Identification of a Novel Estrogen Receptor-α Variant and Its Upstream Splicing Regulator

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Alternative splicing of precursor mRNA is a fundamental mechanism to generate multiple proteins from a single gene. Although constitutive and alternative mRNA splicing is temporally and spatially regulated, deregulation of mRNA splicing could cause development, progression, and metastasis of tumors. Through yeast two-hybrid screening of a human breast cDNA library using estrogen receptor-α (ERα) as bait, we identified a novel nuclear receptor box containing full-length protein, nuclear protein E3-3 (NPE3-3). Our results revealed that NPE3-3 associates with not only ERα but also with splicing factors, serine/arginine-rich protein (SRp)-30c, SRp40, and splicing factor SC-35, suggesting that NPE3-3 is likely to be involved in regulation of mRNA splicing. Accordingly, transient expression of NPE3-3 in cells resulted in expected splicing of the CD44 control minigene. We also discovered that NPE3-3-overexpressing clones produced a novel, previously unrecognized, alternatively spliced variant of ERα (termed ERαV), which had a molecular size of 37 kDa composed of only exons 1, 2, 7, and 8. ERαV was expressed and sequestered in the cytoplasm in MCF-7 cells stably overexpressing NPE3-3, suggesting its involvement in nongenomic hormone signaling. NPE3-3 clones exhibited up-regulation of ERK1/2 signaling, cyclin D1, and cathepsin D and enhanced tumor cell proliferation, migration, and tumorigenicity. Moreover, direct expression of the ERαV in breast cancer cells stimulated ERK1/2 up-regulation and cyclin D1 expression. We found that ERαV physically interacted with MAPK kinase (MEK)-1/2, and thus, an ERαV and MEK1/2 complex could lead to the activation of the ERK1/2 pathway. Interestingly, NPE3-3 was up-regulated in human breast tumors. These findings revealed a role for NPE3-3 in alternative splicing and suggest that ERα is a physiological target of NPE3-3, leading to a constitutive nongenomic signaling pathway in breast cancer cells. **(*Molecular Endocrinology* 24: 914–922, 2010)**

mRNA splicing is a process that cells use to remove intron sequences and ligate exon sequences, and involves a multicomponent ribonucleoproteotein complex known as a spliceosome. Through dynamic RNA-protein, protein-protein, and RNA-RNA interactions, the spliceosome defines splice sites in mRNA and catalyzes a two-step *trans*-esterification reaction to produce mature mRNA. Alternative splicing of mRNA is the fundamental mechanism to generate multiple proteins from a single gene. Genomic analyses indicate that up to 70% of human genes might undergo alternative splicing (1), suggesting that alternative splicing plays an essential role to produce proteome complexity and functions in every aspect of cellular events.

Although constitutive and alternative splicing of mRNA is temporally and spatially regulated in cells and

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Abbreviations: AF, Activation function; E2, estrogen; ERα, estrogen receptor-α; GBD, Gal4-binding domain; hERα36, human ERα36; IP, immunoprecipitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; MEK, MAPK kinase; NPE3-3, nuclear protein E3-3; NR, nuclear receptor; p-, phospho-; PARP, poly ADP-ribose polymerase; SRp30c, serine/arginine-rich protein-30c; WB, Western blotting.

tissues, mutations of *cis*-acting splicing elements in the genes of tumor suppressors, oncogenes, and adhesion molecules and the inactivation of *trans*-acting splicing regulators induce deregulation of mRNA splicing, causing development, progression, and metastasis of tumors. Deregulation of mRNA splicing has been associated with different features in cancers, including breast cancer. For example, mutations of *cis*-acting splice elements of the *BRCA1* and *MDM2* genes result in an abnormal splice site selection (2–4), leading to inactivation of a tumor suppressor and activation of an oncogenic product, respectively. Furthermore, specific alternatively spliced isoforms of CD44 have been found only in highly aggressive and metastatic breast tumors. These splice variants of CD44 have established roles in mediating oncogenic signaling via cross talk with human epidermal growth factor receptor-2 (5), Rho kinase (6), c-Src kinase (7), and matrix metalloproteinase (MMP)-9 (8). The functional interactions of CD44 with these molecules promote tumor cell growth, migration, and invasion. Despite the important role of CD44 splice variants in breast cancer tumorigenesis, the driving force of aberrant CD44 alternative splicing in breast cancer cells remains unknown. Because there are no mutations in *cis*-acting splicing elements of CD44 in breast tumors, the mechanisms of abnormal CD44 alternative splicing is likely to involve deregulated participation of *trans*-acting splicing regulatory factors. In fact, abnormal activity of *trans*-acting splicing factors has been implicated in breast cancer initiation and progression by modulating target gene alternative splicing (9).

Alternative splicing of the estrogen receptor-α (ERα) has been purported to be involved in breast tumor growth and progression (10–13). For example, human ERα36 (hERα36) lacks both transcriptional activation function (AF)-1 and AF-2 but retains the DNA-binding and ligand-binding domains (14). hERα36 acts as a dominant-negative effector of estrogen (E2)-dependent and -independent *trans*-activation by full-length ERα and an E2- and anti-E2-dependent activator of MAPK/ERK in membrane-initiated E2 signaling pathway (15). hERα36 has been found to be expressed in both ER-positive and -negative breast cancer cell lines and tissues (13, 15). Another variant, hERα46, lacks the N-terminal A/B domains, and thereby, AF-1 antagonizes the proliferative function of full-length ERα in breast cancer cells by competition (16). Despite the prevalence of multiple spliced ER isoforms, the nature of an upstream splicing factor that could target the endogenous *ER* gene to generate a specific spliced isoform remains unknown.

In the current study, through yeast two-hybrid screening of a human breast cDNA library, we found a nuclear receptor (NR)-box-containing full-length protein, called nuclear protein E3-3 (NPE3-3), which was able to bind to ERα. The rat homolog of NPE3-3 has been previously shown to be a binding partner of serine/arginine-rich protein-30c (SRp30c) in yeast (17). Although the function of NPE3-3 is unclear, we considered that NPE3-3 might play a role in mRNA splicing. We provide evidence that NPE3-3 promotes ERα splicing, leading to a previously unrecognized spliced ERα in breast cancer cells.

Results

NPE3-3 binds to ERα and splicing factors

A NR-box-containing full-length protein, NPE3-3 was identified through a yeast two-hybrid-based screening of a human breast cDNA library using ERα as bait (Fig. 1A). NPE3-3 might interact with N-terminal (amino acids 1-263) and C-terminal (amino acids 264-552) regions of ERα. To validate the interaction between these two proteins using a mammalian system, we next transfected MCF-7 cells with Flag-NPE3-3 and T7-ERα. Flag-NPE3-3 was found to be effectively coimmunoprecipitated with T7-ERα (Fig. 1B). Moreover, endogenous NPE3-3 and ERα could be also coimmunoprecipitated from ZR-75 or MCF-7 cells (Fig. 1C). Confocal microscopic analysis also confirmed the colocalization of NPE3-3 with ERα in MCF-7 cells (Fig. 1D). A previous study has shown the association of NPE3-3 with a member of the SRp family, splicing factor SRp30c (17). We next showed that, indeed, NPE3-3 not only associated with SRp30c but was also weakly bound to SRp40 using coimmunoprecipitation (co-IP) (Fig. 1E). Because splicing factor SC-35 is known to display a characteristic speckled pattern that occupies a portion of the nucleoplasm, we examined whether NPE3-3 and ERα also colocalized with the SC-35 speckles. Confocal scanning microscopy data demonstrated that, indeed, NPE3-3 and ERα colocalized with the splicing factors hot spots, SC-35 speckles (Fig. 1, F and G). These results suggest that NPE3-3 might be involved in mRNA splicing regulation.

NPE3-3 promotes splicing from a minigene

Previous studies suggest that tumor-associated CD44 splicing variants could play an important role in initiation and progression of breast tumor (18). These observations, along with the fact that SRp30c was initially characterized as a splicing factor capable of influencing the CD44 alternative splicing decision *in vivo* upon mitogenic stimuli (19) reminded us that NPE3-3 might contribute to breast cancer cellular alternative splicing of CD44. To test the possibility that NPE3-3 might affect splicing, we next used a CD44 minigene system illustrated in Fig. 2 (20). Transient expression of NPE3-3 in Cos-7 cells resulted in

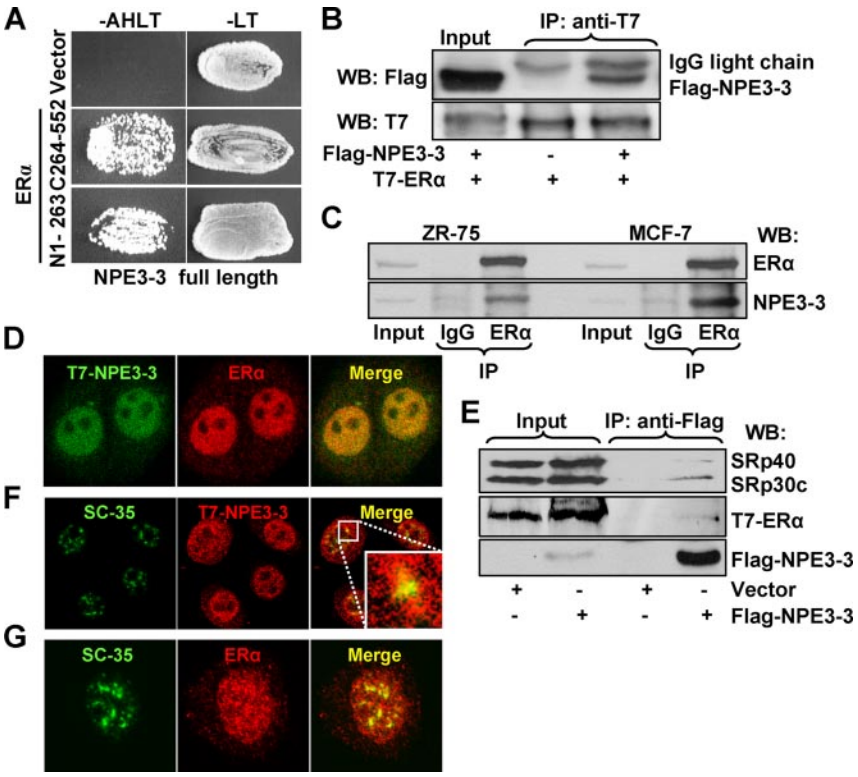

FIG. 1. Identification of NPE3-3 as a NR-binding protein. A, Yeast cells were cotransfected with GAD-NPE3-3 and the GBD control vector, or GBD-ERα (amino acids 1-263) or GBD-ERα (amino acids 264-552). Cotransformants were plated on selection plates lacking leucine and tryptophan (−LT) or lacking adenine, histidine, leucine, and tryptophan (−AHLT) with or without the presence of E2 (10^{-9} M). Growth was recorded after 72 h. B, Cell lysates from Cos-7 cells cotransfected with Flag-NPE3-3 and T7-ERα were immunoprecipitated with anti-T7 antibody and probed with anti-Flag and anti-T7 antibodies. C, ZR-75 and MCF-7 cells were transfected with Flag-NPE3-3, immunoprecipitated with anti-ERα antibody, and probed with anti-ERα anti-FLAG antibodies. D, Colocalization of NPE3-3 with ERα in the nuclear compartment. MCF-7 cells transfected with T7-NPE3-3 were cultured on coverslips for 24 h, fixed, and labeled with anti-T7 and anti-ERα antibodies after transfection. Then, cells were labeled with Alexa fluor 488- and Texas Red-conjugated secondary antibodies and analyzed by confocal microscopy. E, Cell lysates from Cos-7 cells cotransfected with Flag-NPE3-3 and T7-ERα were immunoprecipitated with anti-T7 antibody and probed with anti-SRp, anti-FLAG, and anti-T7 antibodies. F, To check whether NPE3-3 localized in SC-35 speckles, MCF-7 cells were transfected with T7-NPE3-3 and labeled with anti-SC-35 and anti-T7 antibodies. G, MCF-7 cells were labeled with anti-SC-35 and anti-ERα antibodies.

different splicing patterns of the CD44 minigene, showing that a product with both exon V4 and V5 (termed 2 exons inclusion in Fig. 2, *top band* in lane 3) in addition to a PCR product with one of exon V4 and V5 (termed 1 exon inclusion, *second band*) and a PCR product without both exons (termed skipping, *third band*) were amplified in the presence of exogenous NPE3-3. The combined transfection of NPE3-3 with ERα induced a marked increase in two exons inclusion (*top band* in lane 4), whereas ERα alone did not affect CD44 splicing patterns (lane 5). Interestingly, estrogen treatment caused a further change in the CD44 splicing pattern but resulted in no exon skipping (lane 6). In brief, these findings suggested that NPE3-3, a newly identified ERα interacting protein, is capable of splicing in the minigene system.

NPE3-3 promotes aberrant splicing of ERα

To better understand the function of NPE3-3, we next generated pooled MCF-7 clones expressing NPE3-3 (MCF-7/NPE3-3) (Fig. 3A). While examining the status of the transfected T7-NPE3-3 in clones, we unexpectedly observed a distinct reduction in the net amount of ERα (66 kDa) as well as the appearance of a new 37-kDa (Fig. 3A, *arrow*) protein band that cross-reacted with the anti-ERα antibody used here, suggesting that NPE3-3 might promote the expression of the ERα isoform. Interestingly, the 37-kDa protein band (presumably, a variant ERα) was present in all MCF-7/NPE3-3 clones but not in the MCF-7/pcDNA clone. To confirm the identity of the potential ERα isoform, the protein was immunoprecipitated and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Fig. 3B, *arrowhead*). This analysis identified four authentic peptides of ERα (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Next, we evaluated the identity of missing exons in the ERα isoform using RT-PCR and direct DNA sequencing. Primers in exons 1 and 8 amplified a smaller-size ERα band (Fig. 3C, *double asterisks*) in the NPE3-3 clones but not in the pcDNA control clone (Fig. 3C). Additionally, primers for a known hERα36 isoform (14) were used as a negative control, and these primers amplified a single band (Fig. 3C, *asterisk*) in both pcDNA and NPE3-3 clones. These sequencing data showed that we have identified a novel ERα isoform, termed ERαV, composed of only exons 1, 2, 7, and 8 (Supplemental Fig. 2). To validate these findings at the level of protein and to firmly establish the distinctiveness of ERαV from hERα36, we next loaded two sets of samples on a SDS-PAGE gel, blotted, cut into two portions, and probed with anti-hERα36 or ERα antibodies. hERα36-specific antibody recognized a single band in pcDNA and all NPE3-3 clones, whereas the ERαV band was smaller in size than that of hERα36 and was detected only in NPE3-3 clones (Fig. 3D). In addition, the antibody recognizing amino acids 2-185 of ERα also detected ERαV

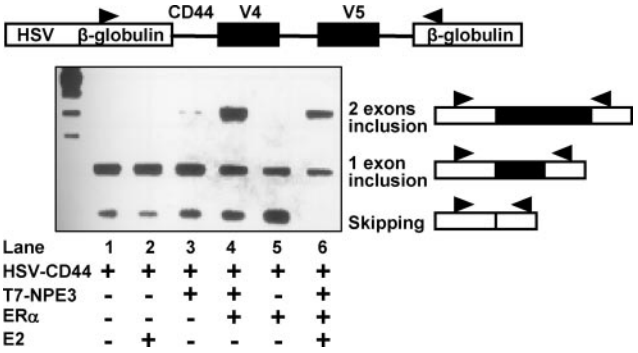

FIG. 2. NPE3-3 affects splicing patterns of CD44 minigene with ERα. Cos-7 cells were cotransfected with HSV-CD44 with or without T7-NPE3-3 and ERα with or without E2 (10^{-9} M). RNA was prepared from the transfected cells after 48 h and RT-PCR was performed to detect splicing patterns of CD44 minigene.

in all the stable NPE3-3 clone cells but not in pcDNA clone cells as the antibody recognizing amino acids 550-595 of ERα detects the variant in those clones in Fig. 3D (Supplemental Fig. 3).

ERα is known to function differently in nuclear (genomic) *vs.* cytoplasmic (nongenomic) signaling. Therefore, the intracellular localization of this ERα isoform is important to consider for understanding its function. A fractionation analysis revealed that ERαV was detected only in the cytoplasmic fraction compared with the presence of intact wild-type ERα mainly in the nuclear fractions (Fig. 3E). In addition, ERαV was shown to localize in the cytoplasmic compartment of MCF-7/NPE3-3 cells (Fig. 3F).

NPE3-3-mediated generation of ERαV participates in cytoplasmic signaling

The cytoplasmic localization of ERαV raised the possibility of cytoplasmic signaling in the NPE3-3 clones. We found that NPE3-3 overexpression activated the phosphorylation of ERK1/2 and the expression of ER-target gene products, such as cyclin D1 and cathepsin D (Fig. 4A). The hyperphosphorylation of ERK1/2 and the elevated expression of cyclin D1 were also caused by transient transfection of ERαV (Fig. 4B), suggesting that NPE3-3 might activate cytoplasmic signaling pathway by producing ERαV. Knockdown of NPE3-3 in MCF-7 cells by NPE3-3 siRNA decreased the constitutive level of phospho- (p-)ERK1/2 (Supplemental Fig. 4).

To determine the responsiveness of pcDNA and NPE3-3 clones to estrogen, cells were treated with estrogen. Phosphorylation of ERK1/2 in the control cells was maximally stimulated within 5 min of estrogen treatment, followed by a gradual return to near-basal level (Fig. 4C). In contrast, the levels of phosphorylated ERK1/2 were high in the stable clone and remained elevated throughout the time of estrogen treatment. These results suggested that cytoplasmic localization of ERαV may result in constitutive stimulation of nongenomic ER signaling, such as ERK1/2 stimulation.

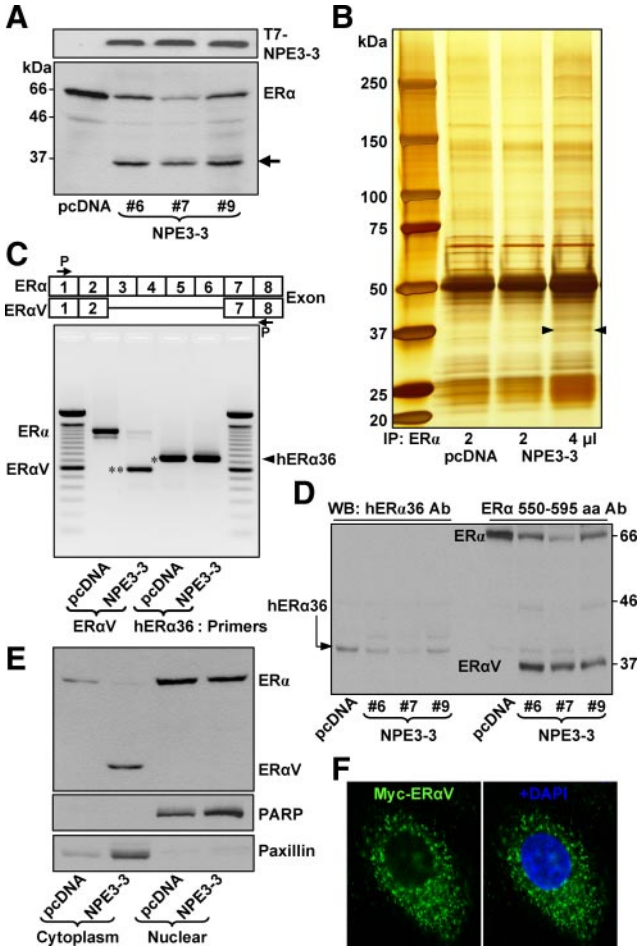

FIG. 3. NPE3-3 mediates aberrant splicing of ERα. A, Cell lysates from stable NPE3-3/MCF-7 and pcDNA/MCF-7 clones were subjected to SDS-PAGE and probed with anti-ERα and anti-T7 antibodies. *Arrow* indicates a previously unrecognized ERα band with molecular size of 37 kDa. B, Cell lysates (2 mg) from stable NPE3-3/MCF-7 and pcDNA/MCF-7 clones were immunoprecipitated with anti-ERα antibody, subjected to SDS-PAGE, and silver stained. The protein band (*arrowhead*) specific in stable clones was cut, trypsinized, and subjected to LC-MS/MS analysis. C, RT-PCR using primers designed in exons 1 and 8 of ERα and primers to amplify hERα36. Note that a novel alternative splicing form of ERα, termed ERαV (*double asterisks*) was present only in NPE3-3 clone cells, whereas hERα36 (*asterisk*) was equally amplified in both pcDNA and NPE3-3 clone cells. D, WB using two antibodies specific to hERα36 and recognizing the C terminus (550-590 amino acids) of ERα. E, Proteins prepared from cytoplasmic and nuclear fractions of stable NPE3-3/MCF-7 and pcDNA/MCF-7 clone cells were analyzed by WB using anti-ERα, anti-PARP, and antipaxillin antibodies. F, MCF-7 cells were transfected with Myc-ERαV, labeled with anti-Myc antibody, and analyzed by confocal microscopy.

To understand the role of ERαV, NPE3-3, or ERα in stimulating growth-promoting ERK1/2 activation and to determine whether there is any contribution of NPE3-3 or ERα in the noted ERαV-mediated ERK1/2 activation, we next transfected ERαV, NPE3-3, or ERα into ERα-negative cell line MDA-MB-231 cells and examined the status of p-ERK1/2 (Supplemental Fig. 5A). p-ERK1/2 expres-

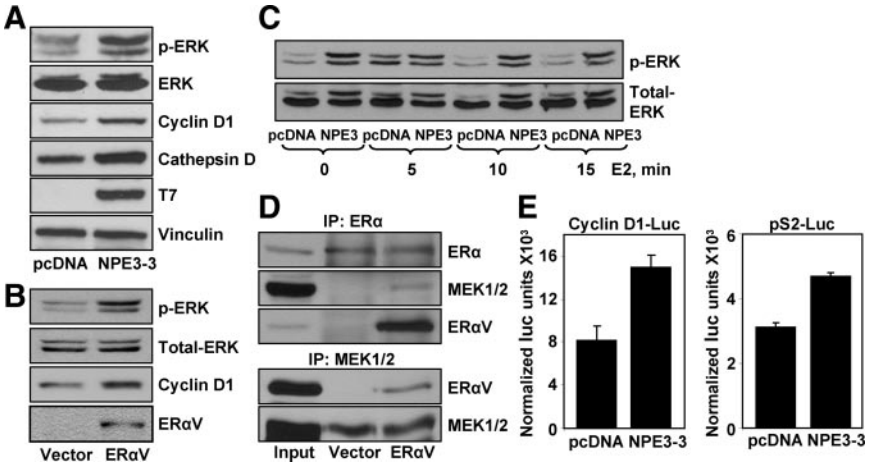

FIG. 4. NPE3-3 constitutively activates ERα nongenomic signaling pathway via its production of ERαV. A, The expression levels of p-ERK1/2, cyclin D1, and cathepsin D, which were involved in ERα nongenomic signaling pathways in stable pcDNA and NPE3-3 clone cells, were determined by WB. B, Myc-ERαV or empty vector was transfected into MCF-7 cells, and the phosphorylation of ERK1/2 and the expression of cyclin D1 were determined. C, Stable pcDNA/ and NPE3-3/MCF-7 clone cells were serum starved for 48 h and treated with E2 (10^{-8} M). ERK1/2 phosphorylation in those cells was determined. D, Cytoplasmic fractions from MCF-7 cells transfected with vector or ERαV were immunoprecipitated with anti-ERα or anti-MEK1/2 antibodies and probed with antibodies as indicated. E, Stable pcDNA and NPE3-3 clone cells were transfected with cyclin D1- or pS2-luciferase plasmids, and luciferase activities were determined.

sion was elevated in the ERαV-transfected MDA-MB-231 cells compared with control nontransfected cells (shown by a *dotted white line*). However, the levels of p-ERK1/2 were not significantly affected in NPE3-3- or ERα-transfected and nontransfected cells. Similarly, the transfection of ERαV, but not vector, NPE3-3 or ERα into MDA-MB-231 cells activated Elk-1-promoter-luc reporter activity, presumably due to p-ERK1/2 activation (Supplemental Fig. 5B). Furthermore, ERαV overexpression enhanced the cell proliferation of MDA-MB-231 compared with control cells (Supplemental Fig. 5C).

To gain insights of the potential mechanism by which ERαV contributes to elevated activation of ERK1/2, we explored the possibility that ERαV could interact with MAPK kinase (MEK)-1/2, an upstream activator of ERK1/2. MCF-7 cells were transfected with ERαV or pcDNA vector, and cytoplasmic fractions were immunoprecipitated with an anti-ERα antibody that effectively recognizes both the full-length ERα and ERαV. We found that equal amounts of ERα were present in cells transfected with ERαV or pcDNA (Fig. 4D). However, MEK1/2 was coimmunoprecipitated only in the ERαV-transfected MCF-7 cells but not in the empty vector-transfected MCF-7 cells. In a reverse experiment, MCF-7 cell lysates were immunoprecipitated with an anti-MEK1/2 antibody. We found that ERαV was coimmunoprecipitated with MEK1/2 only in the ERαV-transfected MCF-7 cells but not in the empty vector-transfected MCF-7 cells (Fig. 4D). These results suggest that ERαV physically interacts with MEK1/2 in a physiologically relevant setting, and such an interaction might lead to the constitutive stimulation of ERK1/2 signaling. As an additional readout of enhanced cytoplasmic signaling in NPE3-3-expressing cells, we next showed increased cyclin D1 and pS2 promoter activity in MCF-7/NPE3-3 cells as compared with MCF-7/pcDNA cells (Fig. 4E). In brief, NPE3-3-mediated generation of ERαV promotes cytoplasmic nongenomic ER signaling.

NPE3-3 expression enhances proliferation, migration, anchorage-independent growth, and tumorigenicity

To understand the biological implications of increased NPE3-3 levels in breast cancer cells, we next measured the growth and invasiveness of the stable clones. As shown in Fig. 5, NPE3-3-expressing cells exhibited increased proliferation, migration, and anchorage-independent growth as compared with control vector-expressing MCF-7 cells (Fig. 5, A and B). Likewise, injection of MCF-7/NPE3-3 cells in mice resulted in undifferentiated carcinoma with significant size of tumor in seven of nine mice, whereas only one of nine controlled mice developed tumors (Fig. 5C). As expected, tumors resulted from the MCF-7/NPE3-3 cells also exhibited elevated expression of Ki-67, cyclin D1, and p-ERK1/2 (Fig. 5D). These findings when combined with the results presented in the preceding sections suggest that NPE3-3 overexpression promotes tumorigenic characteristics of breast cancer cells, possibly due to increased cytoplasmic signaling.

Elevated expression of NPE3-3 in human breast tumor cells

To determine the status of NPE3-3 levels in breast tumors, we next generated an antipeptide antibody against NPE3-3. The antibody was characterized for its specificity to detect NPE3-3. We found that NPE3-3 could be easily detected in the nuclear proteins from both ER-positive and -negative breast cancer cells (Fig. 6A). Using a small number of lysates from human breast tumors, we also found relatively increased levels of NPE3-3 in five of eight cases of the tumor tissue samples (Fig. 6B). Although NPE3-3 levels might be up-regulated in both ER-positive and ER-negative cells, we next wished to examine the natural prevalence of its target ERαV in a small collection of well characterized human tumor samples, as

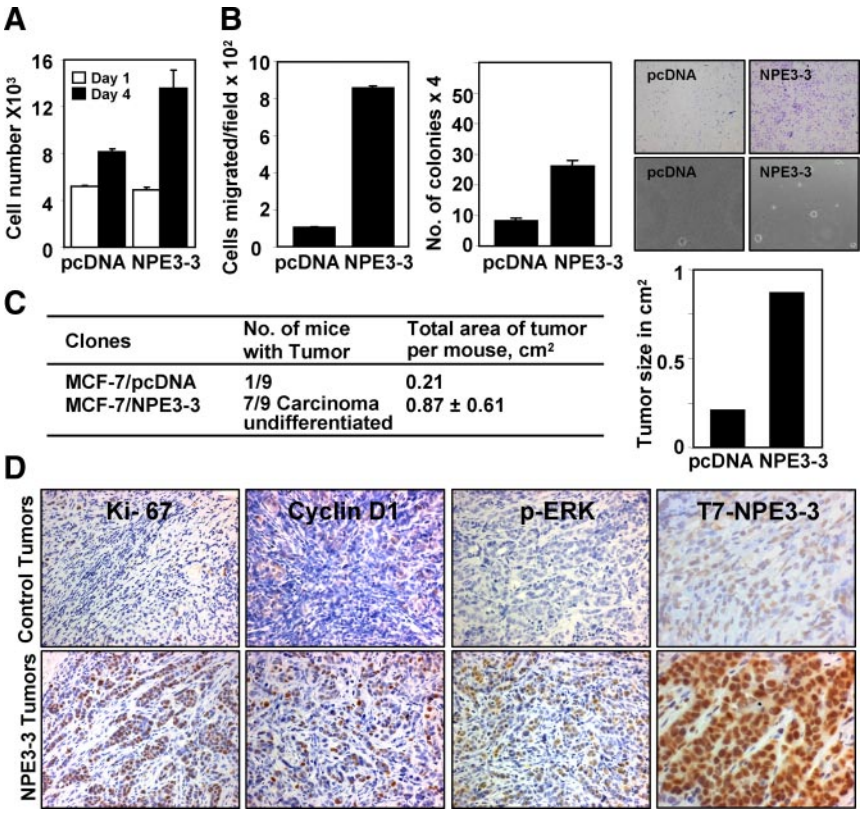

FIG. 5. NPE3-3 enhanced tumor cell proliferation, migration, and colony formation and *in vitro* tumorigenicity. A, Cell numbers of pcDNA and NPE3-3 clones were counted after d 4. B, pcDNA or NPE3-3 clone cells (1×10^5 cells) were added on the lower side of a Boyden chamber and incubated for 16 h. Then the cells that passed through the filters were fixed, stained, and counted (*left*). pcDNA or NPE3-3 clone cells (1×10^5 cells) mixed with 0.36% Bactoagar solution in DMEM were layered on top of the 0.6% Bactoagar layer. After incubation for 21 d, the colony formation (*middle*) was analyzed. *Right*, Representative pictures of cell migration (*top*) and colony formation (*bottom*). C, pcDNA or NPE3-3 clone cells (1×10^6 cells) were injected into nude mice as shown in *Materials and Methods*. D, Immunohistochemistry of Ki-67, cyclin D1, and p-ERK1/2 in tumor tissues from xenografts.

a proof of concept. As illustrated in Fig. 6C, ERαV was detectable in eight of 13 breast cancer tissues (*asterisks*) by repeating PCR and the identity of the DNA bands was confirmed by direct sequencing (Supplemental Fig. 6).

Discussion

The NPE3-3 rat homolog was identified as a SRp30c-interacting protein (17). Although the protein was expected to be a new component of the spliceosome, this could not be demonstrated because of the failure to detect its interaction with other splicing components and the weakness of its interaction with SRp30c. Therefore, the function of the rat homolog of NPE3-3 remained still unclear until this study was undertaken. Here we found that NPE3-3 distinctively interacted with SRp30c and SRp40 and colocalized with SC-35 in the nucleus. In addition, it was revealed that NPE3-3 promoted alternative splicing of a CD44 minigene and ERα in the breast cancer cells. These results suggested that NPE3-3 plays a role in mRNA splicing events, presumably as a component of the spliceosome.

Earlier studies have shown that components of the spliceosome bind to ERα. For example, breast cancer amplified sequence 2 (BCAS2), which was initially identified as an amplified and upregulated gene in human breast cancer cell lines, was found to be a component of the spliceosome, to interact with ERα, and to potentiate ERα transcriptional activity by acting as an ER coactivator (21). The other spliceosome component, SF3a p130 was shown to physically associate with ERα in a manner dependent on the phosphorylation at Ser-118 (22). In this study, we found that SF3a p130 potentiated ERα-mediated mRNA splicing in a Ser-118 phosphorylation-dependent manner. Although the mechanism through which ERα contributes to mRNA splicing remains to be elucidated, the data from other and our studies suggest that the interaction of spliceosome components with ERα plays an important role in both mRNA splicing and the ERα signaling pathway. It has been recently reported that ERα coordinately regulates alternative splicing of ERα mRNA with homeobox transcription factor BARX2 and elevates the expression of the isoforms hERα46 (23). Therefore, the binding of NPE3-3 with ERα might be a critical step for the generation of ERαV by alternative splicing of ERα.

NPE3-3 also changed the splicing patterns of a CD44 minigene in the presence of ERα. To determine which variant exons (v1–v10) of endogenous CD44 were modified by NPE3-3, we performed RT-PCR using primers to amplify each variant exon. In 10 exons (v1–v10), the inclusion of v3 and v9 exons increased in all NPE3-3/MCF-7 clone cells (data not shown). CD44v3, 8-10 isoform was shown to bind specifically to the cytoskeletal protein ankyrin that forms invadopodia and closely associates with the active form of MMP-9 in the invadopodia (8). Because all these processes participate in breast cancer cell migration and invasion, the CD44 isoforms generated by NPE3-3 may potentially contribute to the oncogenic signaling pathway in breast cancer cells.

ERαV possessing exons 1, 2, 7, and 8 is different from hERα36 with exons 3, 4, 5, and 6. Although it regains a

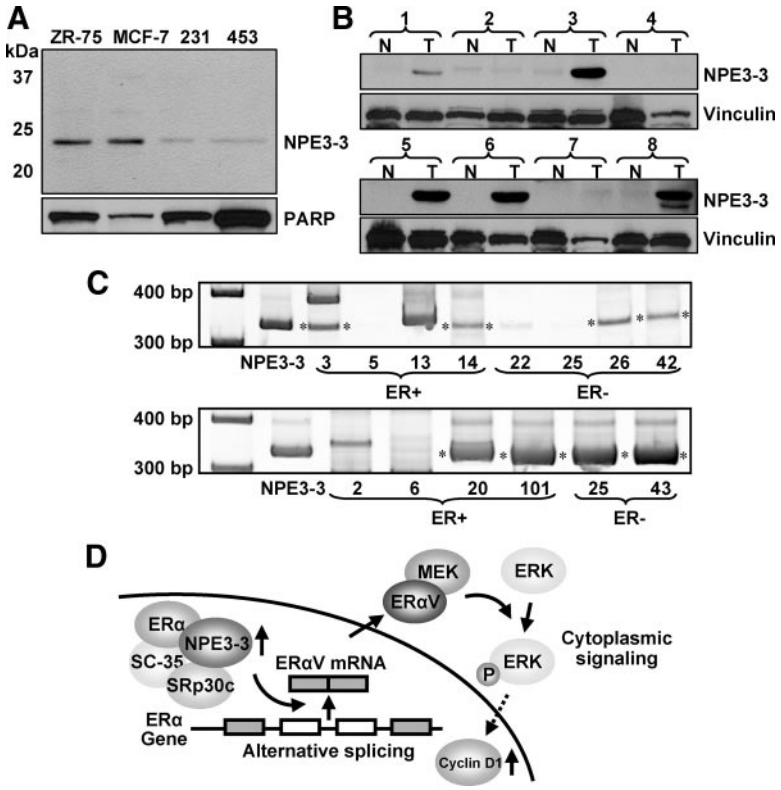

FIG. 6. NPE3-3 expression is promoted in breast tumor cells. A, Antibody against NPE3-3 was generated and its specificity was evaluated using cell lysates from breast cancer cell lines including ZR-75, MCF-7, MDA-231, and MDA-453. B, The expression of NPE3-3 was tested in tumor tissue samples from patients with breast cancer (T) and paired normal breast tissue samples (N) using the anti-NPE3-3 antibody. C, ERαV expression was tested by RT-PCR using RNA prepared from 13 $ER^{+/-}$ breast cancer tissue samples. Bands of the same size same as that of a band in positive control (NPE3-3 lane) were cut, and the PCR products were extracted from gels. Then, PCR was repeated once (*upper panel*) or twice (*lower panel*) using the extracted DNA. The final PCR products were sequenced, and eight of 13 samples were found to be positive (*asterisks*). D, Proposed working model for activation of nongenomic signaling pathway via alternative splicing of ERα by NPE3-3. Increase of NPE3-3 expression promotes alternative splicing of ERα mRNA through its interaction with ERα, Srp30c, and SC-35. ERαV, which is generated as the alternative splicing variant of ERα, localizes to the cytoplasmic compartment in cells and binds to MEK1/2. Then the resulting complex of ERαV and MEK1/2 promotes the activation of ERK1/2 phosphorylation and subsequent up-regulation of cyclin D1 via a nongenomic signaling pathway, leading to the activation of oncogenic signaling including cell proliferation and progression in breast cancer.

ligand-independent AF, hERα36 retains the DNA-binding domain and partial dimerization and ligand-binding domains but lacks both AF-1 and AF-2 (14). hERα36 has three potential myristoylation sites located proximal to the N terminus, leading to its localization at the plasma membrane. From our confocal microscopic and biochemical analyses, ERαV appears to have only one potential myristoylation site and, thus, may localize to the cytoplasm. ERα36 has been shown to act as a dominant-negative effector of both E2-dependent and independent *trans*-activation functions against full-length ERα and promote membrane-initiated E2-dependent activation of ERK1/2. NPE3-3 promoted constitutive ERK1/2 activation in an E2-independent manner, presumably due to the generation of ERαV by NPE3-3. However, this brought us to further question how ERαV could mediate the constitutive activation of ERK1/2. Recently, methylation at arginine 260 in exon 4 of ERα has been shown to regulate E2 rapid nongenomic signaling (24). However, ERαV is lacking this arginine site. In this context, our data revealed that ERαV physically binds to a specific upstream kinase of ERK1/2, MEK1/2, suggesting that increased ERK1/2 activation in ERαV-containing breast cancer cells could be mediated via the interaction of ERαV with MEK1/2. Interestingly, we also found evidence of expression of both NPE3-3 and ERαV in human breast tumors, highlighting the physical relevance of the findings presented here in inherent cytoplasmic signaling.

Although mRNA splicing is widely recognized as a fundamental mechanism to regulate gene expression, the significance of genetic variances in the cellular machinery of splicing in breast cancer is still underestimated. Moreover, although several individual cases of inherited and acquired mutations in *cis*-acting splice elements have been associated with breast cancer, the significance of deregulation of *trans*-acting splicing regulatory factors in the initiation and maintenance of mammary tumor is largely unappreciated. Here, we show that NPE3-3 acts like a *trans*-acting splicing regulatory factor that might mediate oncogenic signaling via its regulation of mRNA splicing in breast cancer (Fig. 6D). Because mRNA splicing is a fundamental tool to regulate gene expression, and the deregulation of NPE3-3 might confer malignancies to the breast cancer cells, our findings offer a novel mechanistic approach to explore the contribution of defects of mRNA splicing in mammary tumorigenesis. Furthermore, delineation of the pathways to regulate ER mRNA splicing by NPE3-3 will represent a valuable target to develop a new effective strategy to treat breast cancer patients.

Materials and Methods

Cell cultures and reagents

Cells were maintained in DMEM-F12 (1:1) supplemented with 10% fetal bovine serum. E2 was purchased from Sigma-

Aldrich (St. Louis, MO). We used the following antibodies: ERα and T7 (Bethyl Laboratories, Montgomery, TX); phospho-p42/p44 ERK/MAPK, (Cell Signaling, Beverly, MA); ERK1, ERK2, and poly ADP-ribose polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA); cyclin D1 and Myc (NeoMarkers, Fremont, CA); and cathepsin D, SC-35, vinculin, and Flag (Sigma-Aldrich). Anti-NPE3-3 antibody was prepared by immunization of NPE3-3 peptide to rabbits. hERα36 antibody was kindly provided by Dr. ZhanoYi Wang (Creighton University Medical School, Omaha, NE). mAb104 was prepared from mouse hybridoma (American Type Culture Collection, Manassas, VA).

Plasmid construction

To clone the ERα variant whole sequence, we amplified DNA from the MCF-7 NPE3-3 stable clone cDNA by using primers GGGGTACCATGACCATGACCCTCCACAC and GGGTTAACTCAGACCGTGGCAGGGAAAC, and the fragment was cloned into pCruz B vector (Santa Cruz).

Two-hybrid library screening

ERα baits were constructed by amplifying amino acids 1-263 and 264-552 of ERα by PCR and by subcloning the products into Gal4-binding domain (GBD) vector pGBD (Clontech, Palo Alto, CA). A mammary gland cDNA library fused to Gal4 activation domain was purchased from Clontech and screened using both the N-terminal (1-263) and C-terminal (264-552) ERα as baits in accordance with the manufacturer's instructions. A total of 2×10^6 clones were screened. Positive interactors were verified by one-on-one transformations followed by selection of agar plates lacking leucine and tryptophan (−LT) or adenosine, histidine, leucine, and tryptophan (−AHLT). Positive clones were isolated and subjected to direct sequencing.

Western blotting (WB) and immunoprecipitation (IP)

WB and IP were performed as described earlier, with some modifications (25). For WB, lysates from whole cells or nuclear extracts were resolved in SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with the antibodies. Immunoblots were developed with the enhanced chemiluminescence reagent (Amersham Pharmacia Biosciences, Piscataway, NJ) according to the manufacturer's instructions. For IP, lysates obtained from ZR-75 cells or MCF-7 cells were subjected to IP with anti-T7, anti-Flag, or anti-ERα, followed by incubation with protein A/G beads (Santa Cruz). After washing, the eluates from the beads were subjected to WB for desired proteins.

Cell proliferation, migration, soft agar, and tumorigenicity assays

For cell proliferation assays, equal numbers of cells were plated in triplicates, and then the proliferation rate of the cells was measured by counting them with a Coulter counter (Beckman Coulter, Fullerton, CA). For the migration assay, cells were loaded onto the upper well of an uncoated Boyden chamber at a concentration of 1×10^5 cells per well. The lower side of the separating filter was filled with a conditioned medium from NIH-3T3 fibroblasts grown in DMEM/F12 medium. We counted the cells that successfully migrated through the filter. Soft agar colony growth assays were performed as described previously (26). Briefly, 1 ml solution of 0.6% Difco agar in DMEM supplemented with 10% fetal bovine serum with insulin was layered onto 60- × 15-mm tissue culture plates. MCF-7 cell (1×10^5 cells) were mixed with 1 ml 0.36% Bactoagar solution in DMEM prepared in a similar manner and layered on top of the 0.6% Bactoagar layer. Plates were incubated at 37 C in 5% CO_2 for 21 d. Experiments were performed in triplicates.

For tumor xenograft studies, 6- to 8-wk-old athymic (nu/nu) mice (Charles River Laboratories, Wilmington, MA; nine mice per group) were given bilateral hind flank injections of MCF-7/pcDNA or NPE3-3 cells (1×10^6 cells) suspended in 30% Matrigel in Hanks' balanced salt solution (Beckton Dickinson, Bedford, MA). Total area of tumor was measured with calipers after 4 wk. At the end of 4 wk, mice were killed by cervical dislocation, and tumors were excised and fixed in 10% buffered formalin. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas M. D. Anderson Cancer Center. Formalin-fixed, paraffin-embedded tumor sections were deparaffinized in xylene, rehydrated in graded ethanol, and stained with the antibodies. Stained tumor sections were examined and photomicrographs captured using an Olympus BX51 microscope and Olympus DPManager software (Olympus America Inc., Melville, NY).

Transfection, immunofluorescence labeling, and confocal microscopy

Transient transfection studies were performed using a FuGENE 6 kit (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the manufacturer's instructions. The cellular localization of proteins was determined by indirect immunofluorescence. Briefly, the cells were grown on sterile glass coverslips, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 10% normal goat serum in PBS. Cells were incubated with primary antibodies, washed three times in PBS, and then incubated with goat antimouse or goat antirabbit secondary antibodies conjugated with 546-Alexa (red) or 488-Alexa (green) from Molecular Probes (Eugene, OR). The blue DNA dye 4′,6-diamidino-2-phenylindole (Molecular Probes) was used as a nuclear stain. Microscopic analyses were performed using an Olympus FV300 laser-scanning confocal microscope using sequential laser excitation to minimize fluorescence emission bleed-through.

Protein identification by LC-MS/MS

Cell lysates from MCF-7 pcDNA and NPE3-3 stable clones were immunoprecipitated with anti-ERα antibody and loaded onto 4–20% SDS-PAGE gradient gels (Bio-Rad, Hercules, CA). The gels were silver stained, and bands were cut from the gel, followed by tryptic digestion and liquid chromatography coupled to electrospray ion trap tandem mass spectrometry (LC-MS/MS) for peptide sequencing.

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