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

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Abstract

Background: TGF-β1 signaling is a potent inducer of epithelial-mesenchymal transition (EMT) in various cancers. Our previous study has indicated that NRP1 was significantly up-regulated and acted as a vital promoter in the metastasis of non-small cell lung cancer (NSCLC). However, the function of NRP1 in regulation of TGF-β1-induced EMT and NSCLC cell migration and invasion remained unclear. Methods: The differential expression level of NRP1 was determined by RT-PCR analysis in human tissue samples with or without lymph node metastasis. Transwell assay and wound healing assay were conducted to determine cell ability of 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 mechanism. Results: Increased expression of NRP1 was found in metastatic NSCLC tissues and can promote NSCLC metastasis in vivo. Transwell assays, wound healing assay and western blot analysis showed that knockdown of NRP1 significantly inhibited TGF-β1-mediated EMT and migratory and invasive capabilities of A549 and H226 cells. Furthermore, overexpression of NRP1 could weak the decreased migratory and invasive capabilities with SIS3 treatment. Co-IP data showed that NRP1 can interact with TGFβR to induce EMT. Conclusion: This is the first time to report that NRP1 can modulate TGF-β1-induced EMT and cell migration and invasion in NSCLC.

1. Background

Non-small cell lung cancer (NSCLC) is a major cause of cancer-related death all over the world with poorer five-year survival rate in patients [1, 2]. In fact, cancer metastasis caused more than 90% of deaths from solid tumor, including lung cancer [3]. Hence, it is critical to understand the mechanisms of NSCLC metastasis for improving survival rate of patients.

Epithelial mesenchymal transition (EMT) is essential in embryonic development and transformation of early-stage tumors into invasive malignancies [4, 5]. There is plenty of evidence that TGF-β1 signaling is a potent inducer of EMT in various cancers, including NSCLC [6-8]. It is well known that the transforming growth factor β1 (TGF-β1) superfamily 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 (TGFβR2) on the cell membrane to form a complex and recruits TGF-β receptor I (TGFβR1) causes its phosphorylation. Activated TGFβR1 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]. TGF-β plays a dual role in tumor progression, it inhibits tumor growth in the early stage and promotes tumor metastasis and invasion by inducing EMT in the late stage. [13, 14]. Recently, a study demonstrated that Neuropilin-1 (NRP1) acts as a TGF-β1 co-receptor and activates latent TGF-β1 and respond in breast cancer [15]. In line with the report above, Kwiatkowski and his colleagues showed that NRP1 acts as a co-receptor with TGFβR2 to enhance TGF-β1 receptor signaling via Smad3 in Glioblastoma [16]. Thus, the relationship between NRP1 and TGF-β signaling pathways in NSCLC requires to be verified.

The neuropilins (NRPs) are involved in multiple processes of cellular biological function, such as immunity, cell development, and cancer. NRP1 and its homolog neuropilin-2 (NRP2) are co-receptor 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]. Our previous study showed that NRP1 is up-regulated in NSCLC tissues and associated with poorer survival in patients. Meanwhile NRP1 can promote NSCLC cells proliferation and migration via EGFR signaling pathway [22]. Taken together, we hypothesized that dysregualted NRP1 can influence TGF-β1-induced EMT.

In this study, we investigated the function of NRP1 in regulation of TGF-β1-induced EMT and NSCLC cell migration and invasion. We first observed up-regulated expression of NRP1 in metastatic NSCLC tissues. In addition, we established A549 and H226 cell lines with stable knockdown of NRP1. Then transwell assays indicated that knockdown of NRP1 suppressed TGF-β1-induced migration and invasion in NSCLC cells. Our findings demonstrate that repression of NPR1 inhibits TGF-β1-induced EMT in NSCLC.

2. Methods

2.1 Tissue samples

Fifty-five NSCLC patient tissues and corresponding para-carcinoma lung tissues were collected between 2012 and 2016 at the respiratory department of First people's Hospital of Soochow University. All participants have been offered with the written informed consent at recruitment. According to the Revised International System for Staging Lung Cancer, all cases have clinically and pathologically confirmed who had not received any other treatment like radiotherapy or chemotherapy before tissue sampling. The tissue samples were frozen at -80°C for storage. This study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

2.2 Cell culture

A549 and H226 were obtained from the Cell bank of the Chinese Academy of Sciences (Shanghai, China) and grown in RPMI1640 medium (Hyclone, South Logan, UT, USA) containing 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA). All cells were cultured in a humidified incubator containing 5% CO_2 at 37˚C. In some conditions, cells were exposed in 5ng/ml TGF-β1, 3μM SIS3 for 48h for further experiment. NRP1 knockdown of A549 and H226 cells were cultured in same medium supplemented 0.5μg/ml puromycin. In addition, NRP1 overexpressed cells were grown with medium containing G418 for positive selection.

2.3 Lentivirus-mediated stable knockdown of NRP1

The human NRP1 specific small interfering RNA fragment (NRP1 shRNA-1: 5'-CCAUACCAGAGAAUUAUGATT-3'; NRP1shRNA-2: 5'-GUAUACGGUUGC

AAGAUAATT-3') were cloned into the lentiviral vector pGMLV-SC5 –Puro (GenePharma, Shagnhai, China) containing the endonucleases EcoR1 and BamH1.Then we co-transfected pGMLV-SC5 –Puro vector along with packaging plasmids into 293T cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). A549 and H226 cells were infected with the packaged lentiviruses at a multiplicity of infection (MOI) of 25 along with 8ug/l polybrene as coadjutant and cultured for 2 days , and cells being selected with 0.4 μg/ml of puromycin (Sigma-Aldrich, St Louis, MO, USA). The Transfection efficiency was evaluated by later western blot and qRT-PCR analysis.

2.4 Plasmid-mediated stable overexpression of NRP1

For plasmid construction, human NRP1 CDS fragment was cloned into PLVX-IRES-Neo vector between EcoR1 and Xbal by Genewiz. H226 and A549 cells were transfected with UPLVX-IRES-Neo-vector or PLVX-IRES-Neo-NRP1 by Lipofectamine 2000 (Invitrogen) and further cultured for2 days before being selected with G418 reagent (Sigma-Aldrich, St Louis, MO, USA).The NRP1-overexpreeion efficiency was evaluated by later qPCR and western blot analysis.

2.5 Wound healing assay

After NRP-knockdown A549 and H226 cells stimulated with TGF-β1or NRP-overexpression tumor cells treatment with SIS3 for 48h, cells were suspended and reseeded into a 6-well plate. At day 2, when cells grown to 80-90% as a monolayer, we gently scratched the monolayer with 10μl pipette tip. The most important thing is to ensure the tip was perpendicular to the bottom of the plate during the operation. The detached cells were removed by washing with PBS gently two times after scratching. Add refresh medium and continue to culture for 24 h. Observe the gap distance with a microscope and take photos, quantitatively evaluate the gap distance with Photoshop.

2.6 Migration and invasion assay

Cell migration and invasion assays were performed with Transwell chambers (Corning, New York, NY, USA). For migration assay, cells were suspended and plated on chambers that were not coated with Matrigel matrix (BD Science, Sparks, MD, USA). For invasion assay, cells were suspended and plated on chambers pre-coated with Matrigel matrix at 37˚C for 2 h first. After NRP-knockdown tumor cells stimulated with TGF-β1 or NRP-overexpression tumor cells treatment with SIS3 for 48h. 800 μl RPMI1640 medium containing 10% FBS was added into each bottom chamber, meanwhile cells were collected and $5x10^4$ cells diluted in 200ul medium containing 1% FBS were seeded into the upper chamber. After

incubation for 24h, cells were fixed with methanol for 30 min and non-invasive cells were removed, air-dried for 15 min, the left cells were stained with 0.1% crystal violet for 1 h and washed with PBS three times. In the end, the cells were photographed and counted. Each experiment was performed in triplicate.

2.7 Western blot assay

Using cold PBS to wash Cells two times and then lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing phosphatase inhibitor and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). 10% SDS-PAGE was used to separate proteins which then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) , 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. Two hours later, we performed our detection by chemiluminescence (Pierce, Rockford, IL, USA) after washing three times with TBST. All antibodies used in this research including NRP1(sc-5307, santa cruze biotechbology), anti-p-Smad3(Ser423/425), anti-Smad3(C67H9), anti-Snail (C15D3), anti-MMP2 (D8N9Y), and anti-MMP9 (603H) (Cell Signaling Technology, Danvers, MA, USA), anti-N-cadherin, anti-Vimentin (RV202) (BD Biosciences, USA), anti-β-actin and anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology).

2.8 Tumor metastasis model

To establish experimental lung metastasis model, we resuspended cells in PBS ($1x10^6$ cells/100μL /mouse) and injected cells into each mouse (6 weeks old, female, BALB/c,) via the tail vein on day 0. The mice were then injected with TGF-β1 drugs（400ng/μL）into their abdominal cavity every five days, and the total injection was 5 times. All mice were sacrificed 50 days after tail vein injection, and the numbers of pulmonary metastasis nodules were counted under microscope after the appropriate tissues were stained with hematoxylin and eosin (H&E).

2.9 Statistical analysis

All results we obtained are presented as mean ± standard deviation (SD). Statistical significance was analyzed with Student's ttest and $P < 0.05$ was regarded as significant. All statistical analyses were performed using SPSS 7.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1 High expression of NRP1 promotes NSCLC metastasis in vitro

We first elucidate the role of NRP1 in NSCLC metastasis. A549 and H226 cell lines were stably knocked down NRP1. The mRNA and protein expression of NRP1 was significantly reduced in A549 and H226 cells transfected with two NRP1 short hairpin RNAs (shRNAs) compared with control group. (Fig. 1A), and knockdown of NRP1 (sh-NRP1) significantly inhibited the expression of Snail, N-cadherin, Vimentin, MMP2 and MMP9. Next, the effect of NRP1 on migration in NSCLC cells was evaluated by wound healing assay. The A549 and H226 cells transfected with sh-NRP1 migrated towards the scratch more slowly than the control cells (Fig. 1B). Further, transwell assays indicated that knockdown of NRP1 considerably inhibited the migration and invasion in NSCLC cells (Fig. 2C).

To further investigate the role of NRP1 in NSCLC cells, we also established NRP1 stably over-expressed 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 control groups. Moreover, the results of transwell and wound healing assays indicated that overexpression of NRP1 promoted the migration and invasion in A549 and H226 cells (Fig. 2B-C).

3.2 High expression of NRP1 promotes NSCLC metastasis in vivo and is associated with TGFβR□

To explore the mechanism of NRP1 promoting the metastasis of NSCLC. We injected stable NRP1-knockdown A549 cells and negative control cells into BALB/c athymic nude mice via the tail vein. As shown in Figure 3A-B, the pulmonary metastasis nodules in mice injected with NRP1-knockdown A549 cells were fewer than control group. Further, we classified 55 lung tissues according to presence or absence of lymph node metastasis and compared the expression of NRP1 mRNA (additional file1, Table S1). We found that mRNA level of NRP1 was up-regulated in NSCLC tissues associated with lymph node metastasis compared to NSCLC tissues without metastasis (Fig. 3C). These results indicate that the expression of NRP1 may contribute to tumor metastasis in NSCLC tissues. Consistent with the hypothesis that NRP1 was correlated with TGF-β1 signaling or several EMT transcription factors in advanced lung cancer. From public data deposited in LinkedOmics (http://www.linkedomics.org/login.php), NRP1 was correlated with SNAI1, SNAI2, MMP2 and TGFBR2 (Fig. 3D-G). Moreover, as a co-receptor of TGF-β1, NRP1 can modulate TGF-β1 receptor signaling in various cell types [21, 16]. In present study, we observed that the interaction between NRP1 and TGFβR□ by co-immunoprecipitation experiments (Fig. 3H), this finding was also reported by Kwiatkowski et al [15].

3.3 Knockdown of NRP1 inhibits TGF-β1-induced cell migration and invasion in NSCLC cells

Next, the function of NRP1 in TGF-β1-induced metastasis in NSCLC was confirmed. The wound healing assay showed that the speed was inhibited in cells stimulated with exogenous TGF-β1 moved towards the scratch in sh-NRP1 group than control group in both A549 and H226 cell lines (Fig. 4C). The transwell assay further indicated that the increased migratory and invasive abilities of cells with exogenous TGF-β1 stimulation for 24h were considerably suppressed in cells with stable knockdown of NRP1 when compared to control group (Fig. 4D-E). In consistence with the phenotype, western blot analysis was conducted to clarify the underlying mechanism. We can obviously find that the TGF-β1-induced increase in the p-Smad3 level was inhibited in the stable cell line with NRP1 knockdown. In addition, the downstream signaling molecules associated with MMPs family like MMP2, MMP9 and EMT markers like Snail, N-cadherin, Vimentin showed similar tendency (Fig. 4A-B).

3.4 NRP1 promotes the metastasis of tumors via SMAD pathway

Based on previous data, we know that knockdown of NRP1 inhibits TGF-β1-induced cell migration and invasion in NSCLC cells. Next, we further validated the association between NRP1 and TGF-β/Smad3 pathway in NRP1 overexpressed cell lines. SIS3 is a permeable, selective Smad3 inhibitor which can inhibit the activation of TGF-β1/Smad3 signaling pathway and thus inhibits cell metastasis. Data from the wound healing assay showed that treatment with SIS3 can slow down the speed in both control and NRP1 overexpressed cells which moved towards the scratch. However, the inhibitory trend was weakened in NRP1 overexpressed cells compared to control cells (Fig. 5C). Transwell assays showed that overexpression of NRP1 attenuated the inhibitory effect of SIS3 on cell migration and invasion compared with the control group (Fig. 5D-E). And western blot analysis confirmed that overexpression of NRP1 noticeably inhibited the SIS3-induced downregulation of p-Smad3, Snail, MMP2, MMP9, N-cadherin and Vimentin in A549 and H226 cells (Fig. 5A-B).

4. Discussion

Neuropilins are a class of cell surface glycoprotein, which consist of two family members NRP1 and NRP2 .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. In addition to TGF-β1, the b1 domain contained a negatively charged cleft, which may account for other ligands or receptors to bind to NRP1 like VEGF and its receptor, hepatocyte growth factor (HGF) and its receptor and so on. And c domain is also named as A5-protein [23-25]. Neuropilins are originally implicated in axon guidance and vascular development on the basis of interaction with semaphorins and VEGF family [23]. Later in development, NRP1 is reported

to be frequently up-regulated in human cancer tissues and functions to contribute to tumor progression via interaction with various extracellular growth factor and its receptors [26-28]. On the one hand, NRP1 has been reported to modulate tumor microenvironment, particularly in regulating the function of dendritic cells (DCs) and regulatory T cells (Tregs) [29, 30].

EMT plays a vital role in tumor metastasis, accompanied by the up-regulated expression of N-cadherin and Vimentin, meanwhile the expression level of E-cadherin is down-regulated [31]. TGFβ/Smad signaling pathway is an important driver in promoting EMT process via activating canonical pathway like Smad family members or non-canonical signaling molecules like Rho kinase [32, 33]. TGF-β1-induced EMT has been implicated in diabetic kidney diseases, fibrosis phenotype and tumor cell metastasis [34-36]. And the functional associated research between NRP1 and TGFβ1-induced EMT has been carried out recently. In the immune system, NRP1 can activate TGFβ latent form to promote regulatory T cell activity [37]. In addition, small molecule NRP1 antagonists can block TGFβ1 production in regulatory T cells [38]. In tumor cells, NRP1 expression is upregulated along with GBM tumor progression. Meanwhile NRP1 can bind with TGFβR2 to activate Smad3 signaling to drive GBM development and the TGFβ/Smad3 signaling is NRP1 dependent during the process [16]. In the central nervous system, NRP1 can balance integrin β8-activated TGFβ signaling to control sprouting angiogenesis [39]. In breast cancer, NRP1 can collaborate with TGFβR1 to capture and activate (LAP)-TGF-β1 [15]. However functions for NRP1 in modulating TGFβ signaling in NSCLC cells have not been extensively investigated.

In current study, NRP1 is identified to be highly expressed 55 paired tissues when compared with corresponding noncancerous lung tissues from patients. After we pay our attention to lymph node metastasis subgroup, it is interesting to demonstrate that NRP1 expression is higher in metastatic NSCLC tissues. Public database also showed that NRP1 in positively correlated with snail, slug, MMP2 and TGFBR□in 515 patients samples. In vitro, we constructed NRP1-stable knockdown cell lines and made transwell and would healing assays. Data indicated that NRP1 overexpression can promote the migratory and invasive abilities of NSCLC cells. We can also observe less pulmonary metastasis nodules in the metastasis model injected with NRP1-silenced cells than control cells. Research into mechanism suggested that snail, N-cadherin, Vimentin, MMP2 and MMP9 was changed, 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. First, we made verification in stable NRP1-knockdown cell lines with exogenous TGF-β1 stimulation. In agreement with previous findings, 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 control group. And the inside mechanism also showed that TGF-β1-induced increase in the p-Smad3 level was inhibited in the stable cell line with NRP1 knockdown. In contrast, cells with NRP1 overexpression were treated with SIS3 inhibitor to block Smad3 phosphorylation. SIS3 treatment blocked TGF-β1 signaling in NSCLC cells and also partially diminished TGF-β1-induced EMT and metastasis in NSCLC cells with NRP1 overexpression compared to NC group. In

addition, the downstream signaling molecules associated with MMPs family like MMP2, MMP9 and EMT markers like Snail, N-cadherin, Vimentin showed similar tendency. Interestingly, co-immunoprecipitation data showed that NRP1 can bind with TGF-βR in tumor cells, which may affects TGF-β1 activation and signaling.

Conclusions

In summary, we showed that NRP1 is overexpressed in metastatic NSCLC tissues. And that NRP1 can contribute to TGF-β1-induced EMT and metastasis in NSCLC cells. In addition, as shown in figure 6, our findings illuminate the inner binding interaction between NRP1 and TGF-βR in NSCLC metastasis, and the affinity for NRP1 to bind with TGFβR may explain, at least in part, how they contribute to cancer metastasis.

Abbreviations

NSCLC: non-small cell lung cancer; EMT: epithelial mesenchymal transition; TGF-β1: transforming growth factor β1; NRPs: neuropilins; NRP1: Neuropilin-1; NRP2: neuropilin-2; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; HGF: hepatocyte growth factor; DCs: dendritic cells; Tregs: regulatory T cells.

Declarations

Ethics approval and consent to participate

All participants were provided with written informed consent at the time of recruitment. And this study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZLD, WWD, and ZL performed the experiments. JJZ and YYZ collected the patient's data, and provided pathologic evaluation. YZ and SJW analyzed all data. ZYL and JAH contributed to the design of this work and ZYL participate in drafting this article. All authors read and approved the final manuscript.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin. 2017; 67(1):7-30.

2. Wang T, Nelson RA, Bogardus A, Grannis FW, Jr. Five-year lung cancer survival: which advanced stage non-small cell lung cancer patients attain long-term survival? Cancer. 2010; 116(6):1518-1525.

3. Gupta GP, Massague J. Cancer metastasis: building a framework. Cell. 2006; 127(4):679-695.

4. Aiello NM, Kang Y. Context-dependent EMT programs in cancer metastasis. J Exp Med. 2019; 216(5):1016-1026

5.Gloushankova NA, Zhitnyak IY, Rubtsova SN. Role of Epithelial-Mesenchymal Transition in Tumor Progression. Biochemistry (Mosc). 2018; 83(12):1469-1476.

6. Cui Q, Ren J, Zhou Q, Yang Q, Li B. Effect of asiatic acid on epithelial-mesenchymal transition of human alveolar epithelium A549 cells induced by TGF-beta1. Oncol Lett. 2019; 17(5):4285-4292.

7. Liu Y, Xue M, Du S, Feng W, Zhang K, Zhang L, Liu H, Jia G, Wu L, Hu X ,et al. Competitive endogenous RNA is an intrinsic component of EMT regulatory circuits and modulates EMT. Nat Commun. 2019; 10(1):1637.

8. Zhang Y, Li JH, Yuan QG, Cao G, Yang WB. Upregulation of LASP2 inhibits pancreatic cancer cell migration and invasion through suppressing TGF-beta-induced EMT. J Cell Biochem. 2019; doi.org/10.1002/jcb.28638.

9.Batlle E, Massague J. Transforming Growth Factor-beta Signaling in Immunity and Cancer. Immunity. 2019; 50(4):924-940.

10. Chiu HC, Li CJ, Yiang GT, Tsai AP, Wu MY. Epithelial to Mesenchymal Transition and Cell Biology of Molecular Regulation in Endometrial Carcinogenesis. J Clin Med. 2019; 8(4); doi: 10.3390/jcm8040439.

11.Derynck R, Budi EH. Specificity, versatility, and control of TGF-beta family signaling. Sci Signal. 2019; 12(570); doi.org/10.1126/scisignal.aav5183.

12. Ahmadi A, Najafı M, Farhood B, Mortezaee K. Transforming growth factor-beta signaling: Tumorigenesis and targeting for cancer therapy. J Cell Physiol. 2019; 234(8):12173-12187.

13.Korkut A, Zaidi S, Kanchi RS, Rao S, Gough NR, Schultz A, Li X, Lorenzi PL, Berger AC, Robertson G, et al. A Pan-Cancer Analysis Reveals High-Frequency Genetic Alterations in Mediators of Signaling by the TGF-beta Superfamily. Cell Syst. 2018; 7(4):422-437 e427.

14. Chen G, Ye B. The key microRNAs regulated the development of non-small cell lung cancer by targeting TGF-beta-induced epithelial-mesenchymal transition. Comb Chem High Throughput Screen. 2019; doi.org/10.2174/1386207322666190410151945.

15. Glinka Y, Stoilova S, Mohammed N, Prud'homme GJ. Neuropilin-1 exerts co-receptor function for TGF-beta-1 on the membrane of cancer cells and enhances responses to both latent and active TGF-beta. Carcinogenesis. 2011; 32(4):613-621.

16. Kwiatkowski SC, Guerrero PA, Hirota S, Chen Z, Morales JE, Aghi M, McCarty JH. Neuropilin-1 modulates TGF-beta signaling to drive glioblastoma growth and recurrence after anti-angiogenic therapy. PLoS One. 2017; 12(9):e0185065.

17.Matkar PN, Jong ED, Ariyagunarajah R, Prud'homme GJ, Singh KK, Leong-Poi H. Jack of many trades: Multifaceted role of neuropilins in pancreatic cancer. Cancer Med. 2018; 7(10):5036-5046.

18.Prud'homme GJ, Glinka Y. Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity. Oncotarget. 2012; 3(9):921-939.

19.Valdembri D, Regano D, Maione F, Giraudo E, Serini G. Class 3 semaphorins in cardiovascular development. Cell Adh Migr. 2016; 10(6):641-651.

20. Peng K, Bai Y, Zhu Q, Hu B, Xu Y. Targeting VEGF-neuropilin interactions: a promising antitumor strategy. Drug Discov Today. 2019; 24(2):656-664.

21. McGowan SE, McCoy DM. Neuropilin-1 and platelet-derived growth factor receptors cooperatively regulate intermediate filaments and mesenchymal cell migration during alveolar septation. Am J Physiol Lung Cell Mol Physiol. 2018; 315(1):L102-L115.

22. Ding Z, Zhu J, Zeng Y, Du W, Zhang Y, Tang H, Zheng Y, Qin H, Liu Z, Huang JA. The regulation of Neuropilin 1 expression by miR-338-3p promotes non-small cell lung cancer via changes in EGFR signaling. Mol Carcinog. 2019; doi: 10.1002/mc.22990.

23.Jubb AM, Strickland LA, Liu SD, Mak J, Schmidt M, Koeppen H. Neuropilin-1 expression in cancer and development. J Pathol. 2012; 226(1):50-60.

24.Vivekanandhan S, Mukhopadhyay D. Genetic status of KRAS influences Transforming Growth Factor-beta (TGF-beta) signaling: An insight into Neuropilin-1 (NRP1) mediated tumorigenesis. Semin Cancer Biol. 2019; 54:72-79.

25. Jia H, Cheng L, Tickner M, Bagherzadeh A, Selwood D, Zachary I. Neuropilin-1 antagonism in human carcinoma cells inhibits migration and enhances chemosensitivity. Br J Cancer. 2010; 102(3):541-552.

26.Boschetti G, Kanjarawi R, Bardel E, Collardeau-Frachon S, Duclaux-Loras R, Moro-Sibilot L, Almeras T, Flourie B, Nancey S, Kaiserlian D. Gut Inflammation in Mice Triggers Proliferation and Function of Mucosal Foxp3+ Regulatory T Cells but Impairs Their Conversion from CD4+ T Cells. J Crohns Colitis. 2017; 11(1):105-117.

27.Lampropoulou A, Ruhrberg C. Neuropilin regulation of angiogenesis. Biochem Soc Trans. 2014; 42(6):1623-1628.

28.Plein A, Fantin A, Ruhrberg C. Neuropilin regulation of angiogenesis, arteriogenesis, and vascular permeability. Microcirculation. 2014; 21(4):315-323.

29. Roy S, Bag AK, Singh RK, Talmadge JE, Batra SK, Datta K. Multifaceted Role of Neuropilins in the Immune System: Potential Targets for Immunotherapy. Front Immunol. 2017; 8:1228.

30. Chaudhary B, Khaled YS, Ammori BJ, Elkord E. Neuropilin 1: function and therapeutic potential in cancer. Cancer Immunol Immunother. 2014; 63(2):81-99.

31. Sha Y, Haensel D, Gutierrez G, Du H, Dai X, Nie Q. Intermediate cell states in epithelial-to-mesenchymal transition. Phys Biol. 2019; 16(2):021001.

32. Liu S, Hou H, Zhang P, Wu Y, He X, Li H, Yan N. Sphingomyelin synthase 1 regulates the epithelialtomesenchymal transition mediated by the TGFbeta/Smad pathway in MDAMB231 cells. Mol Med Rep. 2019; 19(2):1159-1167.

33. Hu H, Wang M, Wang H, Liu Z, Guan X, Yang R, Huang R, Tang Q, Zou C, Wang G ,et al. MEGF6 Promotes the Epithelial-to-Mesenchymal Transition via the TGFbeta/SMAD Signaling Pathway in Colorectal Cancer Metastasis. Cell Physiol Biochem. 2018; 46(5):1895-1906.

34. Yin J, Wang Y, Chang J, Li B, Zhang J, Liu Y, Lai S, Jiang Y, Li H, Zeng X. Apelin inhibited epithelial-mesenchymal transition of podocytes in diabetic mice through downregulating immunoproteasome subunits beta5i. Cell Death Dis. 2018; 9(10):1031.

35. Kanemaru R, Takahashi F, Kato M, Mitsuishi Y, Tajima K, Ihara H, Hidayat M, Wirawan A, Koinuma Y, Hayakawa D ,et al. Dasatinib Suppresses TGFbeta-Mediated Epithelial-Mesenchymal Transition in Alveolar Epithelial Cells and Inhibits Pulmonary Fibrosis. Lung. 2018; 196(5):531-541.

36. Zhang X, Feng W, Zhang J, Ge L, Zhang Y, Jiang X, Peng W, Wang D, Gong A, Xu M. Long noncoding RNA PVT1 promotes epithelialmesenchymal transition via the TGFbeta/Smad pathway in pancreatic cancer cells. Oncol Rep. 2018; 40(2):1093-1102.

37. Glinka Y, Prud'homme GJ. Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. J Leukoc Biol. 2008; 84(1):302-310.

38. Powell J, Mota F, Steadman D, Soudy C, Miyauchi JT, Crosby S, Jarvis A, Reisinger T, Winfield N, Evans G ,et al. Small Molecule Neuropilin-1 Antagonists Combine Antiangiogenic and Antitumor Activity with Immune Modulation through Reduction of Transforming Growth Factor Beta (TGFbeta) Production in Regulatory T-Cells. J Med Chem. 2018; 61(9):4135-4154.

39.Hirota S, Clements TP, Tang LK, Morales JE, Lee HS, Oh SP, Rivera GM, Wagner DS, McCarty JH. Neuropilin 1 balances beta8 integrin-activated TGFbeta signaling to control sprouting angiogenesis in the brain. Development. 2015; 142(24):4363-4373.

Additional File Legend

Additional file 1: Table S1. Clinicopathological features of NSCLC tissues from 27 patients with non-lymph node and metastasis and 28 patients with lymph node and metastasis.

Figures

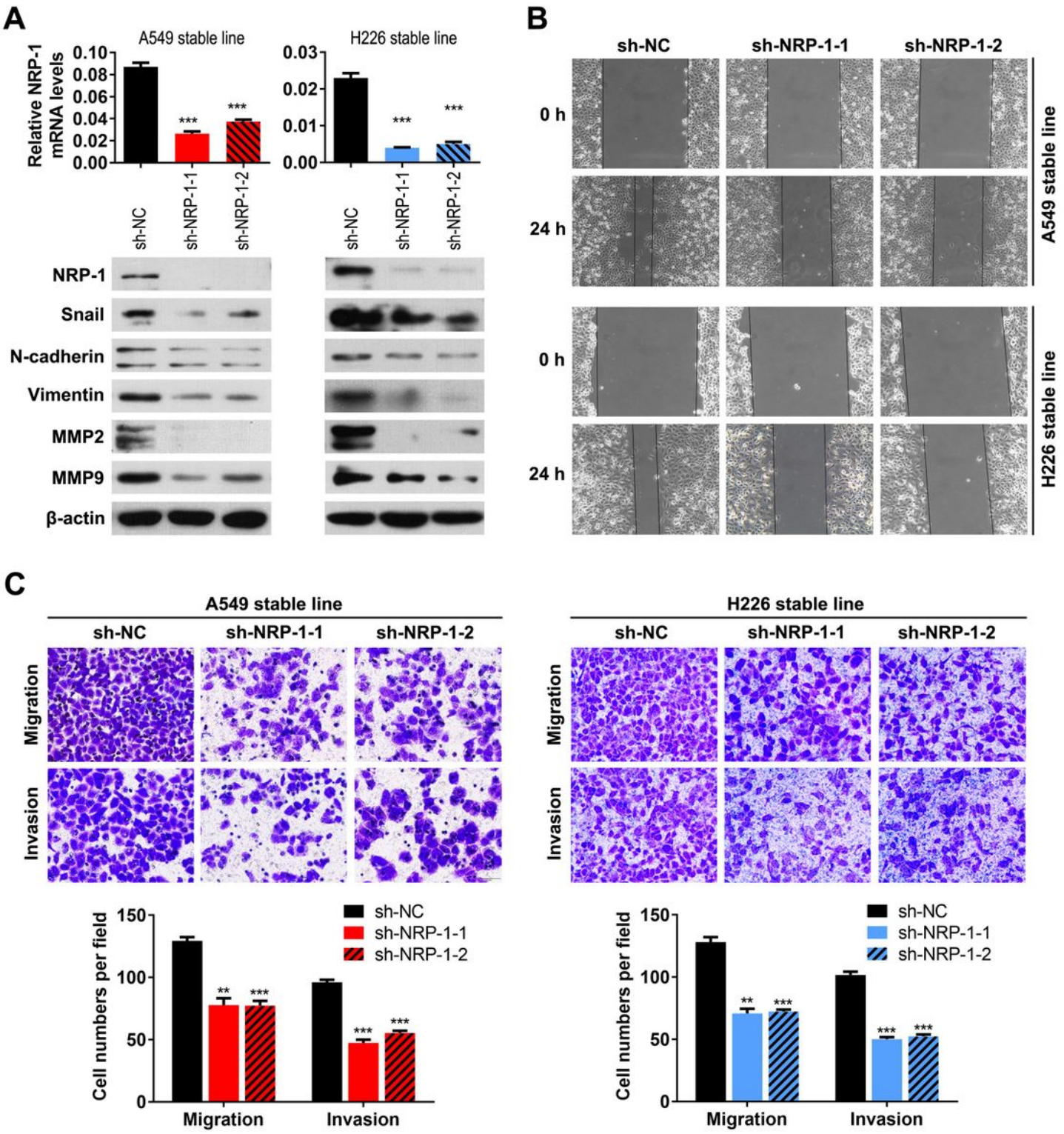

Figure 1

Knockdown of NRP1 inhibits NSCLC cell migration and invasion (A) qRT-PCR and Western blot analysis of NRP1 and downstream signaling protein expression level in A549 and H226 cells after stable knockdown of NRP1 expression. (B) The wound healing assay of cells after stable knockdown of NRP1 expression. (C) Transwell assay of the cell migratory and invasive activity in A549 and H226 cell lines after NRP1 was knockdown.

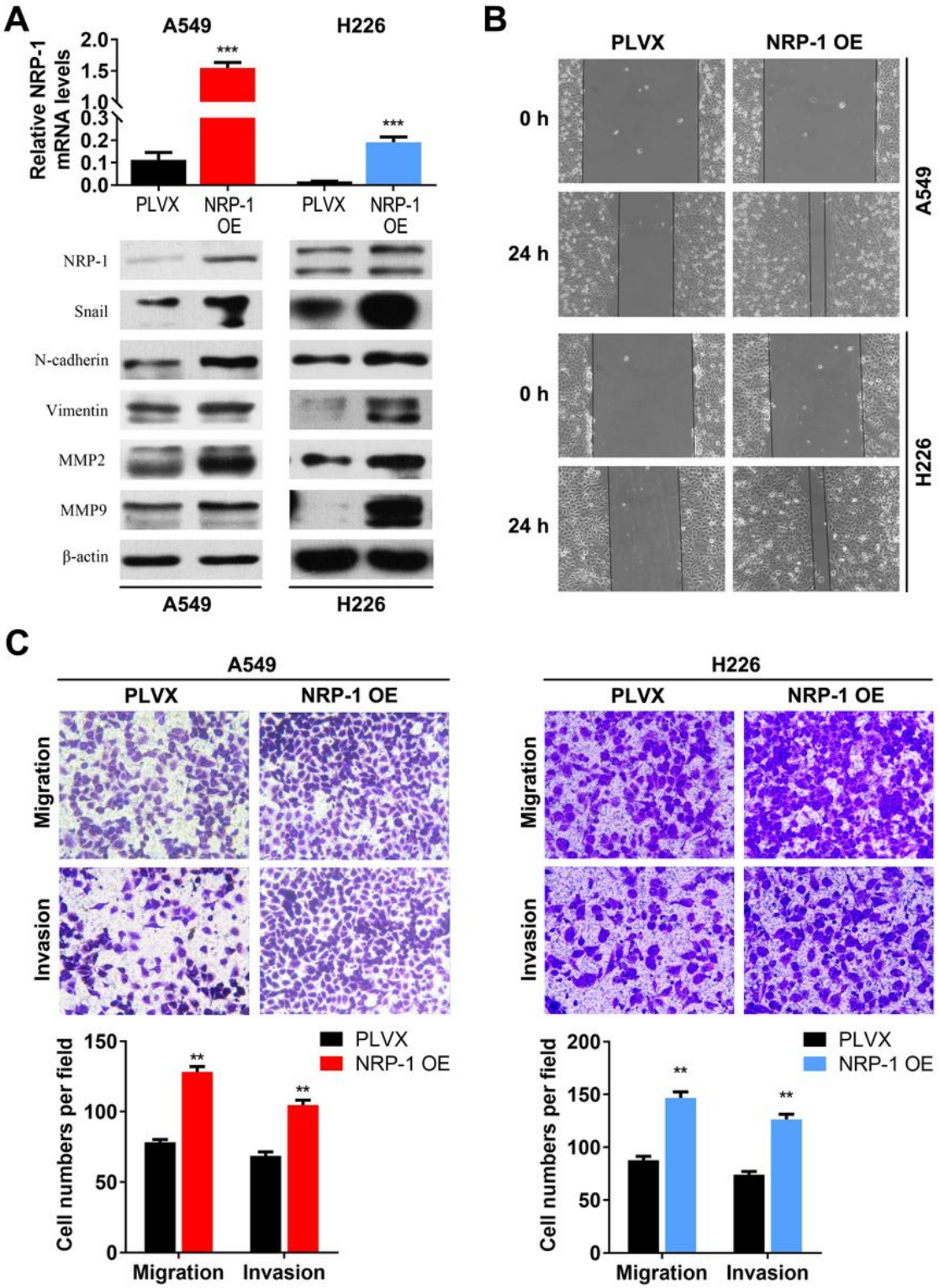

Figure 2

Overexpression of NRP1 promotes NSCLC cell migration and invasion (A) qRT-PCR and Western blot analysis of NRP1 and downstream signaling protein expression level in A549 and H226 cells after stable overexpression of NRP1. (B) The wound healing assay of cells after stable overexpression of NRP1. (C) Transwell assay of the cell migratory and invasive activity in A549 and H226 cell lines after stable overexpression of NRP1.

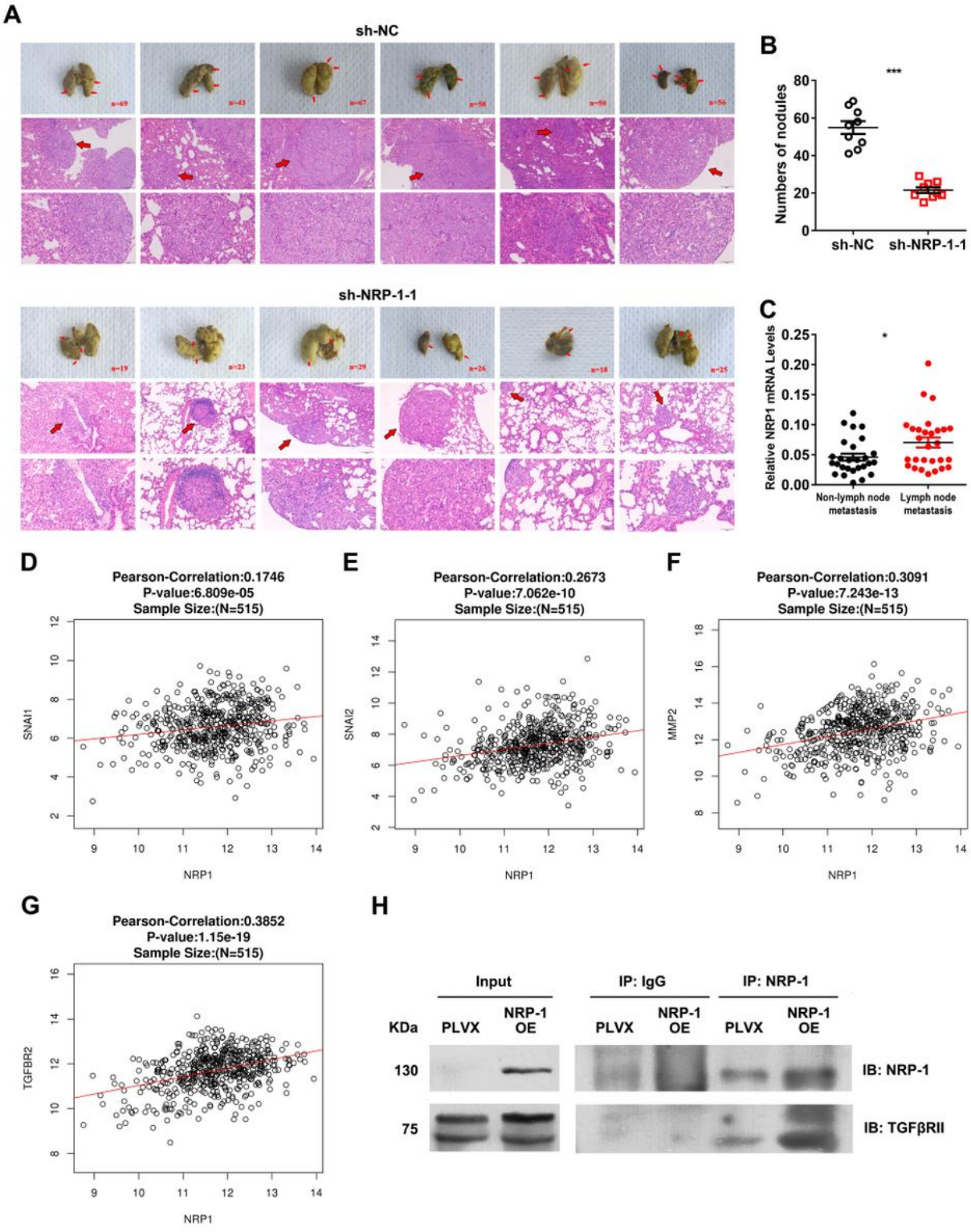

Figure 3

High expression of NRP1 promotes NSCLC metastasis in vivo and is associated with TGFβR (A) The representative images of pulmonary metastasis nodules and HE staining in NRP1-silenced cells compared with control cells. (B) The calculated numbers of nodules in sh-NRP1 and sh-NC group. (C) The NRP1 mRNA expression in 55 NSCLC tissues with or without lymph node metastasis. (D-G) The

correlation of NRP1 expression and SNAI1, SNAI2, MMP2 and TGFBR2 in linkedomics cohort (Pearson's correlation coefficient). (H) Co-IP assay of NRP1 and TGFβR2 interaction

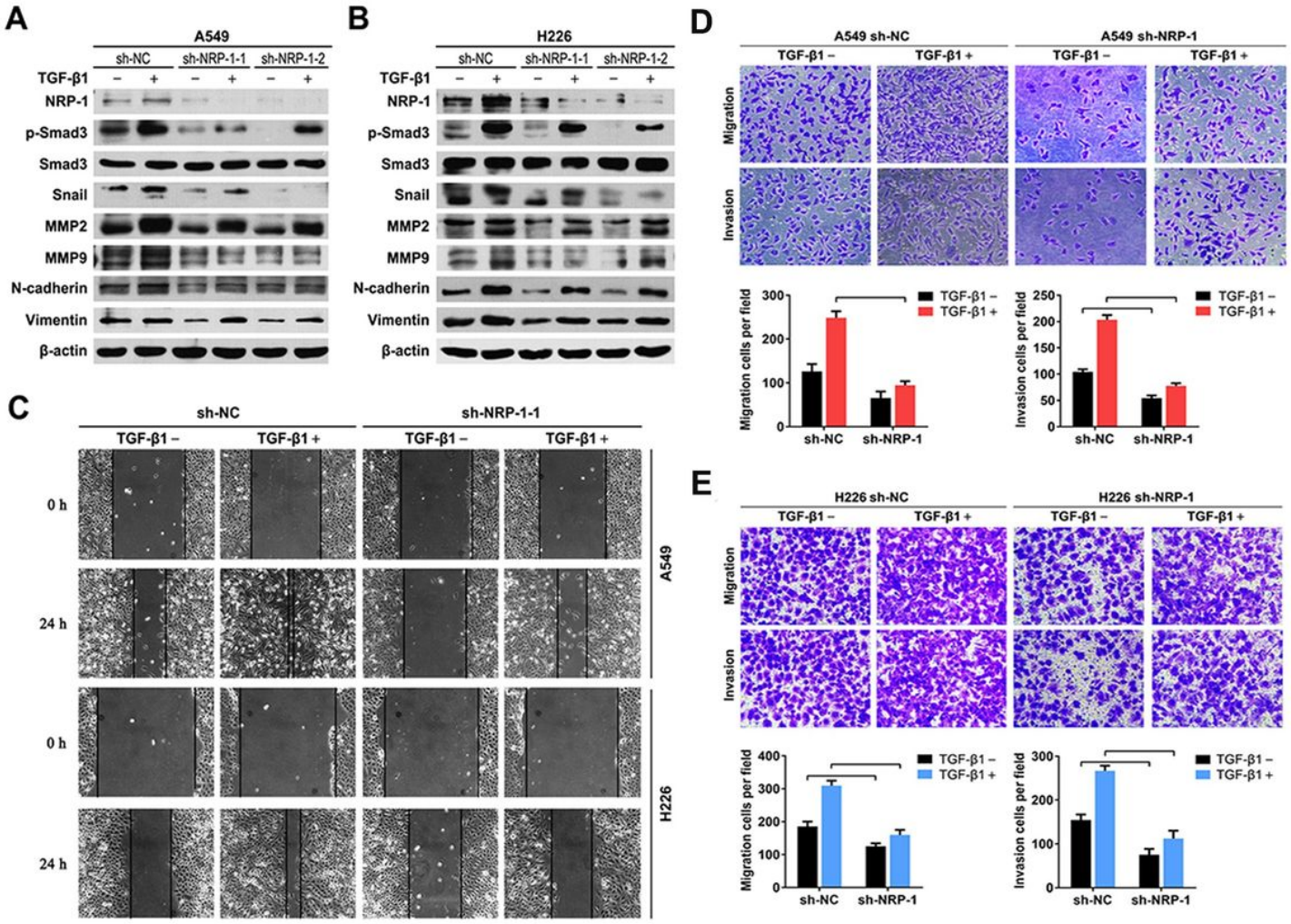

Figure 4

Knockdown of NRP1 inhibits TGF-β1-induced cell migration and invasion in NSCLC cells (A-B) Western blot analysis of associated molecules involved in TGF-β1-induced EMT signaling pathway in sh-NRP1 and sh-NC group. (C) The wound healing assay of cells in sh-NRP1 and sh-NC group with TGF-β1 stimulation for 24h. (D-E) The transwell assay of cells in sh-NRP1 and sh-NC group with TGF-β1 stimulation for 24h.

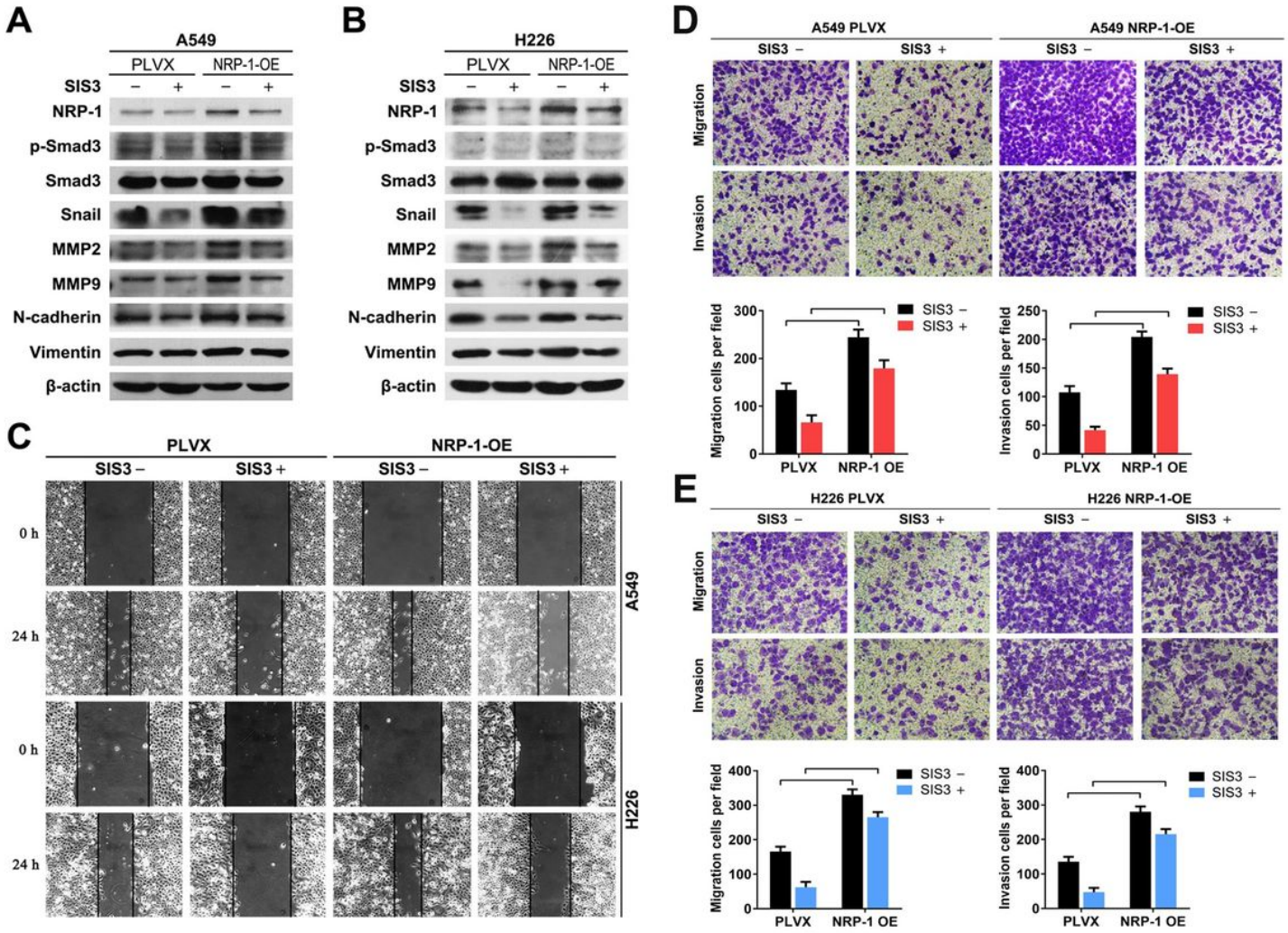

Figure 5

Overexpression of NRP1 inhibits SIS3-blocked cell migration and invasion in NSCLC cells. (A-B) Western blot analysis of associated molecules involved in TGF-β1-induced EMT signaling pathway in NRP1 overexpression cells after SIS3 treatment. (C) The wound healing assay of cells in s NRP1 overexpression cells after SIS3 treatment. (D-E) The transwell assay of cells in NRP1 overexpression cells after SIS3 treatment compared with control group.

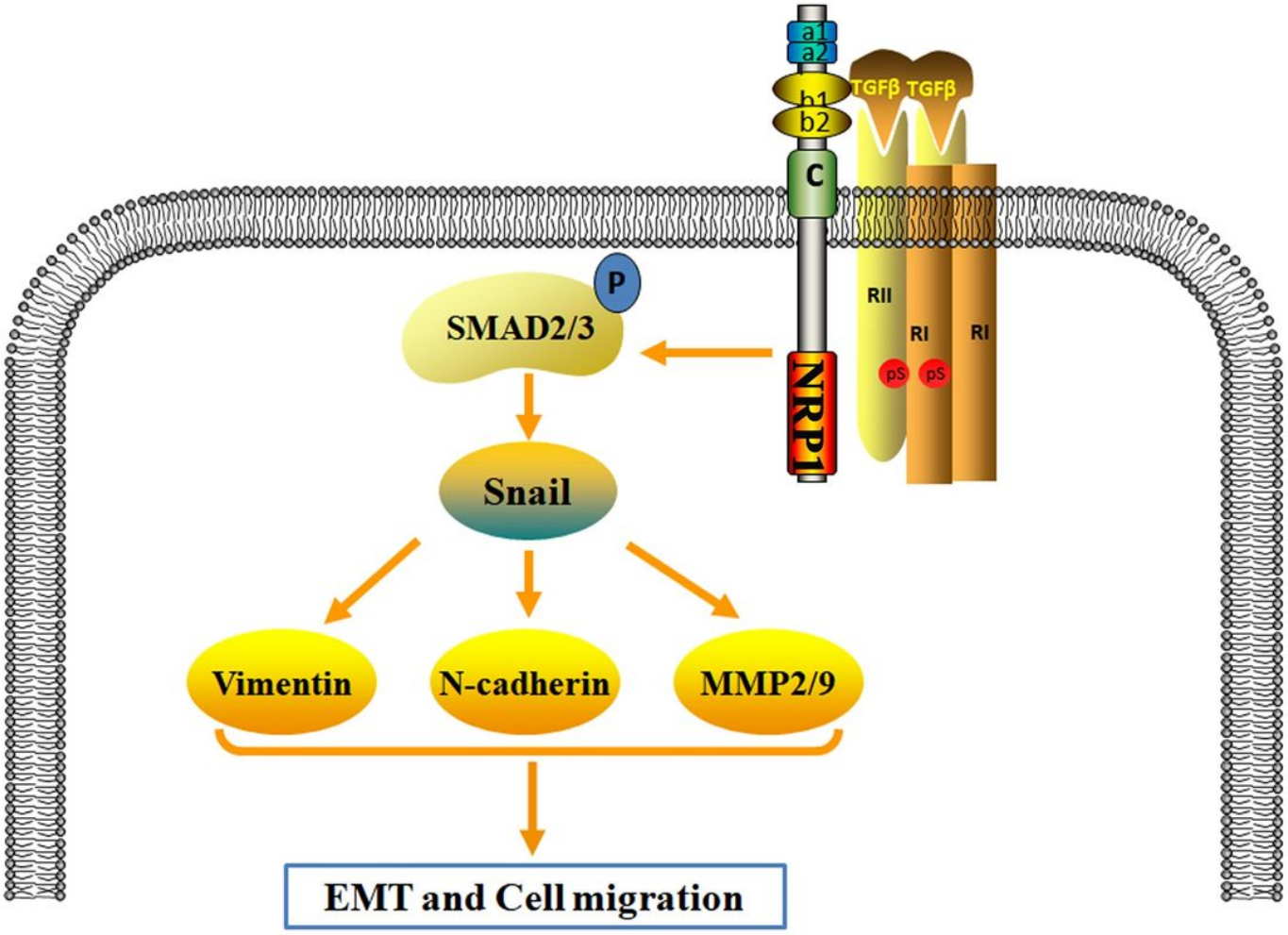

Figure 6

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

Supplementary Files

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