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ORIGINAL ARTICLE

Silencing Neuropilin 1 gene reverses TGF-β1-induced epithelial mesenchymal transition in HGC-27 gastric cancer cell line*

Weiguo Xu[1] (✉), Xin Yang[1], Qiqi Zhan[1], Guanyi Ding[1], Shang Guo[1], Bing Zhu[1], Hong Xu[2], Xiangmei Liu[1, 3]

[1] Department of Surgical Oncology, North China University Of Science and Technology Affiliated Hospital, Tangshan 063000, China
[2] North China University of Science and Technology, Tang Shan 063000, China
[3] Department of Gynaecology and Obstetrics, Liuyang Maternal and Child Health Care Hospital, Liu Yang 410300, China

Abstract

Objective The aim of this study was to determine Neuropilin 1 (NRP1) contribution to transforming growth factor β1 (TGF-β1)-induced epithelial mesenchymal transition (EMT) of HGC-27 gastric cancer cells and study its mechanism.

Methods In this study, TGF-β1 was used to induce EMT in HGC-27 cells. Further, these cells were stably transfected with siRNA targeting NRP1. Wound healing and transwell assays were used to measure cell migration and invasion, respectively. NRP1 and EMT markers were measured using quantitative real time reverse transcription polymerase chain reaction and western blotting.

Results Exposure of TGF-β1 conferred a fibroblastic-like shape to cancer cells and significantly increased the expression of NRP1 in HGC-27 cells. TGF-β1 subsequently promoted migration and invasion of HGC-27 cells. Furthermore, silencing NRP1 inhibited the invasion and migration of TGF-β1-induced cells undergoing EMT.

Conclusion Silencing NRP1 can inhibit cell migration, invasion, and metastasis and reverse the TGF-β1-induced EMT process of gastric cancer.

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Key words: Neuropilin1 (NRP1); epithelial-mesenchymal transition (EMT); gastric cancer; transforming growth fqactor-β1

Gastric cancer (GC) is one of the most important cancers worldwide. It is the fifth most frequently diagnosed cancer and is the third leading cause of cancer death in patients [1].

Epithelial-mesenchymal transition (EMT) is perceived as a significant phenotypic transformation that occurs during early-stage development [2]. It has become progressively certain that EMT has significant roles in malignancy, diminishes the affectability of disease cells to therapeutics, and advances malignant cell growth and metastasis [3]. Lost E-cadherin articulation, which is recognized in gastrointestinal malignancies, is related to a poor prognosis with faster disease progress [4]. The articulation and initiation of the EMT-prompting interpretation factors result as a response to different signaling pathways, including those that are interceded by transforming growth factor β (TGF-β), Wnt, Sonic Hedgehog (Shh), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), bone morphogenetic protein (BMP), Notch and integrin [5–10]. During EMT, cells lose their epithelial attributes with a rise in mesenchymal phenotype before entering systemic circulations during metastasis [11].

Neuropilins are single-pass transmembrane proteins. Neuropilin 1 (NRP1) was described in 1987 as the first member of the family, and later, in 1997, neuropilin 2 (NRP2) was insulated by Chen *et al* [12–13]. In 1998, Soker *et al*. isolated NRP1 from endothelial cells and tumor

✉ Correspondence to: Weiguo Xu. Email: mimimay860@gmail.com
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tissues [14]. Indeed, NRP expression is not just restricted to intra-tumoral vessels, but a vast majority of cancer cells are reported to express NRPs [15]. Since its discovery, NRP1 has been widely used as a selective tumor targeting agent in both preclinical and human studies, and NRPs have rapidly become recognized as key regulators of angiogenesis, lymphangiogenesis, EMT, and tumor progression [16]. Clinical-pathological data seem to indicate a correlation between the increased expression of NRPs and the advanced stage of tumors with poor prognosis, and they are more broadly observed in a large variety of diverse tumor types and the generation of cancer stem cells [17–20]. Non-small cell lung cancer (NSCLC) patients with a high articulation of NRP1 have a shorter disease-free and endurance rate [21]. NRP1 upregulation in gastrointestinal carcinomas appears to correlate with invasive behavior and metastatic potential [22]. It has been shown that NRPs interact with TGF-β, hepatocyte growth factor (HGF), and signal PDGF [23–26].

Few ecological signs have been found to control the NRP's articulation of tumor cells *in vitro*. It is not completely understood how NRPs can control such a wide scope of various signaling receptors. The mechanism by which NRP1 impacts tumorigenesis is not yet thoroughly characterized. In this study, we hypothesized that NRP1 contributes to the responsiveness of the TGF-β1-induced EMT pathway in the HGC-27 cells, which is important for the progression and metastasis of tumors. We also hypothesized the anticipated cure for GC by targeting NRP-1.

Materials and methods

Cell culture

The human GC cell line HGC-27 was bought from the cell bank of the Chinese Academy of Sciences (China). The cells were cultured in RPMI 1640 (Gibco, USA) complemented with 10% fetal bovine serum (FBS) (Jiangsu Ke Te Biological Co., Ltd., China), 100 μg/mL streptomycin, and 100 U/mL of penicillin (Invitrogen, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Establishment of TGF-β1-induced EMT model

Recombinant human TGF-β1 (Invitrogen Biotechnology Co., Ltd., USA) was used to initiate EMT in HGC-27 cells. Cells were simmered for 24 h and seeded in 6-well plates for TGF-β1 incentive, and these cells were cultured with RPMI 1640 accompanied with 2% FBS comprising 5 ng/mL TGF-β1 every 2 d until 7 d in 37 °C incubator with 5% CO_2 to achieve the EMT state. Cell morphology changes were observed using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting to find the mRNA and protein expression of NRP1, E-cadherin, Vimentin, and Snail, respectively, and to screen out the best TGF-β1-induced EMT model.

Gene expression knockdown by RNA interference of HGC-27 cells

In tumor cells, NRP1 expression was suppressed by transfecting targeted siRNA sequences with Lipofectamine 2000 (Invitrogen, USA). The NRP1 siRNA (si-NRP1) and negative control siRNA (si-NC) were purchased from RiboBio (Suzhou RiboBio Co., Ltd., China). The target arrangement was used to down-regulate NRP1 *in vitro*. A nontarget siRNA sequence was utilized as a depressing control. Cells were then grouped and transfected as the CON group (blank control, transfected with the phosphate-buffered saline; PBS), NC group (transfected with the negative control siRNA), and si-NRP1 group (transfected with the NRP1 siRNA). Lipofectamine 2000 were transfected when the cell intensity reached 30% to 50%. Later, cells were handled with the mixed solution and cultivated for 4 to 6 h at 37 °C with 5% CO_2. After incubation, cells were transported to a complete medium and cultivated for 48 h to 72 h. The qRT-PCR and western blotting validated knockdown efficiency.

Wound healing assay

HGC-27 cells were cultured in a 6-well plate at a final density of 1×10^6 cells/well and incubated overnight for adhesion. When the confluence reached 95%, a 200 μL micropipette tip was used to create a vertical linear scratch on the cell monolayer in the 6-well plate. Next, the wells were washed twice with PBS to remove any loose cells, and cells were continuously cultured in medium supplemented with 2% FBS under the standard conditions. The images were taken at different time points, including 0 h, 24 h, and 48 h after the scratch. Using Image J software, the wound closure was analyzed. The 24 h (or 48 h) relative percent of wound closure = (the width at 0 h - the width at 24 h (or 48 h))/the width at 0 h.

Cell migration and invasion assays

For the cell invasion assay, transwell chambers were coated with Matrigel (dilution 1:8; Becton, Dickinson and Company, USA) on the upper side of the membrane and incubated at 37 °C overnight before starting the invasion assay. Three wells per test group were assayed. In the meantime, at a final density of 1×10^5 cells/mL, trypsin-digested cells were combined with RPMI 1640. Then, 600 μL of RPMI 1640 containing 10% FBS were added into the lower chamber, whereas 200 μL of the cell suspension were transferred to the upper chamber. After 24 h of culture, cells that did not enter through the membrane were removed with cotton swabs and detached from the

culture plate. Transferred cells were then fixed with 4% polyformaldehyde for 15 min, then with a 0.1% crystal violet solution for 15 min, and finally cleaned with PBS. Lastly, we observed cells under the microscope. For cell migration, the invasion assay was performed the same as described above, except chambers were not coated with Matrigel. Cell migration and invasion were defined by counting the marked cells in 5 randomly selected fields with a light microscope, and the number of cells was computed with ImageJ software.

qRT-PCR

Using Trizol (Invitrogen, USA) reagent, total RNA was extracted from HGC-27 cell lines. After confirming the concentration and purity of the RNA, a Reverse Transcription Kit (ZR102-1, Zeman Biotechnology Co., Ltd. China) was used for the reverse transcription of 2 μg RNA to cDNA with 20 μL of the reverse transcription system according to the manufacturer. qRT-PCR was directed with a 2 × SYBR qPCR Mix. The sequences of primers are given below: NRP1 (Forward: 5′- GATCTACCCCGAGAGAGCCA -3′ Reverse: 5′- TGAGCTGGAAGTCATCACCTG -3′), E cadherin (Forward: 5′- GGCTGGACCGAGAGAGTTTC -3′ Reverse: 5′- CAAAATCCAAGCCCGTGGTG -3′), Vimentin (Forward: 5′- TCCGCACATTCGAGCAAAGA -3′ Reverse: 5′- TGAGGGCTCCTAGCGGTTTA -3′), Snail (Forward: 5′- CGGCTTTTGCAGTGGACATC -3′ Reverse: 5′- CGGCTTTTGCAGTGGACATC -3′), and GAPDH (Forward: 5′- ACCCAGAAGACTGTGGATGG -3′ Reverse: 5′- TCTAGACGGCAGGTCAGGTC -3′). The mRNA level of the target genes was normalized by the GAPDH mRNA level to quantify gene expression. The gene expression was analyzed using the $2^{-\Delta\Delta CT}$ approach (formula: $\Delta\Delta CT = \Delta CT_{\text{experimental group}} - \Delta CT_{\text{control group}}$. $\Delta CT = CT_{\text{target gene}} - CT_{\text{internal reference}}$).

Western blotting analysis

Overall proteins were mined from cells. PBS-washed cells were lysed with RIPA lysis buffer, which contained 1% phenylmethylsulfonyl fluoride (PMSF) at 4 °C. The whole-cell lysate obtained was centrifuged at 12,000 rpm for 15 min. The protein intensities were calculated using bicinchoninic acid (BCA) method. 30 μg protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) at 90 V and moved to a polyvinylidene fluoride membrane at 120 V for 100 min. The membrane was closed with PBS containing 5% skimmed milk powder for 1 h and incubated with primary antibodies at 4 °C overnight with anti-NRP1 (diluted at 1:1500, ARG59279), anti-E cadherin (diluted at 1:700, A11492), anti-Vimentin (diluted at 1:1000, ARG69199), anti-Snail (diluted at 1:1000, A5544), and anti-Tubulin (diluted at 1:200, GTX11270). The membrane was washed with TBST 5 times. Afterward, the membrane was incubated with the anti-rabbit (1:5000) secondary antibodies for 1 h at 37℃. Washing thrice with TBST for 5 min each afterward, the membrane was incubated with the enhanced chemiluminescence solution, followed by X-film exposure and photographing. The intensities of protein bands were analyzed using Image J software. Gray values of the target bands were normalized with those of the internal reference band (Tubulin). Protein expression was calculated between the target and the internal criteria band.

Statistical analysis

All data were statistically analyzed using IBM SPSS Statistics for Windows (version 20.0; IBM Corp. Armonk, USA). Values were expressed as the mean ± SD. An ANOVA was used for multiple groups, while pairwise evaluations were completed using Student's *t*-tests. All the experiments were repeated in triplicate. $P < 0.05$ was considered significant.

Results

TGF-β1 induced EMT in the GC HGC-27 cells

Through EMT, HGC-27 cells can undergo a phenotypic, reversible switch to fibroblast-like cells. TGF-β1 exposure led to the formation of spindle-shaped cells with elongated cellular processes and diminished cell-to-cell contacts, characteristics of EMT, as compared to the TGF-β1 negative group (Fig. 1a). TGF-β1-induced EMT led to upregulation of migration and invasion of HGC-27 cells (Fig. 1b and 1c). Furthermore, TGF-β1 reduced protein expression of epithelial markers (E-cadherin) and increased expression of Vimentin, Snail and NRP1 (Fig. 1d). qRT-PCR was performed to examine the mRNA levels of EMT-related molecules in HGC-27 cells at 0, 2, 3, 4, and 7 d (Fig. 1e). In conclusion, TGF-β1 could be used to stimulate the HGC-27 cell line to implement the TGF-β1-induced EMT model for further studies.

NRP1 expression and stable knockdown in the GC HGC-27 cell lines

Following the transfection result, qRT-PCR demonstrated that *NRP1* gene expression was significantly downregulated in the siNRP1 group compared to the NC and CON groups, and the differences were statistically significant ($P < 0.05$). Western blotting clearly showed that NRP1 protein was suppressed 0.47-fold in HGC-27 cells transfected with NRP1-siRNA (Fig. 2).

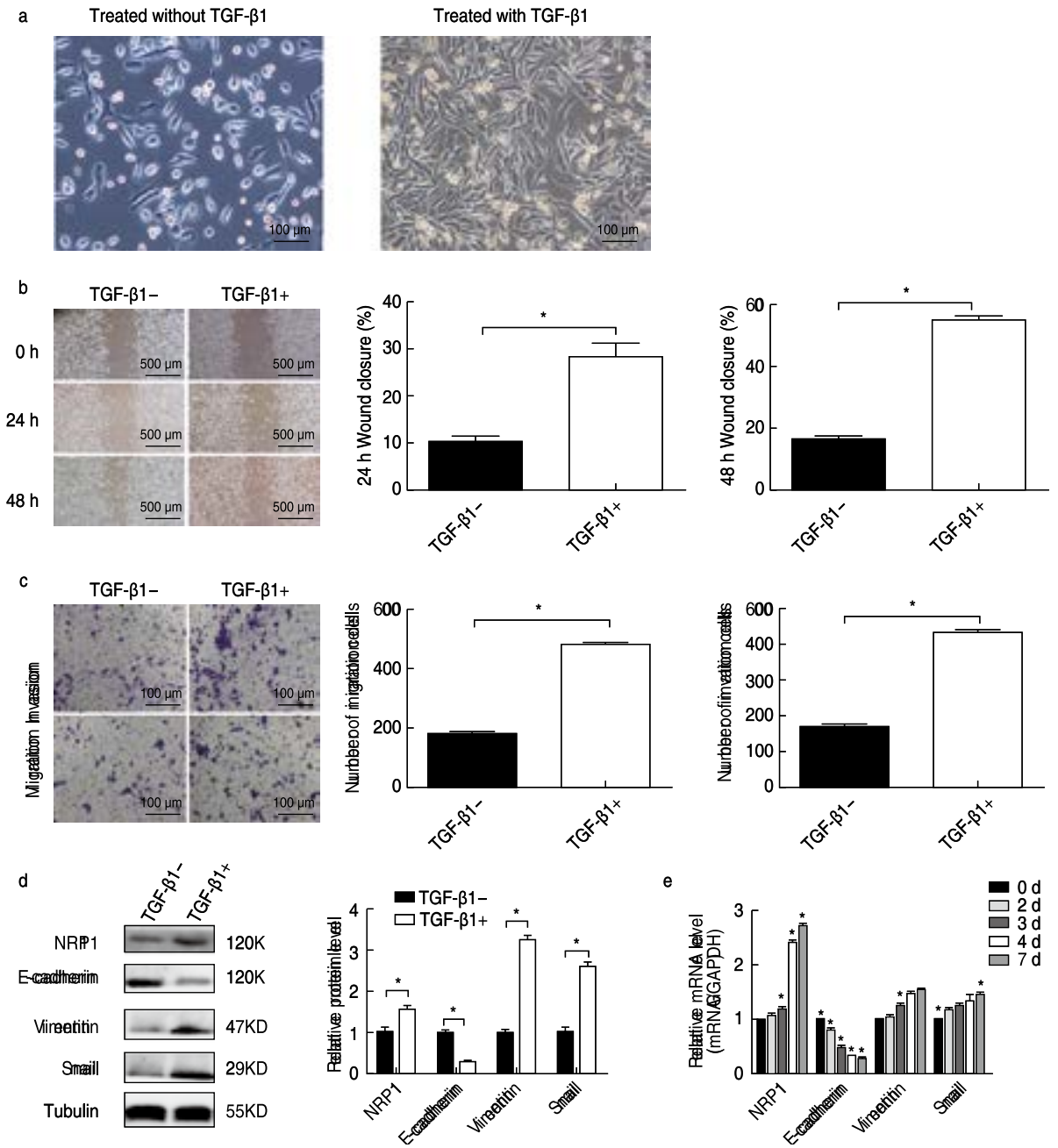

Fig. 1 TGF−β1 induces EMT in HGC-27 cells. (a) Morphological changes. At 200× magnification, scale bar = 100 μm; (b) The results of the wound healing assay. At 40 × magnification, scale bar = 500 μm; (c) The results of the transwell assay. At 100× magnification, scale bar = 100 μm; (d) Western blot analysis; (e) qRT-PCR analysis. The cells were treated with 5 ng/mL TGF−β1 for 0 to 7 d. Data were presented as the mean ± SD; $^{*}P < 0.05$ vs TGF−β1 negative group

Silencing NRP1 diminishes the ability of invasion and migration of HGC-27 via inhibition of TGF-β1-induced EMT

To examine the potential effect of NRP1 on TGF-β1-induced EMT in HGC-27 cells, experiments were divided into four groups: (1) blank group (without treatment), (2) TGF-β1 + CON group (cells treated with TGF-β1 and transfected with the PBS), (3) TGF-β1 + NC group (cells treated with TGF-β1 and transfected with the negative control siRNA), and (4) TGF-β1 + siNRP1 group (cells treated with TGF-β1 and transfected with NRP1-siRNA). As presented in Fig. 3a and 3b, 24 and 48 h after the scratch, the wound of TGF-β1 + siNRP1group exhibited significant closure, and the number of migratory and

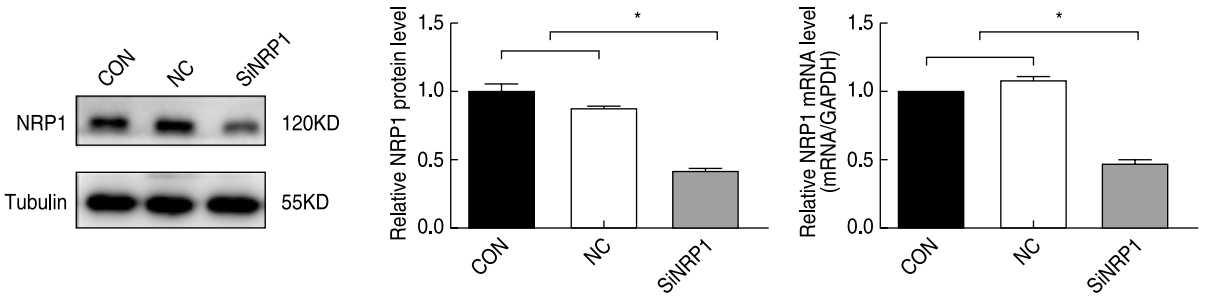

Fig. 2 The NRP1 expression in HGC-27 transfected with siRNA was assessed with Western blotting qRT-PCR. Data were presented as the mean ± SD; $^{*}P < 0.05$

invasive cells in TGF-β1+ siNRP1 group was significantly reduced. These results demonstrated that treatment with TGF-β1 promoted HGC-27 cell invasion and migration, and those effects were greatly weakened after silencing NRP1, which indicates that low expression of NRP1 attenuates TGF-β1-induced cell invasion and migration.

NRP1 contributes to the TGF-β1-induced EMT in GC cells

HGC-27 cells were first treated with 5 ng/mL of TGF-β1 for 96 h to initiate EMT and further treated with siNRP1. Western blot results showed that in the TGF-β1 + siNRP1 group, E-cadherin level was increased to a great extent, the expression of Snail and Vimentin decreased ($P < 0.05$); conversely, no changes were observed in protein expression levels between the TGF-β1 + CON and TGF-β1 + NC groups ($P \geq 0.05$). Therefore, TGF-β1-induced EMT was reversed by silencing NRP1 in HCG-27 cells (Fig. 3c). qRT-PCR detected mRNA expression alterations similar to the western blot trend (Fig. 3d). The loss of NRP1 may efficiently reverse EMT produced by TGF-β1. The outcome expressed that the initiation of EMT markers produced by TGF-β1 was reduced later in epithelial cells by NRP silencing, demonstrating that the NRP1 signaling pathways could be liable for TGF-β1-negotiated EMT. Western blot and qRT-PCR were utilized to identify protein and mRNA expression alterations of NRP1 in the TGF-β1/NRP1 signaling pathway during EMT, rspectively. Results indicated that on exposure with 5 ng/mL TGF-β1, the protein expression of NRP1 steadily reduced, which was considerably lower in TGF-β1+SiNRP1group ($P < 0.05$), and no major change in these protein expression were seen between the TGF-β1+NC and TGF-β1+ CON groups ($P \geq 0.05$) (Fig. 3c and Fig. 3d). Silencing NRP1 may reverse the EMT process by regulating the TGF-β1/NRP1 signaling pathway, indicating that NRP1 acted downstream of the TGF-β1 pathway.

Discussion

Without activating oncogenes, EMT may struggle for a subsistence benefit for cancer cells [27]. Cancer cell invasion, migration, resistance to apoptosis, therapy resistance, and metastasis involves EMT [28–32]. In distant sites of metastasis, the mesenchymal cells coalesce and repolarize through a reverse process known as "the mesenchymal to epithelial transition (MET) form secondary epithelial cells" [33]. Thus, through EMT at the primary site and with MET at secondary sites, the neoplastic epithelial cells gain (through evolution) the ability to invade surrounding tissues and, therefore, can spread to further locations [34]. Interestingly, CD44 (high)/CD24 (low) cells, which are purified from normal and malignant breast cancer tissues, show features of EMT and exhibit stem cell-like properties along with increased metastatic potential [35]. Since the mesenchymal-type cancer cells are more resistant to chemotherapeutic agents than the epithelial-type cancer cells [36–37], the status of the EMT characteristics must, therefore, be reversed to overcome drug resistance, which in turn could lead to the sensitization of drug-resistant cancer cells to conventional chemotherapeutic agents. There is increasing evidence of an association between the acquired resistance to standard chemotherapeutic agents and EMT in gastrointestinal malignancies [38]. In this study, the treatment of TGF-β1 caused EMT in HGC-27 cells, further transfection with siRNA-NRP1 led to upregulation of E-cadherin and downregulation of mesenchymal markers at dissimilar stages, as tested by western blot analysis and qRT-PCR (Fig. 3c and Fig. 3d). On approachable epithelial cell types, EMT-inducing signals can disturb intercellular bond complexes and cause the intermediate loss of apical-basal polarity [39]. Embryos require multiple steps under EMT and MET during complete gastrulation and primitive streak creation, which highlights the reversibility of this procedure [40].

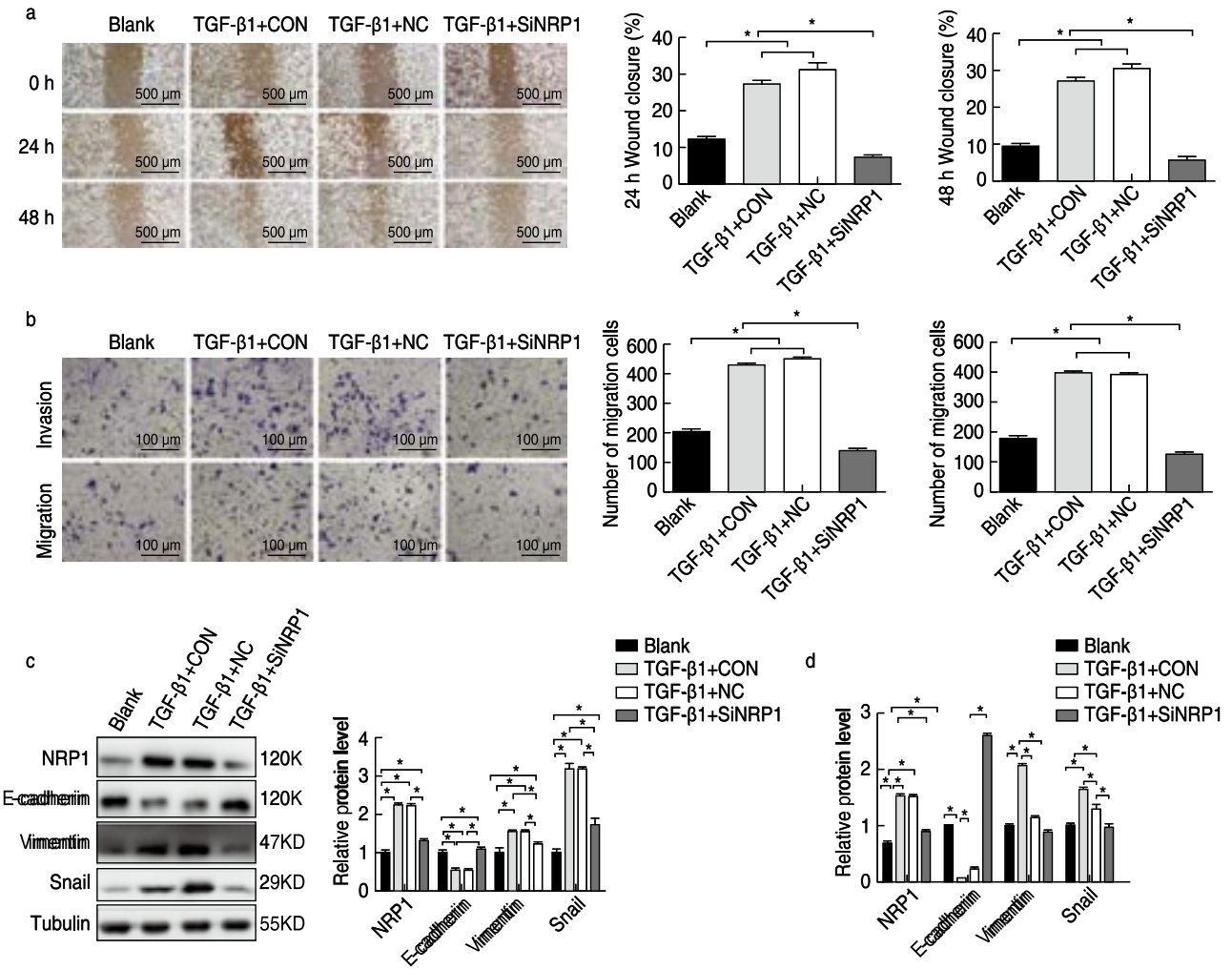

Fig. 3 The potential effect of knockdown NRP1 on TGF−β1-induced EMT in HGC-27 cells. (a) The results of the wound healing assay. At 40× magnification, scale bar = 500 μm; (b) The results of transwell assay, At 100 × magnification, scale bar = 100 μm; (c) Western blot analysis; (d) qRT-PCR analysis. Data were presented as the mean ± SD; $^{*}P < 0.05$

NRP1 is a multifunctional protein that is essential for the development of both neural and vasculature systems. [41]. In most cases, NRP1 acts with a co-receptor and, at times, a multi-protein complex of VEGF and TGF-β, which results in a series of diverse biological roles that encompass angiogenesis, carcinoma, and immunity [41–42]. Thus, NRP1 acts as a receptor hub present on the cell surface that promotes multiple signaling cascades [43–44]. In some studies conducted, the NRP1-binding peptides or knockdown of NRP1 by siRNA inhibited cancer cell growth and increased the sensitivity of cells to chemotherapeutic agents (e.g., 5-FU, paclitaxel, and cisplatin) [45]. Furthermore, the NRP1 may be a valuable target for therapy in glioblastoma, melanoma, and some in forms of leukemia [46–48].

Since TGF-β1 promotes metastasis, this is extremely relevant to cancer biology [23]. Wu *et al* [49] using a meta-analysis of the patients suffering from GC, reported a TGFβ-associated supermodule of stroma-related genes that are associated with diffuse-type histology and poor prognosis in patients with GC. Drugs that inhibit TGF-β1 signaling prevent EMT and block metastases in murine models [50]. TGF-β plays an important role in EMT through regulating the expression of multiple genes and pathways, as recently reviewed by Fuxe *et al* [51]. Upcoming studies, authenticating special NRP1-interfering molecules for this determination that is valid *in vivo* under preclinical models must be conducted. While NRP1 attenuates EMT via TGF-β1 pathway, the inhibition of NRP1 may contribute to a TGF-β1-independent EMT reversal.

Our outcomes recommend that NRP1 guides TGF-β1 constitutive signaling activation and endures a probable

role of NRP1 and TGF-β1 under the EMT. To demonstrate our hypothesis that NRP1 persuades the constitutive activation of the TGF-β1 pathway, we studied the impact of NRP1 on TGF-β1 signaling. In this study, our data revealed that knockdown NRP1 overpowers the EMT and TGF-β1 signaling pathway in GC cells by targeting many markers and proteins in the process. In future, we need *in vivo* experiments to confirm that TGF-β1 promotes the growth of HGC-27 cells and that this effect was weakened on silencing NRP-1 expression. Resultantly, silencing NRP-1 may attenuate TGF-β1-generated EMT in HGC-27 cells, indicating the prominence of the contribution of NRP-1 as a potential marker for GC therapy.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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