Neuropilin 1 modulates TGF-β1-induced epithelial-mesenchymal transition in non-small cell lung cancer

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Abstract. Previously, the authors reported that neuropilin-1 (NRP1) was significantly increased and acted as a vital promoter in the metastasis of non-small cell lung cancer (NSCLC). However, the regulatory mechanism of NRP1 in NSCLC cell migration and invasion remained unclear. The present study aimed to explore the regulatory mechanism of NRP1 in the transforming growth factor-β (TGF-β) 1-induced migration and invasion of NSCLC cells. The expression level of NRP1 was determined by RT-qPCR analysis in human tissue samples with or without lymph node metastasis. Transwell assay and wound healing assay were conducted to determine the cell migration. Lentivirus-mediated stable knockdown and overexpression of NRP1 cell lines were constructed. Exogenous TGF-β1 stimulation, SIS3 treatment, western blot analysis and *in vivo* metastatic model were utilized to clarify the underlying regulatory mechanisms. The results demonstrated that the expression of NRP1 was increased in metastatic NSCLC tissues. NRP1 promoted NSCLC metastasis *in vitro* and *in vivo*. The Transwell assays, wound healing assays and western blot analysis revealed that the knockdown of NRP1 significantly inhibited TGF-β1-mediated EMT and migratory and invasive capabilities of NSCLC. Furthermore, the overexpression of NRP1 weakened the inhibitory effect of SIS3 on the NSCLC migration and invasion. Co-IP assay revealed that NRP1 interacted with TGFβRII to induce EMT. On the whole, the findings of this study demonstrated that NRP1 was overexpressed in metastatic NSCLC tissues. NRP1 could contributes to TGF-β1-induced EMT and metastasis in NSCLC by binding with TGFβRII.

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Introduction

Non-small cell lung cancer (NSCLC) is a major cause of cancer-related mortality worldwide with a poor 5-year survival rate in patients (1,2). In fact, cancer metastasis caused more than 90% of deaths from solid tumor, including lung cancer (3). Thus, it is critical to understand the mechanisms of NSCLC metastasis for improving survival rate of patients.

Epithelial-mesenchymal transition (EMT) plays an essential role in embryonic development and the transformation of early-stage tumors into invasive malignancies (4,5). Previous studies have demonstrated that TGF-β1 signaling is a potent inducer of EMT in various types of cancer, including NSCLC (6-8). It is well known that transforming growth factor β1 (TGF-β1) plays crucial roles in cell differentiation, proliferation, apoptosis and angiogenesis (9-11). In the canonical TGF-β signaling pathway, TGF-β binds tightly to TGF-β receptor II (TGFβRII) on the cell membrane to form a complex and recruits TGF-β receptor I (TGFβRI), inducing its phosphorylation. Activated TGFβRI phosphorylates Smad2 and Smad3, and phosphorylated Smad2/3 forms a transcriptional complex with Smad4 into the nucleus to regulate the transcription of specific target genes (12). The TGF-β signaling pathway plays a dual role in tumor progression, which inhibits tumor growth in the early stages and promotes tumor metastasis and invasion by inducing EMT in the late stages of the disease (13,14). Recently, one study demonstrated that neuropilin-1 (NRP1) acts as a TGF-β1 co-receptor and activates latent TGF-β1 in breast cancer (15). Consistently, Kwiatkowski *et al* reported that NRP1 acts as a co-receptor with TGFβRII to enhance TGF-β1 receptor signaling via Smad3 in glioblastoma (16). Thus, the association between NRP1 and TGF-β signaling pathways in NSCLC remains to be verified.

Neuropilins (NRPs) are involved in multiple processes of cellular biological function, such as immunity, cell development and tumorigenesis. NRP1 and NRP2 are co-receptors that bind to and interact with a variety of growth factors (17,18). NRP1 is a transmembrane glycoprotein that binds to various extracellular ligands, including class III/IV semaphorins (19), certain isoforms of vascular endothelial growth factor (VEGF) (20), TGF-β1 (15), and platelet-derived growth factor (PDGF) (21). A previous study by the authors demonstrated that the expression of NRP1 was high in NSCLC tissues and was associated with a poorer survival of patients (22). In addition, NRP1 can promote NSCLC cell proliferation and migration via the EGFR signaling pathway (22). Taken together, it was thus hypothesized that dysregulated NRP1 can influence TGF-β1-induced EMT.

In the present study, the function of NRP1 in the regulation of TGF-β1-induced EMT and NSCLC cell migration and invasion was investigated. The upregulated expression of NRP1 was first observed in metastatic NSCLC tissues. In addition, A549 and H226 cell lines with stable knockdown of NRP1 were established. Subsequently, Transwell assays indicated that the knockdown of NRP1 suppressed the TGF-β1-induced migration and invasion of NSCLC cells. The findings of this study demonstrate that the suppression of NPR1 inhibits TGF-β1-induced EMT in NSCLC.

Materials and methods

Tissue samples. A total of 55 NSCLC patient tissues and corresponding para-carcinoma lung tissues were collected between March, 2012 and December, 2016 at the Respiratory Department of the First people's Hospital of Soochow University. All the participants provided written informed consent at recruitment. According to the Revised International System for Staging Lung Cancer, all cases have clinically and pathologically confirmed who did not receive any other treatment including radiotherapy or chemotherapy before tissue sampling. The tissue samples were frozen at -80˚C for storage. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Cells and cell culture. A549 and H226 cells were obtained from the Cell bank of the Chinese Academy of Sciences (Shanghai) and grown in RPMI-1640 medium (HyClone) containing 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific) and 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific). The cells were cultured in a humidified incubator containing 5% CO_2 at 37˚C. In some conditions, the cells were exposed to 5 ng/ml TGF-β1 (R&D Systems) or 3 μM SIS3 (Selleck Chemicals) for 48 h for further experiments. The A549 and H226 cells in which NRP1 was knocked down were cultured in the same medium supplemented with 0.5 μg/ml puromycin (Sigma-Aldrich). In addition, NRP1-overexpressing cells were grown with medium containing G418 (Sigma-Aldrich) for positive selection.

Lentivirus-mediated stable knockdown of NRP1. The human NRP1-specific small interfering RNA fragments (NRP1 shRNA-1, 5'-CCAUACCAGAGAAUUAUGATT-3'; NRP1shRNA-2, 5'-GUAUACGGUUGCAAGAUAATT-3') were cloned into the lentiviral vector pGMLV-SC5-Puro (GenePharma) containing the endonucleases *Eco*R1 and *Bam*H1. Subsequently, the pGMLV-SC5-Puro vector was co-transfected along with packaging plasmids into 293T cells (Cell bank of the Chinese Academy of Sciences) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). The A549 and H226 cells were infected with the packaged lentiviruses at a multiplicity of infection (MOI) of 25 along with 8 μg/l polybrene as coadjutant and cultured for 2 days, and cells were selected with 0.4 μg/ml of puromycin (Sigma-Aldrich). The transfection efficiency was evaluated by later western blot analysis and RT-qPCR.

Plasmid-mediated stable overexpression of NRP1. For plasmid construction, the human NRP1 CDS fragment was cloned into PLVX-IRES-Neo vector between *Eco*R1 and *Xba*l (Genewiz). The H226 and A549 cells were transfected with UPLVX-IRES-Neo-vector or PLVX-IRES-Neo-NRP1 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) and further cultured for 2 days prior to selection with G418 reagent (Sigma-Aldrich). The NRP1 overexpression efficiency was evaluated later by RT-qPCR and western blot analysis.

Wound healing assay. Following the knockdown of NRP1, the A549 and H226 cells stimulated with TGF-β1 and the NRP1-overexpressing tumor cells treated with SIS3 for 48 h; the cells were then suspended and re-seeded in a 6-well plate. At day 2, when the cells grew to 80-90% confluency as a monolayer, the monolayer was gently scratched with a 10 μl pipette tip, with the tip being perpendicular to the bottom of the plate during the operation. The detached cells were removed by gently washing with PBS twice after scratching. Fresh medium was added and the cells were cultured for a further 24 h. The gap distance was observed using a microscope (CKX41; Olympus) and images were captured. The gap distance was quantitatively evaluated using Photoshop.

Migration and invasion assays. Cell migration and invasion assays were performed using Transwell chambers (Corning, Inc.). For the migration assay, the cells were suspended and plated on chambers that were not coated with Matrigel matrix (BD Science). For invasion assay, cells were suspended and plated on chambers pre-coated with Matrigel matrix at 37˚C for 2 h first. This was followed by the stimulation of cells in which NRP1 was knocked down with TGF-β1 or the treatment of NRP-overexpressing cells with SIS3 for 48 h. RPMI-1640 medium (800 μl) containing 10% FBS was added into each bottom chamber and the cells were collected. Subsequently, $5x10^4$ cells were diluted in 200 μl medium containing 1% FBS and seeded into the upper chamber. Following incubation at 37˚C for 24 h, the cells were fixed with methanol for 30 min and non-invasive cells were removed, and air-dried for 15 min. The remaining cells were stained with 0.1% crystal violet (Beyotime) at room temperature for 1 h and washed with PBS 3 times. Subsequently, the invasive cells were photographed and counted under a light microscope (Olympus). Each experiment was performed in triplicate.

Western blot analysis. The cells were washed twice in cold PBS and then lysed in RIPA buffer (Cell Signaling Technology)

containing phosphatase inhibitor and protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was measured using the Enhanced BCA Protein Assay kit (Beyotime). Subsequently, 10% SDS-PAGE was used to separate proteins (20 μg) that were then transferred onto nitrocellulose membranes (Millipore), the immunoblots were blocked with 5% skim milk in TBST buffer with 0.1% Tween-20 for 1 h at room temperature and then incubated with corresponding primary antibodies overnight at 4˚C and the appropriate secondary antibodies. After 2 h, detection was performed via chemiluminescence (Pierce) after washing the cells 3 times with TBST. The primary antibodies used in this study included NRP1 (A-12, 1:500, sc-5307; Santa Cruz Biotechnology), anti-p-Smad3 (Ser423/425, 1:1,000, cat. no. 9520), anti-Smad3 (C67H9, 1:1,000, cat. no. 9523), anti-Snail (C15D3, 1:1,000, cat. no. 3895s), anti-MMP2 (D8N9Y, 1:1,000, cat. no. 13132), and anti-MMP9 (603H, 1:1,000, cat. no. 13667) (Cell Signaling Technology), anti-N-cadherin (8C11, 1:1,000, cat. no. 561553), anti-Vimentin (RV202, 1:1,000, cat. no. 550513) (BD Biosciences). Anti-β-actin (13E5, 1:1,000, cat. no. 4970S) and anti-mouse (1:2,000, cat. no. 7076S) or anti-rabbit (1:2,000, cat. no. 7074S) constituted the secondary antibodies (Cell Signaling Technology).

Co-immunoprecipitation assay. A549-PLVX- and A549-NRP1-overexpressing cells were cultured in a 10-cm plate until 95-100% confluence. The cells in each dish were then washed twice with PBS, collected by scraping, and lysed with 1 ml of modified RIPA buffer (Cell Signaling Technology) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min. Cell lysates were collected by centrifugation at 10,000 x g at 4˚C for 15 min. Clear lysates were pre-cleared by addition of 25 μl of protein G bead (Santa Cruz Biotechnology) slurry and incubated at 4˚C overnight with rotation. Supernatants were transferred to a new tube and incubated with 1 μg of rabbit anti-NRP1 antibody (sc5307; Santa Cruz Biotechnology) with rotation overnight in a cold room at 4˚C; this was followed by additional incubation for 3-4 h with protein G beads. The beads were washed 3 times with RIPA buffer (Cell Signaling Technology) and then boiled in 5X SDS protein loading buffer (Sangon Biotech) for 5 min. Samples (20 μl) were loaded on an SDS-PAGE gel for western blot analysis using the anti-NRP1 (A-12, 1:500, sc-5307, Santa Cruz Biotechnology) and anti-TGFβRII (E-6, 1:500, sc-17792) antibody.

Tumor metastasis model. To establish an experimental lung metastasis model, the cells were resuspended in PBS ($1x10^6$ cells/100 μl/mouse) and injected cells into each mouse (6 weeks old, female, BALB/c, n=6/group) via the tail vein on day 0. The mice were then injected with TGF-β1 (Novoprotein Scientific) (400 ng/μl) into their abdominal cavity every 5 days, and the total number of injections was 5 times. Mice were maintained in exhaust ventilated closed system cages in a specific pathogen-free environment, with 55±5% humidity, at 23±2˚C. Food and water were provided *ad libitum*. All the mice were sacrificed 50 days after tail vein injection. Surgically resected mouse lung tissues were fixed in Bouin's fluid (Thermo Scientific) and the number of pulmonary metastasis nodules were counted under a microscope (CKX41; Olympus) after the appropriate tissues were stained with hematoxylin and eosin (H&E; Beyotime) at room temperature for 10 min. All animal experiments were carried out in accordance with the Guide for the Care and Use of Experimental Animals from the Experimental Animal Center of Xuzhou Medical University. Experiments on Animals were approval by the Animal Ethical Committee of Xuzhou Medical University.

Correlation analysis between NRP1 and related genes. The public database LinkedOmics (http://www.linkedomics.org/login.php) was applied to search for the data of genes related to NRP1. The correlation between NRP1 and Snail1, Snail2, MMP2 or TGFβRII was analyzed by LinkFinder analytical module.

Statistical analysis. The results obtained are presented as mean ± standard deviation (SD). Significant differences between the two groups were assessed with the Student's t-test. Significance among multiple groups was obtained using one-way ANOVA followed by Tukey's post-hoc test. The correlations between NRP1 and Snail1, Snail2, MMP2 or TGFβRII were analyzed by Pearson's correlation analysis. All statistical analyses were performed using SPSS 7.0 software (SPSS) and GraphPad Prism 7.0 (GraphPad).

Results

High expression of NRP1 promotes NSCLC metastasis in vitro. Previously, it was demonstrated NRP1 expression in NSCLC tissues and cells was higher than that in adjacent non-cancerous lung tissues and bronchial epithelial cells (22). In this study, to elucidate the role of NRP1 in NSCLC metastasis, NRP1 was stably knocked down in A549 (adenocarcinoma) and H226 (squamous carcinoma) cells. The mRNA and protein expression of NRP1 was significantly decreased in the A549 and H226 cells transfected with two NRP1 short hairpin RNAs (shRNAs) compared with the control group (Fig. 1A). The knockdown of NRP1 (sh-NRP1) significantly inhibited the expression of Snail, N-cadherin, Vimentin, MMP2 and MMP9. Subsequently, the effect of NRP1 on the migration of NSCLC cells was evaluated by wound healing assay. The A549 and H226 cells transfected with sh-NRP1 migrated towards the scratch at a slower rate than the control cells (Fig. 1B). Furthermore, the Transwell assays indicated that the knockdown of NRP1 considerably inhibited the migration and invasion of NSCLC cells (Fig. 2C).

To further investigate the role of NRP1 in NSCLC cells, we also established NRP1 stably overexpressing A549 and H226 cell lines (Fig. 2A). The mRNA and protein expression levels of NRP1 were increased in stable NRP1-overexpressing A549 and H226 cells compared to the control groups. Moreover, the results of Transwell and wound healing assays indicated that the overexpression of NRP1 promoted the migration and invasion of A549 and H226 cells (Fig. 2B and C).

High expression of NRP1 promotes NSCLC metastasis in vivo and is associated with TGFβRII. To confirm that NRP1 promotes NSCLC metastasis stable NRP1-knockdown A549 cells and negative control cells were injected into BALB/c athymic nude mice via the tail vein. As shown in Fig. 3A and B, the pulmonary metastatic nodules in mice

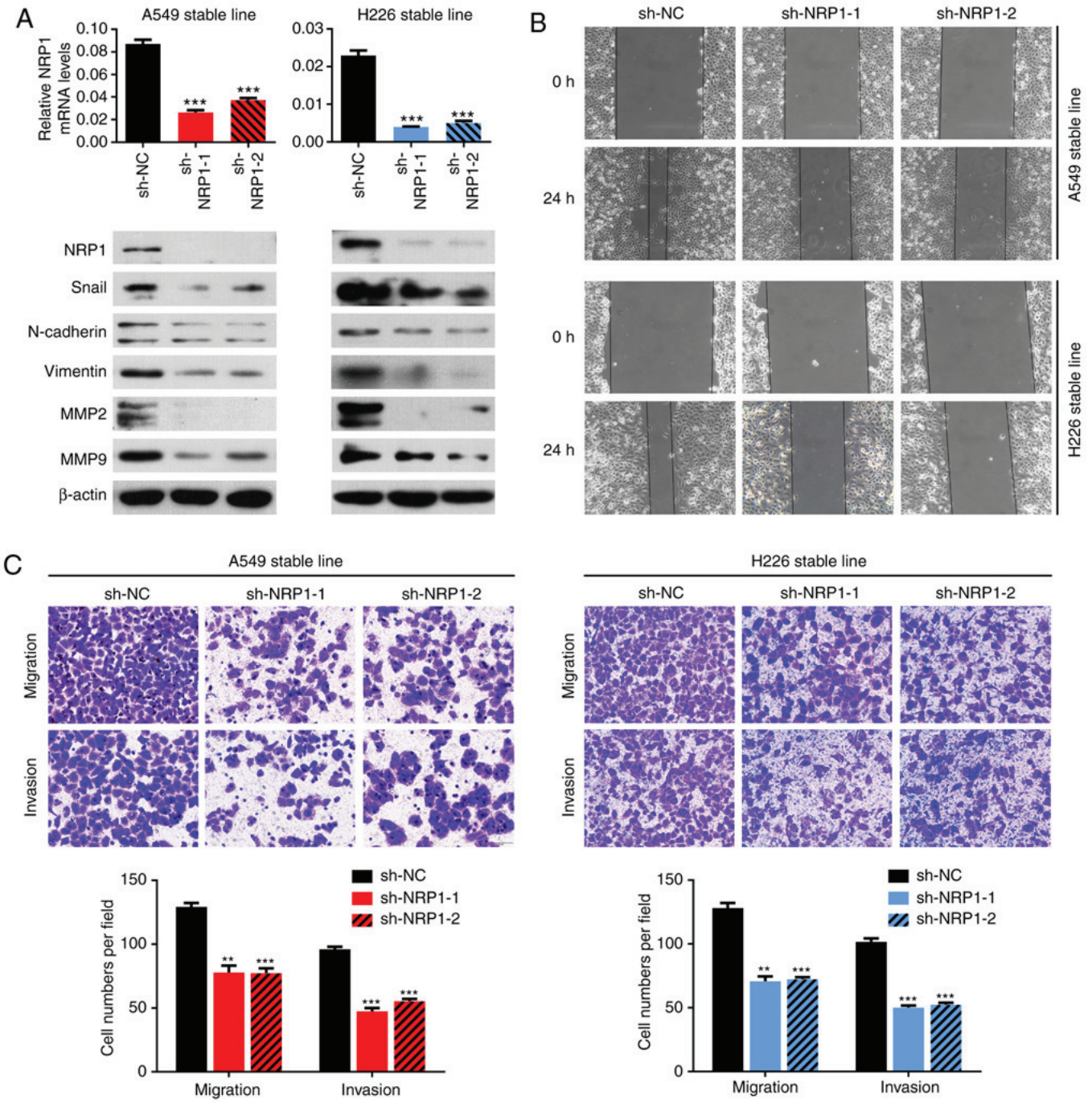

Figure 1. Knockdown of NRP1 suppresses NSCLC cell migration and invasion. (A) The protein expression levels of Snail, N-cadherin, Vimentin, MMP2 and MMP9 in stable NRP1-silenced A549 and H226 cells were detected by western blot analysis. (B) The migratory ability of stable A549 and H226 cells in which NRP1 was knocked down was investigated by wound healing assays (magnification, x200). (C) The migration and invasion of stable NRP1-silenced A549 and H226 cells were investigated by Transwell assays (magnification, x200). Data are shown as the means ± SD. Significantly different from the control (sh-NC) (**P<0.01 and ***P<0.001). NRP1, neuropilin 1; NSCLC, non-small cell lung cancer.

injected with NRP1-knockdown A549 cells were fewer than those in the control group. Furthermore, 55 lung tissues were classified according to presence or absence of lymph node metastasis and analyzed the mRNA expression level of NRP1 (Table SI). The results indicated that the mRNA expression level of NRP1 was high in NSCLC tissues with lymph node metastasis compared to NSCLC tissues without metastasis (Fig. 3C). Previous studies have reported that NRP1, as a co-receptor of TGF-β1, can modulate the TGF-β signaling pathway in various cell types (16,21). In addition, from the public database LinkedOmics (http://www.linkedomics.org/login.php), NRP1 was found to correlate with SNAI1, SNAI2, MMP2 and TGFβRII (Fig. 3D-G). Moreover, the co-immunoprecipitation assay revealed that NRP1 interacted with TGFβRII (Fig. 3H). These results suggested that NRP1 may contribute to tumor metastasis through modulating TGF-β signaling pathway.

Knockdown of NRP1 suppresses the TGF-β1-induced migration and invasion of NSCLC cells. The function of NRP1 in

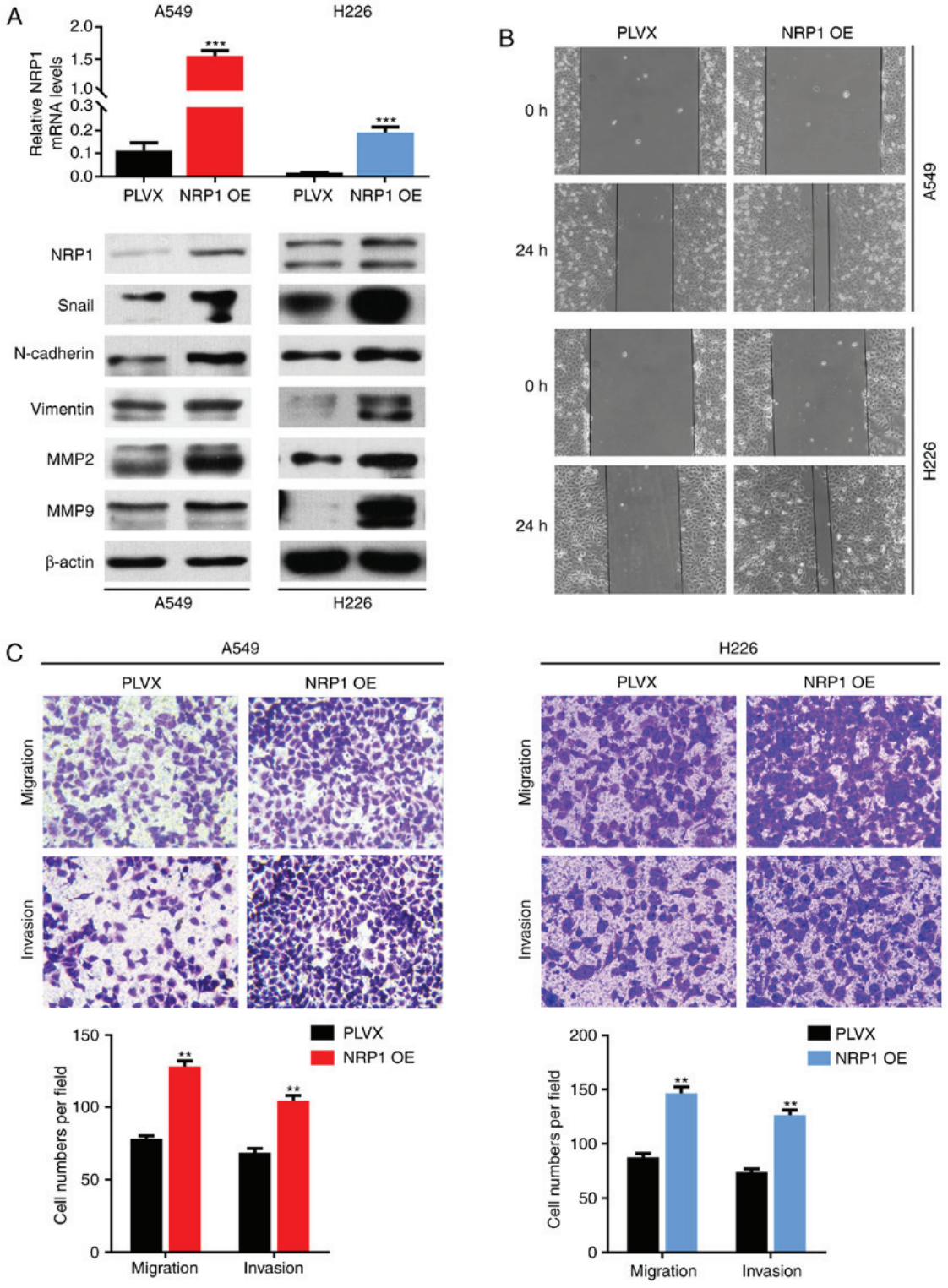

Figure 2. Overexpression of NRP1 promotes NSCLC cell migration and invasion. (A) The protein expression levels of Snail, N-cadherin, Vimentin, MMP2 and MMP9 in stable NRP1-overexpressed A549 and H226 cells were detected by western blot analysis. (B) The migratory ability of stable NRP1-overexpressing A549 and H226 cells was investigated by wound healing assays (magnification, x200). (C) The migration and invasion of stable NRP1-overexpressed A549 and H226 cells were investigated by Transwell assays (magnification, x200). Data are shown as the means ± SD. Significantly different from control (PVLX) (**P<0.01 and ***P<0.001). NRP1, neuropilin 1; NSCLC, non-small cell lung cancer.

TGF-β1-induced NSCLC metastasis was confirmed. The wound healing assay revealed that the knockdown of NRP1 was capable of retarding the movement towards the scratch in both A549 and H226 cells treated with TGF-β1 (Fig. 4C). The Transwell assays further indicated that the increased migratory and invasive abilities induced with exogenous TGF-β1 stimulation for 24 h were considerably suppressed by the knockdown of NRP1 (Fig. 4D and E). In addition, western blot analysis was conducted to clarify the underlying molecular mechanisms. As shown in Fig. 4A and B, the TGF-β1-induced increase in the

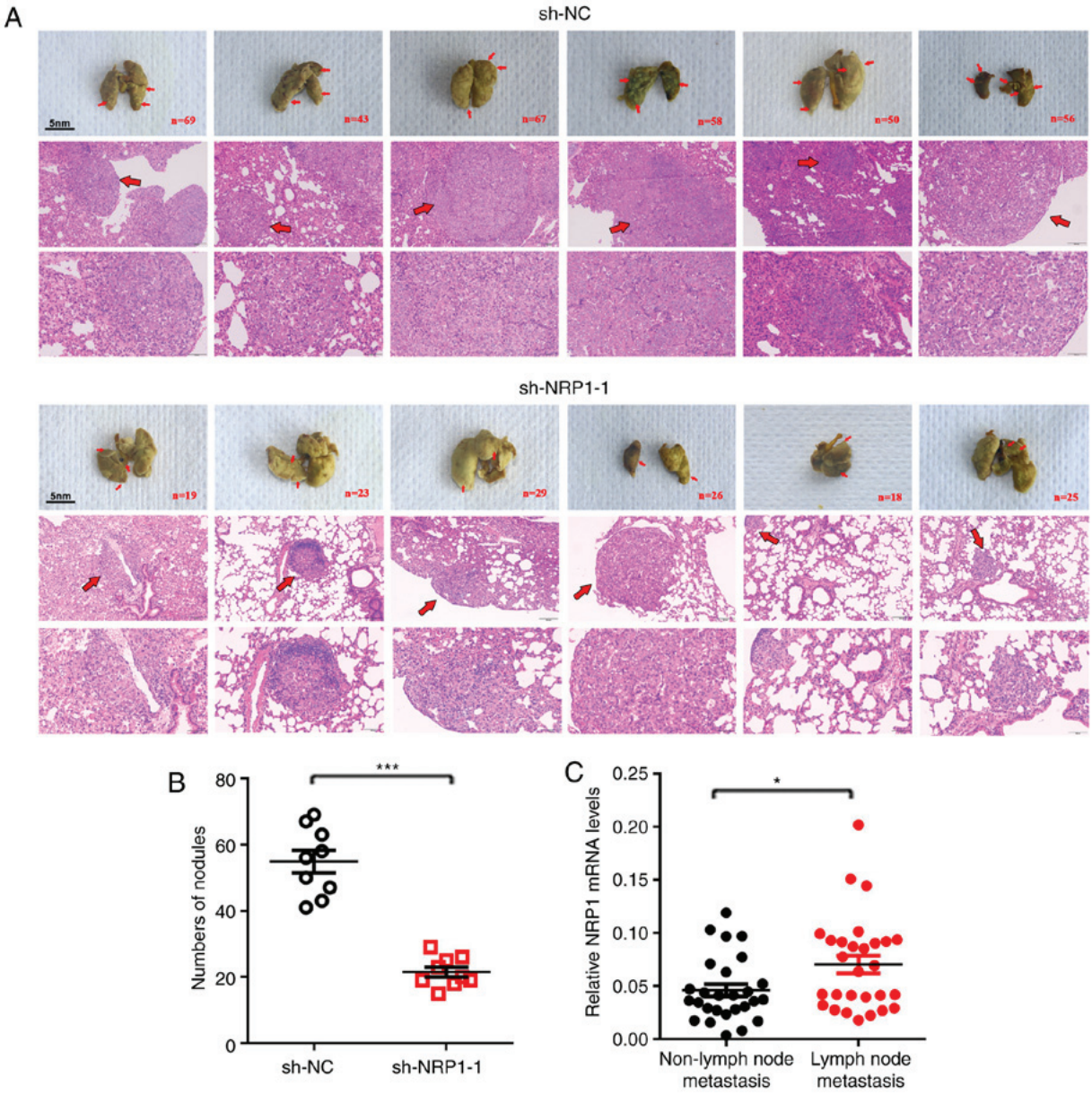

Figure 3. High expression of NRP1 promotes NSCLC metastasis *in vivo* and is associated with TGFβR. (A) Surgically resected mouse lung tissues were fixed in Bouin's fluid. The pulmonary metastatic nodules on the surface of the lung tissue were counted (largest size was 1 mm), and the pulmonary micrometastases were detected by hematoxylin and eosin (H&E) staining; red arrowheads indicate micrometastases (magnification, x100). (B) A comparison of the number of pulmonary metastatic nodules between the sh-NRP1 and sh-NC groups. (C) Comparison of the relative mRNA expression of NRP1 detected by RT-qPCR between the tissues of NSCLC non-lymph node and lymph node metastasis. An unpaired t-test was used and the results were presented as means ± SD. Significantly different from the control (sh-NC or non-lymph node metastasis) (*P<0.05 and ***P<0.001). NRP1, neuropilin 1; NSCLC, non-small cell lung cancer; TGFβR, transforming growth factor-β receptor.

p-Smad3 level was inhibited in the stable cell line with NRP1 knockdown. In addition, the downstream signaling molecules associated with the MMP family, i.e., MMP2 and MMP9, as well as EMT-related markers such as Snail, N-cadherin, Vimentin exhibited a similar tendency (Fig. 4A and B).

NRP1 promotes the metastasis of tumors via the SMAD pathway. The association between NRP1 and the TGF-β/Smad3 pathway was then further validated in NRP1-overexpressing cell lines. SIS3 is a permeable, selective Smad3 inhibitor that can suppress the activation of the TGF-β1/Smad3 signaling pathway and thus inhibits cell metastasis. As shown in Fig. 5C, treatment with SIS3 retarded the speed in both the control and NRP1-overexpressing cells that moved towards the scratch. However, this inhibitory trend was weakened in NRP1-overexpressing cells compared to the control cells (Fig. 5C). Transwell assays revealed that overexpression of NRP1 attenuated the inhibitory effect of SIS3 on cell migration and invasion compared with the control group (Fig. 5D and E). Additionally, western blot analysis confirmed that the overexpression of NRP1 markedly inhibited the SIS3-induced downregulation of p-Smad3, Snail, MMP2, MMP9, N-cadherin and Vimentin in A549 and H226 cells (Fig. 5A and B). Taken together, these findings reveal the interaction between NRP1 and TGFβRII, which activated the downstream SMAD3/Snail

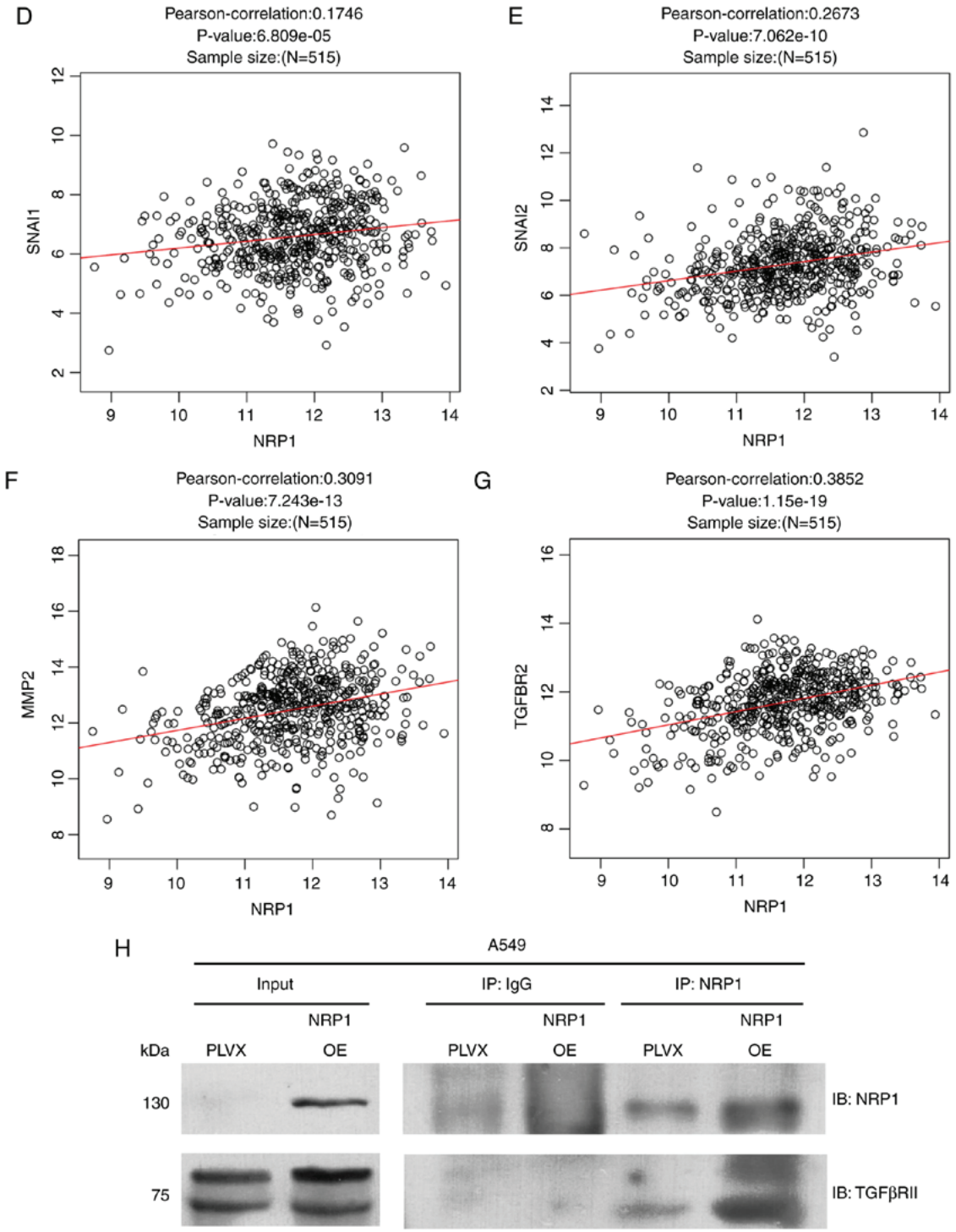

Figure 3. Continued. (D-G) Correlation of NRP1 expression and SNAI1, SNAI2, MMP2 and TGFBR2 in linkedomics cohort (Pearson's correlation coefficient). (H) Interaction between NRP1 and TGFβRII was verified by co-immunoprecipitation assay. NRP1, neuropilin 1; NSCLC, non-small cell lung cancer; TGFβR, transforming growth factor-β receptor.

signaling pathway to promote the EMT and metastasis in NSCLC (Fig. 6).

Discussion

Neuropilins are a class of cell surface glycoproteins, which consist of two family members NRP1 and NRP2 (23,24). The extracellular structure of NRP1 can be divided into three individual components. The a1/a2 domains can function as cubilin homology domain, b1/b2 domain contained TGF-β1 binding site and thus can function as a co-receptor for TGF-β1 (15). In addition to TGF-β1, the b1 domain contained a negatively charged cleft, which may account for other ligands or receptors that bind to NRP1-like VEGF and its receptor, hepatocyte growth factor (HGF) and its receptor (25,26). In addition, c domain is also named as A5-protein (26-28). Neuropilins were

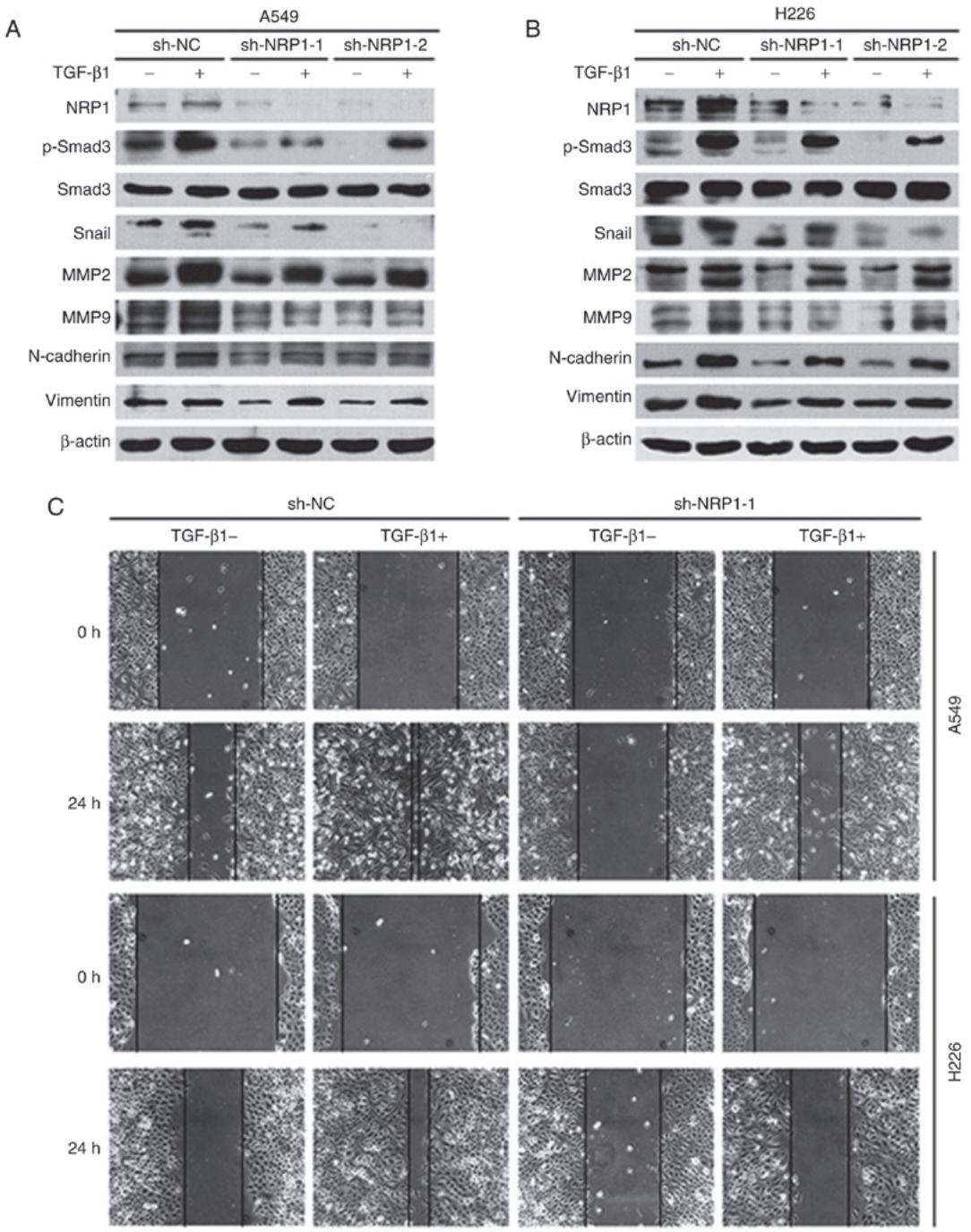

Figure 4. Knockdown of NRP1 suppresses the TGF-β1-induced cell migration and invasion of NSCLC cells. (A and B) Following serum starvation for 24 h, stable NRP1-silenced A549 and H226 cells were treated with or without TGF-β1 (5 ng/ml) for 48 h. The expression of p-Smad3, Smad3, Snail, MMP2, MMP9, N-cadherin, Vimentin was analyzed by western blot analysis. (C) Stable NRP1-silenced A549 and H226 cells were treated with or without TGF-β (5 ng/ml) for 48 h, respectively, and the migratory ability of the cells was then investigated by wound healing assays. NRP1, neuropilin 1; NSCLC, non-small cell lung cancer; TGF-β, transforming growth factor-β.

originally implicated in axon guidance and vascular development on the basis of interaction with semaphorins and VEGF family (26). Later in development, NRP1 was reported to be frequently upregulated in human cancer tissues and functions to contribute to tumor progression via interaction with various extracellular growth factors and its receptors (29-31). Of note, NRP1 has been reported to modulate tumor microenvironment, particularly in regulating the function of dendritic cells (DCs) and regulatory T cells (Tregs) (32,33).

EMT plays a vital role in tumor metastasis as is evident by the upregulated expression of N-cadherin and Vimentin, while the expression level of E-cadherin is downregulated (34). TGF-β/Smad signaling pathway is an important driver in promoting the EMT process via activating the canonical pathway as with Smad family members or non-canonical signaling molecules such as Rho kinase (35,36). TGF-β1-induced EMT has been implicated in diabetic kidney diseases, fibrosis phenotype and tumor cell

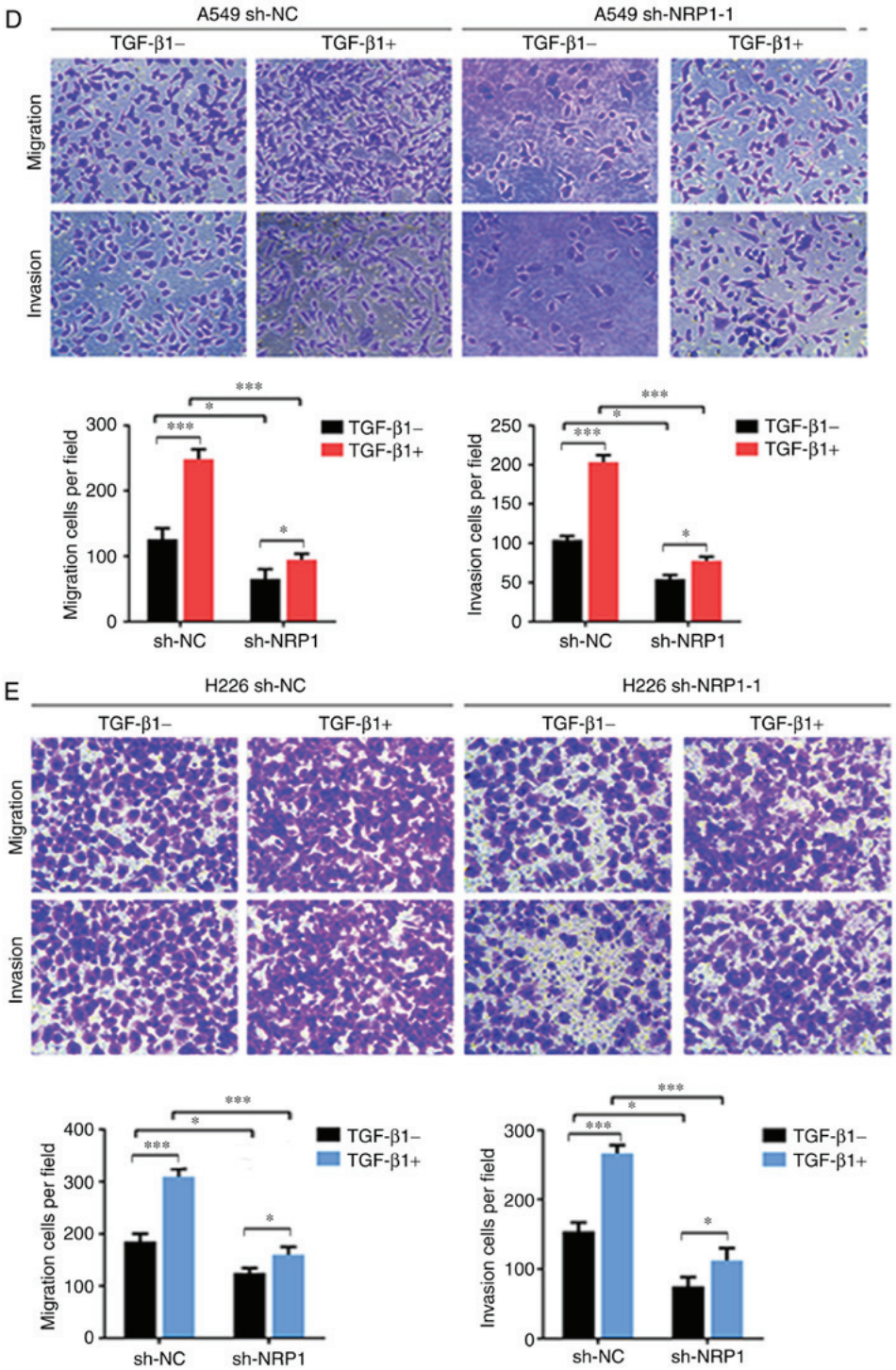

Figure 4. Continued. (D and E) Stable NRP1-silenced A549 and H226 cells were treated with or without TGF-β (5 ng/ml) for 48 h, respectively, and allowed to migrate through 8-μM pore Transwell inserts. The migrated cells were stained and counted in at least three microscopic fields (magnification, x100). The cells were allowed to invade through Matrigel-coated Transwell membranes, and invasive cells were stained and counted under a light microscope. Data are shown as the means ± SD. Significantly different from the control (*P<0.05 and ***P<0.001). NRP1, neuropilin 1; NSCLC, non-small cell lung cancer; TGF-β, transforming growth factor-β.

metastasis (37-39). Functional associated research between NRP1 and TGFβ1-induced EMT has also been carried out. In the immune system, NRP1 can activate TGF-β latent form to promote regulatory T cell activity (40). In addition, small molecule NRP1 antagonists can block TGF-β1 production in regulatory T cells (41). In tumor cells, NRP1 expression is upregulated along with GBM tumor progression. Additionally, NRP1 can bind with TGFβRII to activate Smad3 signaling to drive GBM development and the TGF-β/Smad3 signaling is NRP1-dependent during the process (16). In the central nervous system, NRP1 can balance integrin β8-activated TGF-β signaling to control sprouting angiogenesis (42). In breast cancer, NRP1 can collaborate with TGFβRI to capture and activate (LAP)-TGF-β1 (15). However, functions for

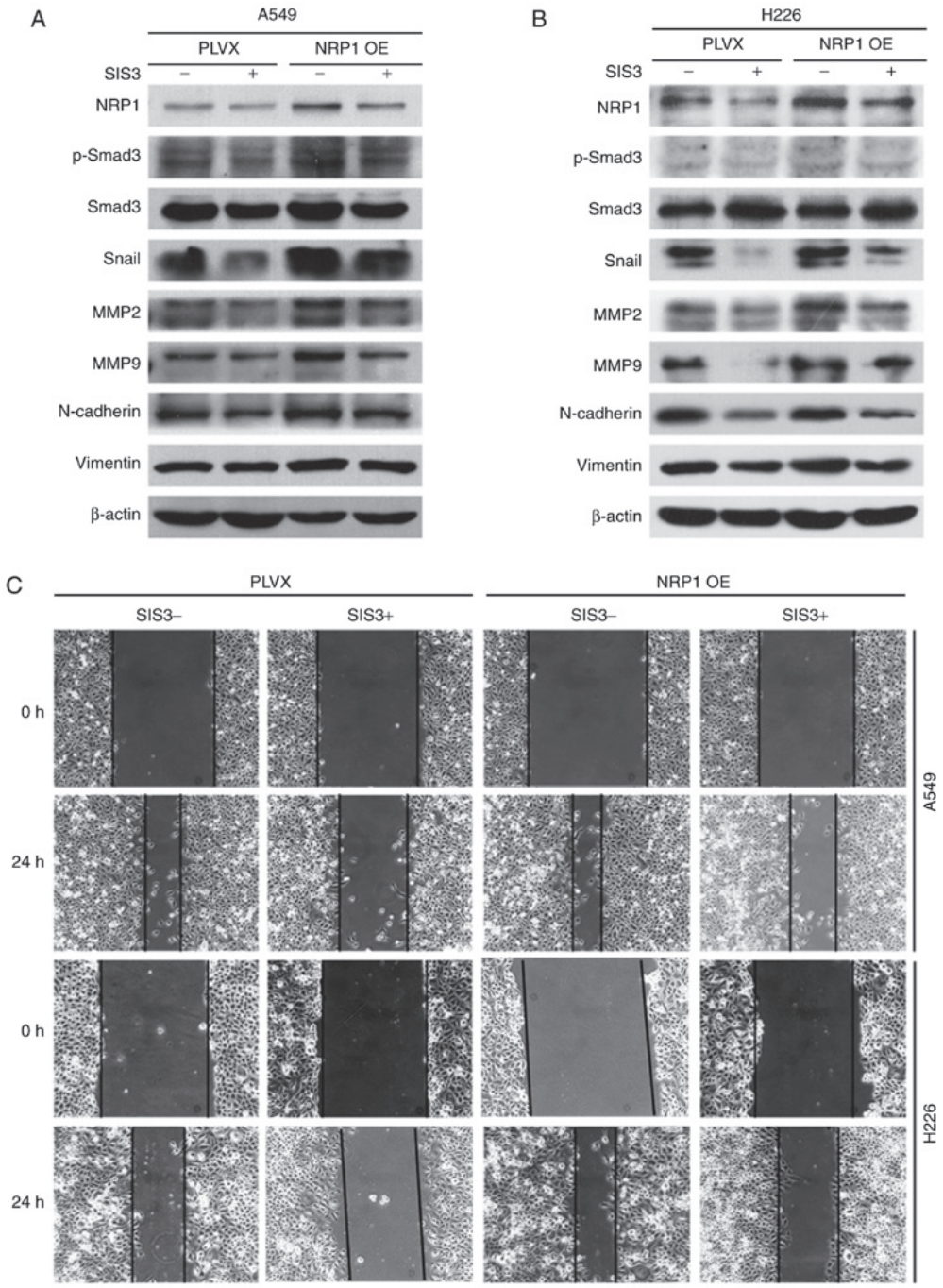

Figure 5. Overexpression of NRP1 suppresses the SIS3-induced decrease in the migration and invasion of NSCLC cells. (A and B) Following serum starvation for 24 h, stable NRP1-silenced A549 and H226 cells were treated with or without SIS3 (3 μM) for 48 h. The expression of p-Smad3, Smad3, Snail, MMP2, MMP9, N-cadherin, Vimentin was analyzed by western blot analysis. (C) Stable NRP1-silenced A549 and H226 cells were treated with or without SIS3 (3 μM) for 48 h, respectively, and the migratory ability of the cells was then investigated by wound healing assays. NRP1, neuropilin 1; NSCLC, non-small cell lung cancer.

NRP1 in modulating TGF-β signaling in NSCLC cells have not been extensively investigated.

In the current study, NRP1 was identified to be highly expressed in 55 paired tissues when compared with corresponding non-cancerous lung tissues from patients. Following examination of the lymph node metastasis subgroup, it was found that NRP1 expression was higher in metastatic NSCLC tissues. A public database LinkedOmics (http://www.linkedomics.org/login.php) also showed that NRP1 was positively correlated with Snail, Slug, MMP2 and TGFβRII in 515 patient samples. *In vitro*, we constructed NRP1-stable knockdown cell lines and carried out Transwell and would healing assays. Data indicated that NRP1 overexpression can promote the migratory and invasive abilities of NSCLC cells. We also observed less pulmonary metastasis nodules in the metastastic model injected with NRP1-silenced cells compared to the control cells. Examination of the underlying mechanism suggested that snail, N-cadherin, Vimentin, MMP2 and MMP9 was altered, displaying that NRP1 can induce EMT to promote NSCLC metastasis.

Given that NRP1 can induce EMT in NSCLC cell metastasis, we next analyzed links between NRP1 and TGF-β1-induced EMT following activating or blocking TGF-β1/Smad3 pathway.

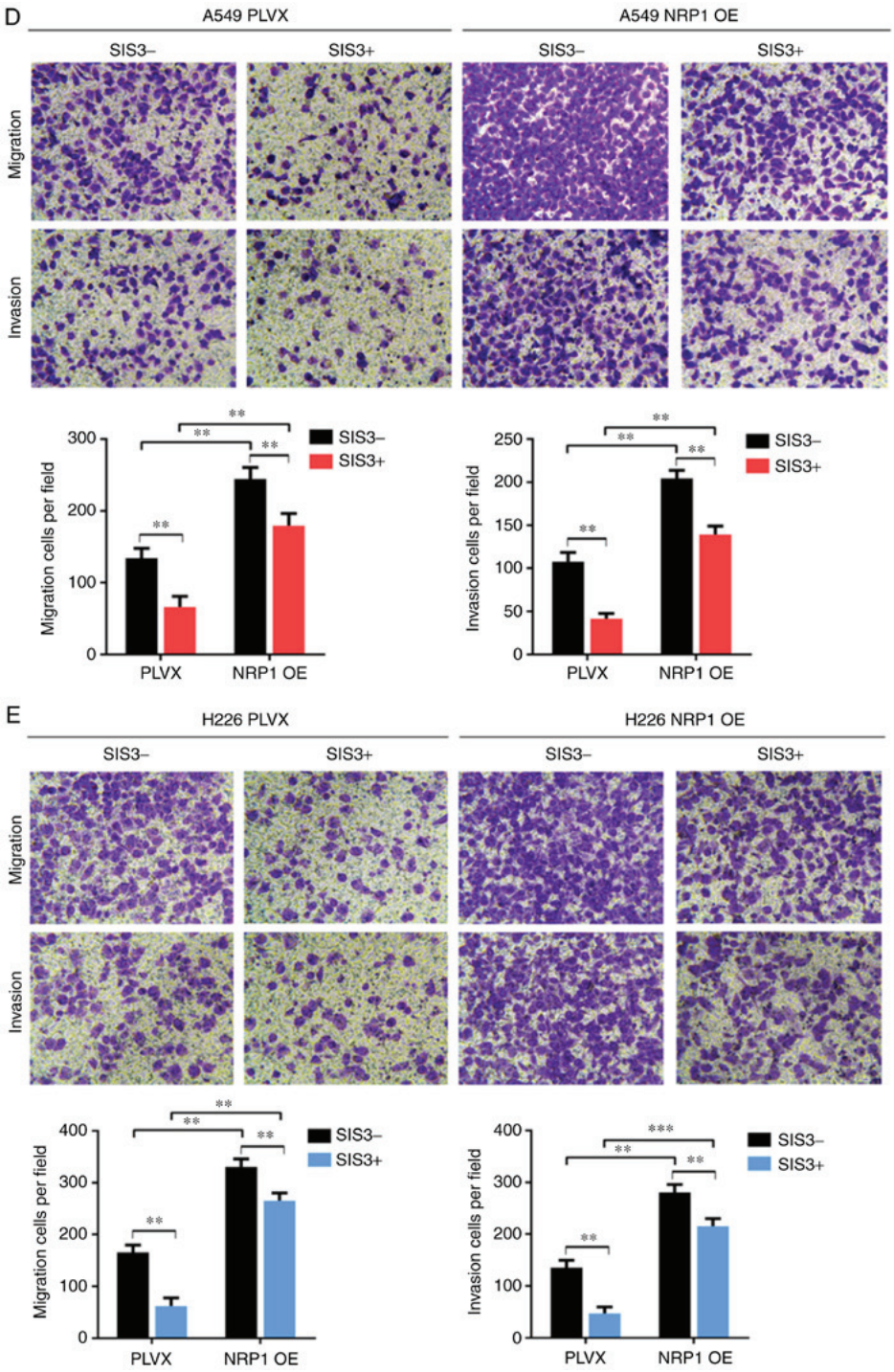

Figure 5. Continued. (D and E) Stable NRP1-silenced A549 and H226 cells were treated with or without SIS3 (3 μM) for 48 h, respectively, and allowed to migrate through 8-μM pore Transwell inserts. The migrated cells were stained and counted in at least three microscopic fields (magnification, x100). The cells were allowed to invade through Matrigel-coated Transwell membranes, and invasive cells were stained and counted under a light microscope. Data are shown as the means ± SD. Significantly different from the control (PVLX) (**P<0.01 and ***P<0.001). NRP1, neuropilin 1; NSCLC, non-small cell lung cancer.

First, we verified the stable NRP1-knockdown cell lines with exogenous TGF-β1 stimulation. The Transwell assay and wound healing assay indicated that the increased migratory and invasive abilities of cells with exogenous TGF-β1 stimulation were considerably suppressed in cells with stable knockdown of NRP1 when compared to the control group. The underlying mechanism also showed that TGF-β1-induced increase in the p-Smad3 level was inhibited in the stable cell line with NRP1 knockdown. By contrast, cells with NRP1 overexpression were treated with SIS3 inhibitor to block Smad3 phosphorylation. SIS3 treatment blocked TGF-β1 signaling in NSCLC cells and partially reduced TGF-β1-induced EMT and metastasis in NSCLC cells with NRP1 overexpression compared to the NC group. In addition, the downstream signaling molecules associated with the MMPs family including MMP2 and MMP9 as well as EMT markers such as Snail, N-cadherin, Vimentin

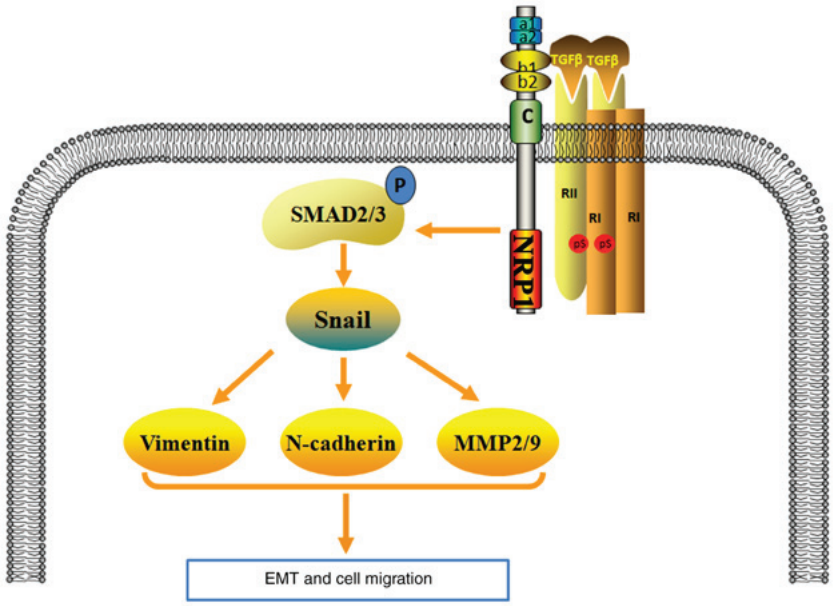

Figure 6. A working model of the mechanistic interaction of NRP1 and TGF-β1-induced EMT involved in NSCLC metastasis.

also showed a similar tendency. Interestingly, co-immunoprecipitation data showed that NRP1 can bind with TGF-βRII in tumor cells, which may affect TGF-β1 activation and signaling. Similarly, Grandclement *et al* reported that NRP2 interacts with TGFβRI to phosphorylate Smad2 to promote EMT in colorectal cancer (43), possibly due to the co-expression of NRP1 and NRP2 as well as overlapping functions of NRP1 and NRP2, suggesting that NRP1 and NRP2 are closely related. Thus, the relationship between NRP1 and NRP2 and the precise mechanism by which NRP1 and NRP2 jointly participate in the EMT process in NSCLC needs further investigation.

In summary, results of the present study showed that NRP1 was overexpressed in metastatic NSCLC tissues. Furthermore, NRP1 can contribute to TGF-β1-induced EMT and metastasis in NSCLC cells. As shown in Fig. 6, the findings reveal the inner binding interaction between NRP1 and TGFβRII in NSCLC metastasis, and the affinity for NRP1 to bind with TGFβRII may explain, at least in part, how they contribute to cancer metastasis. Therefore, this study provides a theoretical basis for NRP1 to become a clinical detection marker and a target for molecular-targeted therapy of NSCLC.

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Availability of data and materials

All data generated or analysed during the study are included in this published article.

Authors' contributions

ZD, WD and ZLei performed the experiments. JZ, YZeng and YZheng collected the patient data and provided pathological evaluation. YZhang and SW analyzed all data. ZLiu and JAH contributed to the design of this study and participated in the drafting of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All participants provided written informed consent at the time of recruitment. This study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. All animal experiments were carried out in accordance with the Guide for the Care and Use of Experimental Animals from the Experimental Animal Center of Xuzhou Medical University. Experiments on Animals were approval by the Animal Ethical Committee of Xuzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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