate NGEP-L function because it is more differentiated than the PC3 line as evidenced by its production of the prostate-specific antigen and its response to androgen (12).

Two cell lines producing NGEP-L were established by transfecting a full-length NGEP-L cDNA into LNCaP cells. Western blots showed that these cell lines produced NGEP-L protein with the expected molecular weight and the levels were comparable with NGEP-L protein levels in prostate cancer specimens (Fig. 2C). Examination of the location of NGEP-L by confocal microscopy using the antibody to NGEP-L showed that NGEP-L was located in the plasma membrane and that its concentration was increased at cell contact regions. Furthermore, as the cells grew to high density, large aggregates of cells formed, suggesting that NGEP-L has an important role in promoting cell:cell interactions. These large

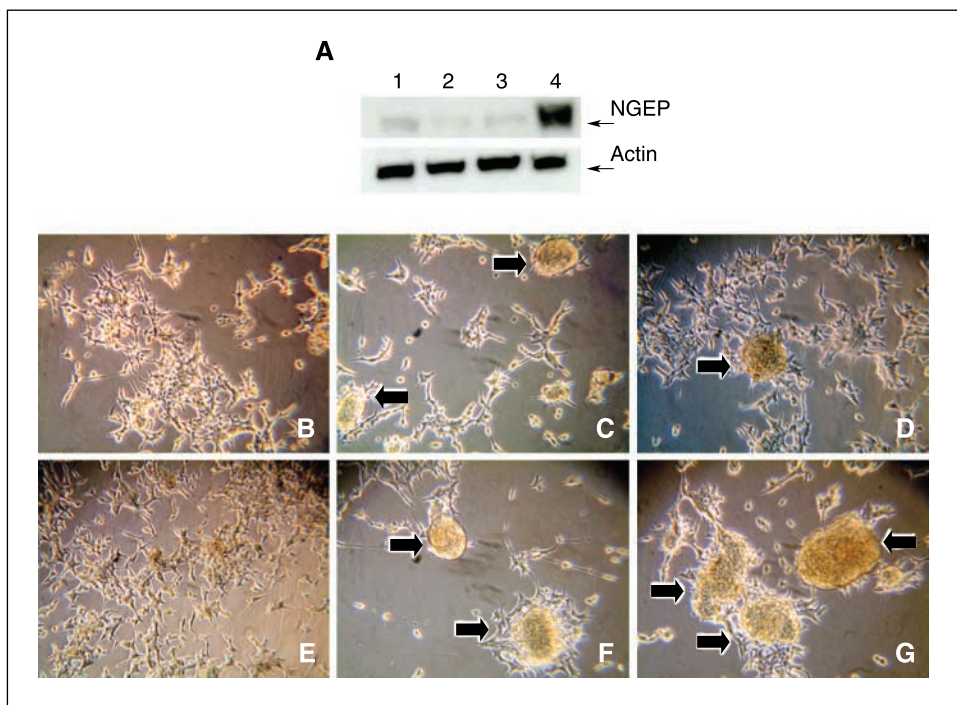


Figure 6. NGEP-L siRNA mediates gene silencing in LNCaP-CL-2 cells. A, Western blot analysis of the NGEP-L protein expression was done after 2, 4, and 6 d of transfection with RNA duplexes. A control siRNA (GL2-Luc) was used in parallel to test potential nonspecific effects of the short RNA duplexes. The unaffected actin expression was used as internal standard of protein concentration in each lane. Twenty micrograms of cell lysates after 2 d of siRNA-NGEP transfection (lane 1); 4 d of siRNA-NGEP transfection (lane 2); 6 d of siRNA-NGEP transfection (lane 3); and 6 d of siRNA-GL2-Luc transfection (lane 4) were loaded onto a gel and Western blot analysis was done using rabbit NGEP antibody (1:1,000). B to G, NGEP-L knockdown induces alteration in the morphology of the LNCaP-CL-2 cells. Phase-contrast photographs of growing LNCaP-CL-2 cells during *NGEP* gene silencing. LNCaP-CL-2 cells transfected with siRNA-NGEP at day 4 (B) and day 6 (E). LNCaP-CL-2 transfected with control siRNA (GL2-Luc) at day 4 (C) and day 6 (F). LNCaP-CL-2 cells at day 4 (D) and day 6 (G). Arrows, clump of cells present in the LNCaP-CL-2 and in the LNCaP-CL-2 cells transfected with nonspecific siRNA. The clumps disappeared in cells transfected with siRNA specific to NGEP-L.

aggregates were not observed in cells transfected with an empty vector or in nontransfected LNCaP cells. To confirm that aggregation was due to presence of NGEP-L protein, we showed that a RNAi for NGEP-L prevented the formation of large cellular aggregates.

We also examined the location of NGEP-L in both normal prostate and prostate cancers tissues using formalin fixed tissues obtained from prostatectomy samples. Figure 4 shows one example; the other three showed very similar results. In both normal and cancers, NGEP-L was localized on the apical and the lateral surfaces of the epithelial cells of prostate. This location is consistent with the finding with transfected LNCaP cells where accumulation of NGEP at cell contact regions was observed. The similar cell:cell contact location of NGEP-L in the LNCaP cells and in the prostate tissue suggests that NGEP-L may have an important role in the cell:cell interactions.

Connexins are gap junction proteins ubiquitously present in epithelial cells, including prostate, and have an important role in intercellular communication allowing the transfer of small molecules between cells. In many epithelial cancers, including prostate cancer, the levels of connexin are low or even absent (13–15). Connexin levels are low in LNCaP cells (16, 17) and overexpression of connexins has been shown to cause aggregation of LNCaP cells similar to that observed with NGEP-L–

transfected LNCaP cells (16). However, unlike NGEP-L, connexins are not differentiation antigens with a specific role in the prostate. It is possible that NGEP-L, perhaps cooperating with connexins, has an important role in the formation of the prostate gland. We are currently making NGEP knockout mice to examine this hypothesis.

NGEP-L is a polytopic protein but its precise orientation of the protein in the membrane has not been established. NGEP-S, which shares a common NH₂ terminus with NGEP-L, is a cytosolic protein and it is likely that the NH₂ terminus of NGEP-L is also inside the cell (6). The COOH terminus of NGEP-L is probably also inside the cell because the antibodies described in this article that react with the COOH terminus only detect NGEP in permeabilized cells. We are currently producing mAbs to other regions of NGEP to learn more about its function and its orientation in the plasma membrane. mAbs to an extracellular portion of NGEP could be useful in the immunotherapy of prostate cancer.

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