Neuropilin-2 Is Upregulated in Lung Cancer Cells during TGF-β1–Induced Epithelial–Mesenchymal Transition

Patrick Nasarre1, Robert M. Gemmill1, Vincent A. Potiron1, Joëlle Roche1, Xian Lu4, Anna E. Barón5, Christopher Korch2, Elizabeth Garrett-Mayer2, Alessandro Lagana6, Philip H. Howe3, and Harry A. Drabkin1

Abstract

The epithelial–mesenchymal transition (EMT) and its reversal, mesenchymal–epithelial transition (MET), are fundamental processes involved in tumor cell invasion and metastasis. SEMA3F is a secreted semaphorin and tumor suppressor downregulated by TGF-β1 and ZEB1-induced EMT. Here, we report that neuropilin (NRP)-2, the high-affinity receptor for SEMA3F and a coreceptor for certain growth factors, is upregulated during TGF-β1–driven EMT in lung cancer cells. Mechanistically, NRP2 upregulation was TβRI dependent and SMAD independent, occurring mainly at a posttranscriptional level involving increased association of mRNA with polyribosomes. Extracellular signal–regulated kinase (ERK) and AKT inhibition blocked NRP2 upregulation, whereas RNA interference-mediated attenuation of ZEB1 reduced steady-state NRP2 levels. In addition, NRP2 attenuation inhibited TGF-β1–driven morphologic transformation, migration/invasion, ERK activation, growth suppression, and changes in gene expression. In a mouse xenograft model of lung cancer, NRP2 attenuation also inhibited locally invasive features of the tumor and reversed TGF-β1–mediated growth inhibition. In support of these results, human lung cancer specimens with the highest NRP2 expression were predominantly E-cadherin negative. Furthermore, the presence of NRP2 staining strengthened the association of E-cadherin loss with high-grade tumors. Together, our results demonstrate that NRP2 contributes significantly to TGF-β1–induced EMT in lung cancer. Cancer Res; 73(23); 7111–21. ©2013 AACR

Introduction

Lung cancer accounts for nearly one-fifth of cancer deaths worldwide, with invasion, metastases, and drug resistance representing major barriers to cure. The epithelial–mesenchymal transition (EMT), by which epithelial cells acquire a mesenchymal and invasive phenotype, contributes significantly to these barriers (1). TGF-β1 is a major inducer of the EMT (2), affecting lung cancer cells, acting either alone or in combination with cytokines or growth factors (3–5). In canonical TGF-β signaling, binding to the type I/II receptor (TβRI/TβRII) complex leads to phosphorylation of SMAD-2/3, their interaction with SMAD-4 and nuclear translocation (6). In contrast, non-canonical signaling is SMAD independent involving AKT, extracellular signal–regulated kinase (ERK), and other pathways (7, 8). In lung cancer, as with many malignant diseases, upregulation of TGF-β is a poor prognostic marker (9). Among other activities, TGF-β secretion by tumor cells and stromal components stimulates fibroblasts and extracellular matrix formation, while it inhibits antitumor immune responses (9). However, proliferation is also inhibited by TGF-β, suggesting why some tumors mutate or downregulate key TGF-β signaling components (10–12). The EMT process impairs growth of metastatic deposits, whereas its reversal, mesenchymal–epithelial transition (MET), is associated with increased proliferation and tumor growth (13). In human breast cancer, a partial EMT phenotype may be sufficient to facilitate invasion and vascular dissemination, as evidenced by circulating tumor cells and tumor cell clusters coexpressing epithelial and mesenchymal markers (14).

We previously demonstrated that SEMA3F, a secreted tumor suppressor isolated from a recurrent 3p deletion region in lung cancer (15), was downregulated by the EMT transcription factor, ZEB1 (16). The secreted class 3 semaphorins (SEMA3a) were discovered as inhibitors of nerve growth cone migration, and subsequently shown to function in various developmental and pathologic processes, including cancer (17). Neuropilin (NRP)-1 and 2 are high-affinity receptors for the SEMA3s, with SEMA3F binding predominantly to NRP2 (18, 19). In addition, both neuropilins were identified as coreceptors for...
VEGF and other selected growth factors (18, 20, 21). Importantly, neuropilins are overexpressed in several cancers, including lung, and their expression correlates with increased invasion and poor prognosis (22).

Because SEMA3F is downregulated during EMT in lung cancer cells, we asked what happens to NRP2. In contrast with SEMA3F, NRP2 is upregulated by TGF-β and contributes significantly to the changes associated with induction of the EMT phenotype. Mechanistically, NRP2 upregulation was predominantly translational, involving increased mRNA binding of polyribosomes. Initial NRP2 upregulation was SMAD independent and blocked by ERK and AKT inhibitors, whereas ZEB1 knockdown reduced steady-state NRP2 levels. In a xenograft model, we found that NRP2 contributes significantly to the invasive phenotype of lung cancer cells, as well as TGF-β1-mediated growth inhibition, which seems to be, at least in part, cell autonomous. In resected human lung cancers, high NRP2 staining together with low E-cadherin was associated with increased tumor grade. Moreover, tumors with the highest NRP2 scores were predominantly E-cadherin negative. Together, these results indicate that NRP2 upregulation plays an important role in TGF-β1-induced EMT.

Materials and Methods
Cell lines, reagents, and expression constructs
Non–small cell lung carcinoma (NSCLC) cell lines HCC-4006, NCI-H441, NCI-H358, and A549 were obtained from the Colorado Lung Cancer SPORE Cell Repository. Verification of NSCLC cell lines (by comparison with American Type Culture Collection data) was carried out using microsatellite genotyping analysis performed by the University of Colorado DNA Sequencing and Analysis Core (Aurora, CO). Cells were grown in RPMI-1640 medium with 10% fetal calf serum and antibiotics (Invitrogen). Cells were cultured in a humidified 5% CO2 incubator at 37°C.

Recombinant human TGF-β1 (5 or 10 ng/mL) was from R&D Systems. Inhibitors were from the following suppliers: SB431542 (StemGenet), U0126 (Promega), cycloheximide (Calbiochem), MKK-2206 (Selleckchem), actinomycin-D (Sigma-Aldrich), hygromycin, blasticidin, puromycin, and doxycycline were from Invitrogen. Doxycycline was used at 1–100 ng/mL. SMAD7 in pRK5 was stably cotransfected with viral particles (Sigma-Aldrich; Supplementary Table S1).

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Stable integration of TGF-β1 under the control of a tetracycline/doxycycline-inducible promoter was obtained with the Flp-In T-Rex System from Invitrogen. Empty vector or TGF-β1–transfected cells were selected and grown with 100 µg/mL hygromycin and 5 µg/mL blasticidin. Doxycycline was used at 100 ng/mL. SMAD7 in pBSK5 was stably cotransfected with pcDNA3 and selected with 500 µg/mL G418. Stable short hairpin RNA (shRNA) transfections were generated from lentiviral particles (Sigma-Aldrich; Supplementary Table S1). Transfection was performed using 8 µg/mL polybrene, and cells were selected with 2 µg/mL puromycin.

RNA and protein analysis
Total RNA extraction protocol and gene expression analysis by quantitative real-time PCR (qRT-PCR) analysis were described previously (4). Data were expressed as the percentage of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are provided in Supplementary Table S2.

Immunoblot analyses were performed as described (4). Primary antibodies included: anti-vimentin (3932S), anti-phospho SMAD2 (3101S), anti-phospho SMAD3/1 (9514S), anti-phospho SMAD1/5 (9516S), anti-phospho ERK1/2 (T202, Y204; 9101S), anti-AKT (4691S), and anti-phospho AKT (S473; 4060S; 1:1000) from Cell Signaling Technologies; anti-NRP1 (sc-7239) and anti-ZEB1 (sc-25388) antibodies (1:1000) from Santa Cruz Biotechnology; anti-NRP2 (AF2215; 1:1000) from R&D Systems; anti-E-cadherin (610181; 1:1000) from BD Biosciences; anti-N-cadherin (ab21221; 1:1000) from Abcam; anti-Myc tag (M4439) and anti-β-actin (A1978; 1:3000) from Sigma-Aldrich; anti-EpCAM (MS-144-PABX; 1:1000) from Neo-markers; anti-SMAD7 (42-0400; 1:1000) from Invitrogen and anti-ERK (V114A; 1:1000) from Promega. Secondary antibodies included: horseradish peroxidase (HRP)-conjugated anti-rabbit (1858415) and anti-mouse (1858413) from Thermo Fisher Scientific Inc; HRP-conjugated anti-goat (81-1620) was from Invitrogen. Detection was performed with Western Lightning Plus ECL reagent (PerkinElmer). E-cadherin and β-actin were detected using an Alexa™-conjugated anti-mouse antibody (A21200; Invitrogen) and signal was recorded using a Typhoon 9400 Image system (GE Healthcare). Band quantitation was performed on Typhoon images or by using stained images (926-322104) and CW680 (926-322212) IRDye-conjugated secondary antibodies and a LI-COR Odyssey fluorescence scanner (LI-COR Biosciences). Autoradiographic bands were quantified using ImageJ freeware or LI-COR analysis software for IR fluorescence.

Immunofluorescence and immunohistochemistry
For immunofluorescence, cells were processed as described (23). Slides were incubated with anti-ZEB1 or anti-NRP2 at 1:100 dilution. Alexa™-conjugated anti-goat (A10155) and Alexa™-conjugated anti-rabbit (A11072) secondary antibodies (1:200) were from Invitrogen. 4’, 6-diamidino-2-phenylindole (DAPI; 1:50,000) was from Sigma-Aldrich. Stained slides were mounted in Dako Fluorescent Mounting Medium (Dako). Images were captured with IPLab software on a BD CARVII spinning disc confocal microscope (BD Biosciences).

Immunohistochemistry procedure, analysis, and scoring methods were performed as described (4). Primary antibodies were mouse anti-human E-cadherin (1:50), goat anti-human NRP2 (1:100), rabbit anti-human Ki67 (18-01917; 1:50), and rabbit anti-human pan-cytokeratin (18-0059; 1:50) from Invitrogen. Secondary antibodies (Vector Laboratories) were biotinylated rabbit anti-goat (BA-5000), goat anti-rabbit (BA-1000), and goat anti-mouse (BA-9200) antibodies. For the tissue microarray (TMA) analysis, scores equal or below 10, for both NRP2 and E-cadherin staining, were considered negative. For the proliferation analysis in tumor xenografts, three fields/tumor were counted in four representative tumors per cohort.

Polyribosome separation on sucrose gradients was performed as described (24).

Migration/invasion assays
Transwell assays were performed using the BD Biocoat system following the manufacturer’s instruction (BD Biosciences). Briefly, the lower chamber was filled with 5% fetal calf serum
containing RPMI-1640 as an attractant. Fifty-thousand H358 cells or 25,000 A549 cells were suspended in 0.5 mL serum free RPMI-1640 and added to the upper chamber followed by the addition of TGF-β or control solutions. H358 cells were incubated for 48 hours and A549 cells for 12 hours at 37°C, 5% CO₂. Cells were then fixed with 4% paraformaldehyde, stained with crystal violet, rinsed, and cells remaining on the upper side of the filter were removed. After allowing the filters to dry overnight, cells were counted under a SMZ1500 stereomicroscope (Nikon). Each condition was performed in triplicate and four fields were counted per filter. ShControl transfectants were included in each experiment to assess reproducibility.

Proliferation assay
Control or NRP2 knockdown H358-Tr-TGF-β cells (5 × 10⁴ cells/plate) were cultured in the presence or absence of doxycycline (100 ng/mL), trypsinized and counted at days 2 and 4. Doubling time was calculated using the following formula: $T = t \times \ln(2)/(\ln(N4)-\ln(N2))$ in which $T$ is the doubling time, $t$ is the time between two measurements, $N4$ is the final number of cells at day 4, and $N2$ is the number of cells measured at day 2. This experiment was repeated three times.

Tumor xenografts and human lung tumor microarrays
One million H358-shControl, H358-shNRP2-B, H358-Tr-TGF-β1-shControl, or H358-Tr-TGF-β1-shNRP2-B cells suspended in 200 µL of 50% Matrigel were injected subcutaneously in the right flank of 6-week-old female athymic nude mice (10 mice per group; Hsd: Athymic Nude-Foxn1nu, Harlan). After sacrifice, tumors were removed and weighed. Half of each tumor was placed into optimal cutting temperature (OCT) compound and the remainder fixed in 4% paraformaldehyde overnight, then incubated at 70% ethanol overnight before paraffin embedding. All animal experiments were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (Charleston, SC).

TMA slides containing 109 lung tumor samples and 10 normal lung tissues were obtained from US Biomax (ref: BC041115). These were processed for immunohistochemistry as described above.

Statistical analysis
Descriptive statistics were computed on TMA expression scores for all genes, as well as clinical characteristics. One-way ANOVA was used to test the association between EMT status (NRP2+/Ecadherin- or NRP2-/Ecadherin+) and clinical characteristics that are categorical variables namely sex, histology, and tumor-node-metastasis stage. Correlations between the EMT status and age or tumor grade were analyzed using Fisher exact test. In addition to analyzing expression scores as continuous variables, each gene’s expression level was also dichotomized: a score of 0 to 10 was considered negative and a score above 10 was considered positive; McNemar test was used to compare the dichotomous gene expression. Correlations between genes were analyzed using Spearman statistics. The naïve $P$ values of these correlations were adjusted for multiple comparisons controlling for false discovery rate. Statistical analyses were performed using SAS/BASE and SAS/STAT software, Version 9.2 of the SAS System for Windows (SAS Institute, Inc.). To determine the relative effects of NRP2 shRNAs on migration/invasion, the log of the number of migrating cells per field was modeled as a function of shRNA (e.g., shNRP2-B vs. shNRP2-Y vs. shControl) and TGF-β (yes vs. no) using linear regression modeling. Model estimates were used to derive estimates of fold-changes comparing conditions, $P$ values for testing statistical significance of fold-change, and calculating 95% confidence intervals for fold-changes. Appropriateness of linear regression assumptions was assessed using residual plots. The significance of the differences between proliferation levels in xenografts and between E-cadherin scores in the TMA was assessed using the Student $t$ test.

Results
NRP2 is upregulated by TGF-β1 in lung cancer cell lines
To determine whether NRP1/2 levels were affected by EMT, we treated four NSCLC cell lines containing epithelial features with exogenous TGF-β1 for 1 to 3 days. Varying levels of increased NRP2 protein were evident along with expected changes in EMT markers (Fig. 1A). In contrast with NRP2, no consistent changes were observed in NRP1 and it was not further examined.

To explore NRP2 upregulation in more detail, we focused on two cell lines, H358 and A549. Although both are epithelial in nature, A549 is partially shifted toward mesenchymal differentiation, as evidenced by lower E-cadherin, higher N-cadherin, and higher ZEB1 levels (4). Moreover, the morphologic response and downregulation of E-cadherin to TGF-β occur more rapidly in A549 cells. As expected for a cell surface receptor, increased NRP2 protein was observed at the plasma membrane (Supplementary Fig. S1A). Consistently, NRP2 upregulation was observed by 8 hours (Supplementary Fig. S1B), and with repeated exogenous TGF-β1, the effect was persistent at 10 days (Supplementary Fig. S1C). Chronic TGF-β1 exposure was obtained by stable transfection in H358 cells (H358-Tr-TGF-β1), which while doxycycline inducible, exhibited significant leaky expression. In the absence of doxycycline, these cells produced 33% more activated TGF-β1 than vector controls (Supplementary Fig. S1D). This level was sufficient to upregulate NRP2 protein, as shown in Fig. 1B, whereas the addition of doxycycline further increased both TGF-β1 and NRP2 (see Fig. 2A below and Supplementary Fig. S1D).

Compared with controls, steady-state NRP2 protein in H358-Tr-TGF-β1 cells was increased by approximately 8-fold, whereas mRNA was only 1.5-fold higher (Fig. 1C). Similar results were obtained with short-term exogenous TGF-β1, although the magnitude of the NRP2 protein upregulation was less (i.e., 2–4-fold). To gain further insight, we examined mRNA and protein stability following TGF-β1 stimulation and the addition of actinomycin-D or cycloheximide, respectively. However, neither was increased (Supplementary Fig. S1E and S1F). We then asked whether TGF-β1 affected protein translation using
Noncanonical TGF-β signaling includes ERK and AKT pathways (7, 8). In A549 cells, inhibition of ERK or AKT with U0126 and MKK-2206, respectively, impaired NRP2 upregulation by TGF-β1, whereas combining the inhibitors was more effective (Figs. 2C and Supplementary Fig. S2C for full time course). Similar results were obtained with the individual inhibitors in H358 cells, although the combination did not result in a greater effect.

We previously reported that ZEB1 inhibits expression of the tumor suppressor, SEMA3F, which uses NRP2 as its high-affinity receptor (16). ZEB1 is also upregulated by TGF-β1 in NSCLC cell lines (e.g., as shown in Fig. 2A) and is the EMT transcription factor best correlated with steady-state mesenchymal features (4, 25). In A549 cells, shRNA targeting of ZEB1 led to reduced steady-state levels of NRP2 (Fig. 2D). ZEB1 knockdown also reduced NRP2 levels after treatment with exogenous TGF-β1 in A549 cells (Figs. 2E and Supplementary Fig. S2D), and after 5 days of doxycycline induction in H358-Tr-TGF-β1 cells (Supplementary Fig. S2E). However, in short-term experiments up to 8 hours, NRP2 upregulation occurred without a detectable change in ZEB1 (Supplementary Fig. S2C). Moreover, ZEB1 levels were unaffected by the MEK and AKT inhibitors that blocked NRP2 upregulation. These results suggest that although ZEB1 may not be involved in the initial phase of NRP2 upregulation, it contributes to NRP2 maintenance. Although SNAIL reportedly contributes to ZEB1 upregulation (26), blocking SNAIL with SMAD7 had no effect on ZEB1 levels after 24 hours of TGF-β1 (Fig. 2B).
NRP2 knockdown impairs downstream TGF-β1 responses

To assess the effects of NRP2 on TGF-β1 activities, we stably transfected control and NRP2-targeting shRNAs into H358 and A549 cells (Supplementary Fig. S3A and S3B). Morphologically, NRP2 knockdown inhibited the mesenchymal transformation of A549 cells (Fig. 3A); similar results were obtained in H358 cells treated with exogenous TGF-β1 and H358-Tr-TGF-β1 induced with doxycycline (Supplementary Fig. S4). Using Transwell assays, NRP2 knockdown inhibited both baseline and TGF-β1–stimulated migration (Fig. 3B). Likewise, invasion through Matrigel-coated membranes was impaired.

The effect of NRP2 knockdown on TGF-β1–induced changes in gene expression and signaling was also examined. NRP2 knockdown blunted the suppression of epithelial genes, as well as upregulation of mesenchymal genes (Supplementary Fig. S3C). As indicated above, TGF-β1 signaling led to ERK and AKT phosphorylation. Although the kinetics differed, NRP2 knockdown consistently inhibited ERK phosphorylation (Fig. 3C and Supplementary Fig. S3D). NRP2 knockdown also inhibited AKT phosphorylation, but only in H358 cells. In hepatic stellate cells, NRP1 knockdown was shown to shift SMAD phosphorylation from SMAD2/3 to SMAD1/5, and NRP2 was stated to have a similar effect (27). However, in multiple experiments under a variety of conditions, NRP2 knockdown had no effect on SMAD2/3 or SMAD1/5 phosphorylation in these lung cancer cells (Supplementary Fig. S3E). In contrast, NRP1 knockdown did enhance SMAD1/5 phosphorylation without affecting phospho-SMAD2/3, although long exposure times were required to detect this (Supplementary Fig. S3G). Together, we

![Figure 2. Induction and maintenance of NRP2 depends on noncanonical TGF-β signaling and involves ZEB1. A, left, H358 cells were treated with 10 μmol/L SB431542 (SB) or vehicle for 60 minutes before TGF-β1 exposure for the indicated times. Right, H358-Tr-TGF-β1 cells were cultured with or without doxycycline (dox) for 3 days, and for the final 24 hours exposed to SB431542, as indicated. Protein lysates were analyzed by Western blot. B, background band in H358 samples. C, left, control- or SMAD7-transfected A549 cells were exposed to TGF-β1 or vehicle for 24 hours and analyzed by Western blot. Right, SNAIL mRNA levels were measured by qRT-PCR in the same cultures. C, Western blot analysis of lysates from A549 cells pretreated for 30 minutes with vehicle, U0126 (10 μmol/L), MKK-2206 (2 μmol/L) or both, then exposed to TGF-β1 for indicated times. The full time course is shown in Supplementary Fig. S2C. D, A549 cells stably knocked down for ZEB1 with independent shRNAs (ZEB1-1 and ZEB1-2) analyzed for NRP2 and the indicated EMT markers. E, control shRNA. E, shZEB1-1 and shControl A549 cells were exposed to TGF-β1 for 48 hours and analyzed by confocal microscopy for NRP2 (green) and ZEB1 (red). Nuclei were stained with DAPI. Scale bar, 100 μm.](https://cancerres.aacrjournals.org/content/73/23/7115)
conclude that NRP2 knockdown inhibits morphology, cell migration/invasion, gene expression, and noncanonical signaling changes induced by TGF-β1, whereas SMAD phosphorylation was unaffected, at least under the conditions studied.

**TGF-β1 suppression of tumor growth is reversed by NRP2 knockdown**

To explore the consequences of NRP2 knockdown, we used subcutaneous xenografts of control and uninduced H358-Tr-TGF-β1 cells stably transfected with nontargeting or NRP2 shRNAs. In the presence of wild-type NRP2 levels, the growth of H358-Tr-TGF-β1 xenografts was substantially slowed (Fig. 4A). This is consistent with the ability of TGF-β1 to inhibit epithelial cell proliferation while driving the EMT process (6, 28). Ki67 staining demonstrated that proliferation was significantly inhibited by TGF-β1 (Fig. 4B). NRP2 knockdown, which was confirmed by immunohistochemistry (Supplementary Fig. S5), completely restored growth of H358-Tr-TGF-β1 xenografts, while it had no effect on the growth of control tumors. To determine whether growth inhibition was autocrine in nature, at least in part, we examined the consequences of TGF-β exposure and NRP2 knockdown in vitro. These results paralleled the in vitro findings demonstrating that NRP2 knockdown relieved the growth inhibition resulting from TGF-β1 (Fig. 4C).

In control and H358-Tr-TGF-β1 tumors expressing nontargeting shRNA, NRP2 staining was intensified in isolated tumor cells and tumor cell clusters in the stroma (Fig. 4D and Supplementary Fig. S5). These cells showed reduced staining for the epithelial marker, pan-cytokeratin, suggesting that they had undergone a partial mesenchymal transition and were invasive. As anticipated from our morphologic observations in vitro (Fig. 3A and Supplementary Fig. S4), NRP2 knockdown resulted in more regularly shaped tumor aggregates and fewer cells that appeared invasive. Thus, we conclude that NRP2 is important for TGF-β1-mediated effects on proliferation and migration. However, although the changes induced by TGF-β1 are autocrine, at least in part, we cannot exclude additional paracrine effects from the tumor microenvironment.

**Increased NRP2 expression correlates with tumor grade and E-cadherin in patient samples**

In previous studies, we showed that E-cadherin, an epithelial marker frequently lost during the EMT process, is absent in 10% to 30% of lung tumors (4, 29). However, this likely underestimates the number of tumors transiently progressing through the EMT. In other studies, we reported that NRP2 levels significantly increase in early lung lesions (30). On the basis of our current findings and previous results, we hypothesized that tumors with high NRP2 and low E-cadherin would be more aggressive. This was examined using a microarray containing 109 resected human lung cancers. Overall, NRP2 and E-cadherin were expressed in 83 (76%) and 69 (63%) tumors, respectively. Of 22 NRP2(+)Ecad(−) tumors, nine were grade 3, whereas the rest were grade 2, and the association with tumor grade was significant (Fisher exact test, $P = 0.0049$; Table 1; Fig. 5A and Supplementary Fig. S6A). Conversely, all NRP2(−)/Ecad(+) tumors were grade 1 or 2. In both scenarios, the use of NRP2 as a marker strengthened the association of E-cadherin with tumor grade. We also hypothesized that high NRP2 scores would correlate with E-cadherin loss. In fact, tumors with the highest NRP2 scores (>100) were predominantly E-cadherin negative (7/9, $P = 0.0252$), whereas tumors with NRP2 scores less than 100 had higher mean...
E-cadherin staining (53 ± 7 vs. 15 ± 10, P = 0.0043; Table 2; Fig. 5B and Supplementary Fig. S6B). Thus, the upregulation of NRP2 during TGF-β–induced EMT in lung cancer cell lines, and its association with E-cadherin loss and more aggressive tumors in the lung cancer TMAs may reflect the same underlying biology.

Discussion

Our results demonstrate that NRP2 is specifically upregulated in TGF-β–responsive lung cancer cells and that it contributes significantly to the development of the EMT phenotype. NRP2 induction was rapid, stable with continued TGF-β, and present at the plasma membrane. Changes in NRP2 were evident primarily at the protein level with modest increases in mRNA. Experiments with cycloheximide or actinomycin-D treatment indicated that neither NRP2 protein nor mRNA were stabilized by TGF-β. Instead, we observed a reproducible increase in the association of NRP2 mRNA with fractions containing the heaviest polyribosomes, consistent with increased translation. In comparison, the polysomal association of GAPDH mRNA was not altered by TGF-β.

Mechanistically, how increased NRP2 protein translation occurs is unknown. The translation of many proteins is regulated by microRNAs (miR; ref. 31). Using the miRiam target Table 1.

<table>
<thead>
<tr>
<th>Grade</th>
<th>NRP2 (−) E-cad (−) n (%)</th>
<th>NRP2 (−) E-cad (+) n (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>2 (17)</td>
<td>0 (0)</td>
</tr>
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<td>13 (59)</td>
</tr>
<tr>
<td>3</td>
<td>0 (0)</td>
<td>9 (40)</td>
</tr>
<tr>
<td>Total</td>
<td>12 (100)</td>
<td>22 (100)</td>
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Table 2. NRP2 score versus E-cadherin status

<table>
<thead>
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<th>NRP2 score</th>
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<th>E-cad (+) n (%)</th>
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<td>&gt;100</td>
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</tr>
<tr>
<td>≤100</td>
<td>34 (34)</td>
<td>66 (66)</td>
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prediction tool (32), we identified 17 miRs or miR families and 39 high-probability binding sites in the 3′ untranslated region (UTR) of the major NRP2 isoform (Supplementary Fig. S7A). However, using overlapping luciferase reporter constructs for the NRP2 3′UTR, we failed to detect consistent changes after exposure to exogenous TGF-β. Nevertheless, predicted binding sites for the TGF-β–regulated miR-15b and miR-16 were present, and although both were expressed at quite different levels, they were inhibited by TGF-β. Further studies will be required to determine their significance. In addition, TGF-β–regulated protein translation during EMT has been shown to occur through heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), which binds a structural element in the 3′UTR of Dab2 and ILEI (33). Stable knockdown of hnRNP E1 in NMuMG cells was associated with NRP2 upregulation (Supplementary Fig. S7B). More work will be required to determine whether hnRNP E1 or another hnRNP has a direct role in the observed NRP2 upregulation.

In the present study, we found that early NRP2 upregulation was SMAD independent. This is based on the inability of SMAD7 to block NRP2 increases, whereas a known SMAD target gene, SNAI1, was inhibited. Among noncanonical signaling pathways activated by TGF-β, we found that ERK and AKT inhibitors impaired NRP2 upregulation, and this effect was greater when the inhibitors were combined. Mechanistically, both TßRI and TßRII can be phosphorylated on tyrosine residues that serve as Shc recruitment sites, leading to activation of ERK (34). ERK activation is an essential component in TGF-β–mediated EMT (35), leading in part to increased FRA-1/c-JUN heterodimers and increased AP-1 activity (36). In subsequent studies demonstrated that both ZEB proteins bind activated R-Smads (48, 49). Our studies do not exclude a possible role for SMAD–ZEB interactions in the maintenance of NRP2 or the possibility that maintenance and induction involve different TGF-β–dependent pathways.

Changes in gene expression, a hallmark of EMT, result in part from the upregulation of transcriptional repressors, including ZEB1, that bind E-box elements in genomic DNA (43). Previously, we found that ZEB1 was the repressor best correlated with EMT features and EGF receptor inhibitor resistance in NSCLC cell lines (4, 44). In addition, we reported that ZEB1 bound E-box sites in the SEMA3F promoter and suppressed its expression (16). Recently, we found that ZEB1 preferentially inhibits acetylation of histone H3 lysine 27 in the promoter region of ZEB1 target genes (45). In the current study, ZEB1 knockdown inhibited NRP2 expression. However, ZEB1 levels were unchanged during early NRP2 induction, and ERK and AKT inhibitors, which blocked NRP2 upregulation, had no effect on ZEB1. Together, these results suggest that ZEB1 acts as a maintenance factor for NRP2. A similar requirement for ZEB1 in maintenance of a stable mesenchymal phenotype was observed in breast cancer (46). ZEB2 was identified as a Smad-interacting protein (47), and subsequent studies demonstrated that both ZEB proteins bind activated R-Smads (48, 49). Our studies do not exclude a role for SMAD–ZEB interactions in the maintenance of NRP2 or the possibility that maintenance and induction involve different TGF-β–dependent pathways.

The EMT process is characterized by changes in morphology, loss of junctional complexes, increased migration/invasion, decreased proliferation and alterations in gene expression (1). NRP2 knockdown inhibited these TGF-β–induced changes. In hepatic stellate cells exposed to TGF-β, NRP1 knockdown was shown to reduce SMAD2/3 and increase SMAD1/5 phosphorylation (27). These authors stated that NRP2 knockdown elicited a similar response. However, we found that in lung cancer cells, only NRP1 knockdown affected SMAD phosphorylation. Thus, the two NRPs are not equivalent in this context. The absence of a SMAD effect prompted us to examine noncanonical pathways. Importantly, NRP2 deficiency consistently inhibited TGF-β–mediated ERK activation, whereas there was no effect on JNK or p38. The kinetics of ERK activation by TGF-β are known to be context dependent (8) and this was apparent in the NSCLC cell lines. NRP2 also influences ERK signaling in response to class 3 semaphorins or VEGF (23, 50, 51), but to our knowledge, a role in noncanonical TGF-β signaling has not been reported.
In vivo, we found that TGF-β impaired the growth of xenograft tumors by reducing proliferation, as previously reported (25, 52). This was confirmed by in vitro growth assays. Our results indicate that the antiproliferative effect of TGF-β has an autocrine component, at least in part, although we cannot exclude an additional paracrine effect. Reduced levels of TβRII have been described in about 40% of primary NSCLCs, often associated with 5′CpG methylation (53). However, a recent study reported that drug-resistant NSCLCs are linked to high TβRII expression and EMT features, concomitant with reduced proliferation, which may facilitate resistance to cytotoxic therapy (54). Moreover, elevated expression of TGF-β has also been correlated with poor prognosis (9). Thus, the consequences of TGF-β signaling in lung cancer are context dependent.

In xenograft tumors, increased NRP2 staining was present in invasive-appearing isolated tumor cells and small tumor cell clusters in the stroma displaying decreased pan-cytokeratin. Of note, NRP2 knockdown resulted in more regularly shaped invasive-appearing isolated tumor cells and small tumor cell aggregates and fewer cells that appeared invasive. Recent studies have confirmed the importance of the EMT process in tumor cell invasion and dissemination, while demonstrating that EMT reversal is critical for the subsequent proliferation of these cells in metastatic sites (13, 14, 55). Furthermore, a partial EMT phenotype seems sufficient to generate circulating tumor cells and tumor cell clusters (14), reminiscent of what we observed in the stroma. In tumor microarrays from resected human lung cancer specimens, we speculated that high NRP2 expression would be associated with reduced E-cadherin and less differentiation. Indeed, tumors with the highest NRP2 scores were predominantly E-cadherin negative, whereas reduced NRP2 was associated with higher E-cadherin staining. Moreover, the presence of NRP2 staining strengthened the association of E-cadherin loss with high-grade tumors.

The role of NRP2 in tumor growth is almost certainly complex and context dependent. In contrast with our results in lung cancer cells, NRP2 expression was associated with increased proliferation in a colorectal cancer model treated with TGF-β (56). Presumably, these cells had escaped TGF-β-mediated growth inhibition. Other studies have shown that NRP2 knockdown inhibits survival, but not proliferation (57–59). In addition, use of an anti-NRP2 function-blocking antibody reduced tumor lymphatic vessels and the number of lung metastases without affecting primary tumor growth (60). Altogether, our results suggest that in lung cancer cells, NRP2 is upregulated and contributes significantly to TGF-β-mediated EMT. Although NRP2 knockdown inhibited the invasive phenotype, it also rescued growth suppressed by TGF-β. Although this would be a clinically undesired effect, these cells may be more sensitive to cytotoxic chemotherapy or other agents. Finally, it would appear that the suppression of SEMA3F and upregulation of NRP2 by TGF-β represents a coordinated program contributing to the acquisition of a motile, invasive phenotype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Study supervision: P. Nasarre, P.H. Howe, H.A. Drabkin

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References
Correction: Neuropilin-2 Is Upregulated in Lung Cancer Cells during TGF-β1-Induced Epithelial-Mesenchymal Transition

In this article (Cancer Res 2013;73:7111–21), which was published in the December 1, 2013, issue of Cancer Research (1), an article (ref. 27, Cao and colleagues, J Biol Chem 2010;285:31840–8) was cited incorrectly, and the correct citation (Glinka and colleagues, Carcinogenesis 2011;32:613–21) was not included.

The citation of ref. 27 on page 7,115 should read as follows:

In hepatic stellate cells, NRP1 knockdown was shown to shift SMAD phosphorylation from SMAD2/3 to SMAD1/5 (27). However, we have shown that in multiple experiments under a variety of conditions, NRP2 knockdown had no effect on SMAD2/3 or SMAD1/5 phosphorylation in these lung cancer cells.

The citation of ref. 27 on page 7,118 should read as follows:

In hepatic stellate cells exposed to TGF-β1, NRP1 knockdown was shown to reduce SMAD2/3 and increase SMAD1/5 phosphorylation (27). In addition, NRP1 has been shown to activate SMAD2/3 in breast cancer cells, whereas Nrp2 could "activate LAP–TGFβ1 (data not shown)" (61). These authors indicated that "Nrp1 and Nrp2 might be largely interchangeable in their interactions with TGFβ components." However, we found that in lung cancer cells, NRP2 knockdown had no effect on SMAD signaling. Thus, the two NRPs are not equivalent in this context.

The new ref. 61 is as follows:


The authors regret this error.

Reference


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Neuropilin-2 Is Upregulated in Lung Cancer Cells during TGF-β1–Induced Epithelial–Mesenchymal Transition


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