Original Paper

VEGF-A/Neuropilin 1 Pathway Confers Cancer Stemness via Activating Wnt/ β -Catenin Axis in Breast Cancer Cells

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Key Words

Nrp-1 VEGF-A Wnt/ β -catenin CSCs breast cancer doxorubicin

Abstract

Background/Aims: Targeting cancer stem cells (CSCs) is emerging as a promising method for cancer treatment. We previously indicated that knockdown of Neuropilin 1(NRP-1) could inhibit breast cancer cell proliferation. Here, we continue exploring the roles and mechanisms of VEGF-A/NRP-1 axis in breast CSCs formation. **Methods:** qRT-PCR was used to detect the levels of VEGF-A and NRP-1 in breast cancer sphere cells and wild-type cells. Mammospheres formation, flow cytometry, soft agar colony and tumor formation assays were performed to evaluate the effects of VEGF-A/NRP-1 on breast cancer stemness. Further HUVECs tube formation, cell invasion and chemotaxis were carried out to detect the effects of VEGF-A/NRP-1 on breast cancer sphere induced angiogenesis. Finally, Annexin V/PI apoptosis and CCK8 assays were used to detect the effects of VEGF-A/NRP-1 on chemoresistance. **Results:** Overexpression of VEGF-A and NRP-1 conferred CSCs-related traits in MCF-7 cells, while knockdown of VEGF-A or NRP-1 reduced CSCs-related traits in MDA-MB-231 cells *in vitro* and *in vivo*. Notably, VEGF-A and NRP-1 acted in a NRP-1 dependent way. Mechanistically, the VEGF-A/NRP-1 axis conferred CSCs-related traits via activating Wnt/ β -catenin pathway. **Conclusion:** our results suggest that VEGF-A/NRP-1 axis could confer CSCs-related traits and chemoresistance.

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Introduction

Cancer stem cells (CSCs) represent a subset of tumor cells that hold the capacity of self-renew, and induce tumor generation and recurrence [1]. Although many cancer “stemness” markers have been identified, the CSCs-targeting therapies have not been established [2]. Thus, it is urgent to find and target the novel stemness modulators of breast cancer which is a major threat to females worldwide.

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Neuropilin 1 (NRP-1) is expressed in various tumors, such as pancreatic, lung, prostate, ovarian and gastrointestinal cancers [3]. NRP-1 is an angiogenic co-receptor of VEGF-A, and involved in epithelial-to-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma [4]. Additionally, VEGF-A could enhance epidermal cancer stem cell survival, and the formation of aggressive and highly vascularized tumors via NRP-1 [5, 6]. VEGF-A/NRP-1 axis could facilitate breast cancer progression via facilitating EMT [7]. Notably, EMT could enable tumor cells with stemness characteristics [8]. Furthermore, our previous study has indicated that knockdown of NRP-1 could inhibit cell proliferation, and promote cell apoptosis in breast cancer cells [9]. Importantly, further study has shown that NRP-1 expression is increased in MDA-MB-231 cells (higher stemness) relative to the MCF-7 cells (lower stemness) [10]. However, the roles and related mechanisms of VEGF-A/NRP-1 axis in breast cancer stemness and chemoresistance have not been revealed. Thus, we sought to explore the roles and downstream effectors of VEGF-A/NRP-1 axis in breast cancer stemness.

The Wnt/ β -catenin pathway has been proved to be engaged in many roles of tumors [11]. Activation of Wnt/ β -catenin pathway could induce cell motility, cell invasion [12]. Numerous studies have demonstrated that Wnt/ β -catenin pathway is essential for cancer cells formation in tumors [13]. Recent studies have revealed that VEGF-A/NRP-1 axis promotes glioma stem-like cell viability and tumor growth independent on VEGF-A-induced angiogenesis [14, 15]. However, it is still confusing whether Wnt/ β -catenin pathway is involved in the roles of VEGF-A/NRP-1 axis in tumors. Additionally, CSCs could contribute to chemoresistance [16]. The important mechanism contributing to chemoresistance is the high expression of ABC transporters in CSCs, like p-gp and MRP2 [16]. Moreover, CSCs seem preferentially increase their ability to repair DNA in response to drugs-induced DNA damage [17], again highlighting the potential of prohibiting CSCs formation to block cancer progression.

Here, we characterized the expression of VEGF-A and NRP-1 in breast cancer spheres and cells. Subsequently, we employed overexpression of VEGF-A/NRP-1 in MCF-7 cells and inhibition of VEGF-A/NRP-1 axis in MDA-MB-231 cells to test the effects of VEGF-A/NRP-1 on the CSCs formation of breast cancer cells *in vitro* and *in vivo*. Furthermore, we characterized the expression levels of VEGF-A and NRP-1 in MCF-7 adriamycin resistant cells (MCF-7-adr) and MCF-7 cells, and evaluated the roles of VEGF-A/NRP-1 axis in attenuating adriamycin resistance *in vitro*. Mechanistically, we found that the VEGF-A/NRP-1 axis acted dependent on Wnt/ β -catenin pathway. Our data suggest that the effects of VEGF-A/NRP-1 axis on the CSCs formation and chemoresistance may be through activating Wnt/ β -catenin pathway in breast cancer cells.

Materials and Methods

Cell culture and reagents

Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the China Academia Sinica Cell Resource Center (Shanghai). MCF-7 adriamycin resistant cells (MCF-7-Adr) were purchased from KeyGEN Biotech (Nanjing, China). MCF-7 and MCF-7-Adr cells were cultured in DMEM medium (Gibco), and MDA-MB-231 cells were maintained in L-15 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 80 Units/ml penicillin and 0.08 mg/ml streptomycin at 37°C under humidified atmosphere with 5% CO₂. The resistance of MCF-7-Adr cells were confirmed before using. Human recombinant VEGF-A (ab9571) was purchased from Abcam. The inhibitors of VEGF-A/NRP-1, EG00229 (HY-10799) and Wnt/ β -catenin, Wnt-C59 (HY-15659) were purchased from Medchem Express.

Mammospheres formation assay

The mammospheres were generated by seeding the cells in an ultra-low attachment 24-well plate (Corning) at a density of 500 cells/well. For sphere culture, cells were cultured in the MammoCult medium (Stem Cell Technologies, Vancouver, Canada) supplemented with MammoCult Proliferation Supplements (Stem Cell Technologies, Vancouver, Canada) and grown at 37°C under a 5% humidified CO₂ atmosphere for

10 days. Fresh medium was added every three days. The solid spheres were photographed and the size of spheres was quantified using AxioVision software (Zeiss).

Flow cytometry analysis

The expression of breast cancer stem cell markers was distinctly measured in cells with different treatment. PE (phycoerythrin)-conjugated anti-CD24 (BDBiosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-CD44 antibodies were purchased from BDBiosciences. Staining was performed following the manufacturer's instructions and analyzed with flow cytometer (BD Biosciences) using FACSDiVa software (BD Biosciences) in which 20000 cells were examined and quantified by percentage.

Soft agar colony formation assay

A 24 well plate was coated with a 1:1 ratio of 1% Agarose and 2 × medium (DMEM + MCF-7 or MDA-MB-231 for MDA-MB-231 cells), solidify for 30 min. Top portion was prepared with 0.6% agarose and 2 × medium. Cells with cells were plated at a density of 5000 cells/ml. Pictures were photographed after culturing for 14 days.

HUVEC cell angiogenesis assay

96-well plates were coated with 50 µl of 10 mg/ml solution of BD Matrigel (BD Biosciences) and incubated at 37°C for 30 min, in which 10⁵ HUVEC cells were seeded. In this tube formation assay, medium was supplemented with 0 or 300 µg extracts of breast cancer cells. The plates were then incubated for 18 h at 37°C and microscopic images were collected for analysis of junction, segment and node formation.

Cell invasion assay

Transwell invasion assay was performed by 24-well MILLIPORE Hanging Cell Culture inserts 8 µm PET (MILLIPORE) pre-coated with Matrigel matrix gel (BD Biosciences). The detailed procedure was mentioned in the previous study [18].

Lentivirus plasmids construction

We thanked for RIBOBIO Inc. for constructing NRP-1 overexpression (LV-NRP-1) and knockdown (LV-NRP-1-shRNA) lentivirus vectors which were verified by DNA sequencing. MCF-7 and MDA-MB-231 cells were infected with the virus in the presence of 2 µg/ml Polybrene following the concentration recommended by RIBOBIO Inc. After infection for 48 h, the virus were removed and the infected cells were selected with puromycin (Sigma, 1.5 µg/ml for MCF-7 cells and MDA-MB-231 cells) for 3 weeks. qRT-PCR and western blot assays were performed to confirm the stable expressing of genes in cells.

Annexin V/PI apoptosis assay

Cell apoptosis analysis was performed with Annexin V-FITC/PI double staining (BestBio, China) following the manufacturer's instructions. Briefly, cells infected with LV-NRP-1 or LV-NRP-1-shRNA were treated with doxorubicin. After 24 h, cells were washed with cold PBS, and then resuspended in binding buffer containing Annexin V-FITC and PI for 15 min in the dark at room temperature. Cell apoptosis was detected by the FACS flow cytometry (BD Biosciences).

Cell viability assay

CCK8 assay was employed to detect the effects of NRP-1 knockdown on the sensitivity of doxorubicin (Sigma Biochemical) in MCF-7-Adr cells. MCF-7-Adr cells with LV-NRP-1-shRNA were seeded in 96-well plates at 4.0×10³ cells/well and stimulated (or not) with doxorubicin for 48 h. Then cell viability was evaluated via CCK8 assay following the manufacturer's recommendation.

Quantitative Real-Time PCR (qRT-PCR)

mRNA was extracted using Trizol reagent (Sigma) and cDNA was synthesized using the GoScript™ Reverse Transcription System (Promega, Madison, WI) with random primers according to manufacturer's instructions. All qRT-PCR reactions were performed following the protocols of SYBR mix™ kit (Biomix, China) on an ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). GAPDH was used as an internal reference. The relative gene expression levels were analyzed via 2^{-ΔΔCt} method.

Western blot assay

Cells were lysed and protein was extracted using RIPA lysis buffer (Beyotime, China) according to the manufacturer's recommendation. Protein concentration was determined via Bradford protein assay (Beyotime). Equal amounts of protein (30 µg) were subjected to 10 % SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, Massachusetts), following blocking with 10% non-fat milk for 2 h at room temperature. Appropriate primary antibodies were incubated with the membranes overnight at 4°C, and then incubated with IgG-HRP secondary antibody (Bio-Rad, Hercules, California) for 1 h at room temperature. Specific signals were detected using ECL chemiluminescence detection kit (Thermo Scientific). The protein expression level was normalized to β-actin.

Cell line xenograft assay

All experiments involving animals were in accordance with the institutional animal welfare guideline and approved by Institutional Animal Care and Use Committee of Xuzhou Medical University. Six-week-old female nu/nu mice were obtained and maintained in a specific pathologic-free environment. Cancer cells or cell subpopulations suspended in total 100 µl serum-free medium and Matrigel (1:1) were inoculated subcutaneously on the single or both flanks (6 animals per experimental group). In *in vivo* tumorigenic assay, tumor incidence was monitored after cells were inoculated for 30 days.

Immunohistochemistry assay

Immunohistochemical staining procedure was performed using Vectastain ABC HRP-Polymer IHC kit (Maixin Biotech). The immunohistochemistry scores for each antigen of interest were semi-quantitatively evaluated on the basis of staining intensity and distribution.

Statistical analysis

All data were obtained from at least three independent experiments ($n \geq 3$), and were representative images or expressed as the mean $\pm$ SD (standard deviation). Statistical analyses were performed using Student's *t* test except for qRT-PCR. Data from qRT-PCR test were analyzed using one-way analysis of variance (ANOVA). Values were compared for multiple comparisons. The difference in the growth of implanted tumors between groups was detected by repeated ANOVA. $P < 0.05$ or less was considered statistically significant. All statistical analyses were performed using Graphpad Prism5.

Results

VEGF-A and NRP-1 expression is increased in breast cancer sphere cells

As the non-adherent spheres are highly enriched for CSCs [19], MCF-7 and MDA-MB-231 cells were cultured in MammoCult medium supplemented with MammoCult proliferation supplement, and seeded in ultra low attachment plates. Spheroid formation was observed 10 days later (Fig. 1A and 1B). qRT-PCR further confirmed that the expression levels of breast cancer stem markers CD44 and ALDH1 were remarkably upregulated in spheres, demonstrating that spheres were enriched in CSCs (Fig. 1C and 1D). We then evaluated the association between breast CSCs and VEGF-A or NRP-1 expression. As shown in Fig. 1E-G, the expression levels of VEGF-A and NRP-1 were markedly upregulated in spheres than in parental cells, respectively. These results imply that VEGF-A/NRP-1 axis might be involved in breast CSCs formation.

The VEGF-A/NRP-1 axis confers breast cancer cell stemness

The effects of VEGF-A/NRP-1 on CSCs features were assessed with gain- and loss-of-function studies. Previous study has shown that CSCs could enhance tumor angiogenic ability through VEGF-A [20, 21]. When co-cultured with the extracts of parental cells or spheres, tube-formation was enhanced in cells treated with spheres-derived extract compared to that with parental cells (Fig. 2A and 2B), indicating that the extracts derived from spheres are enriched in agents that stimulate angiogenesis. These effects were reduced by treating spheres-derived extracts with anti-VEGF-A, suggesting that VEGF-A is a key pro-angiogenic factor in breast

CSCs. Importantly, the spheres formed large tumors (Fig. 2C and 2D), and vascularization was also increased characterized as the upregulation of CD34 (Fig. 2E and 2F). However, VEGFR1/2 expression were unaffected in spheres-formed tumors, but NRP-1 expression was elevated (Fig. 2G and 2H), these effects suggests that the classic VEGF-A/VEGFR1/2 pathway is not altered in spheres-derived tumors. As the lower stemness of MCF-7 cells and higher stemness of MDA-MB-231 cells, we chose to overexpression NRP-1 in MCF-7 cells and knockdown NRP-1 in MDA-MB-231 cells. qRT-PCR and western blot assays confirmed the stable overexpression or knockdown of NRP-1 in cells (Fig. 3A-D). MDA-MB-231 cells with NRP-1 knockdown or treated with VEGF-A siRNA attenuated cell spheroid formation, but cells treated with VEGFR1- or VEGFR2-siRNA had no impact (Fig. 3E and 3F), while MCF-7 cells with NRP-1 overexpression or treated with recombinant VEGF-A enhanced cell spheroid formation. Fig. 3G results confirmed the knockdown efficiency of siRNAs against VEGF-A, VEGFR1 and VEGFR2 are nearly 100%, 85% and 80%, respectively. Furthermore, the expression levels of stemness marker Oct4 and Nanog were upregulated in MCF-7 cells with NRP-1 overexpression, but downregulated in MDA-MB-231 cells with NRP-1 knockdown (Fig. 4A-C). Notably, flow cytometry analysis showed that overexpression of NRP-1 increased CD44⁺/CD24⁻ subpopulation in MCF-7 cells (Fig. 4D). These results suggest that VEGF-A/NRP-1 axis could be involved in breast cancer cell stemness.

The VEGF-A-NRP-1 interaction is essential for CSCs formation

As we have shown that NRP-1 is essential for VEGF-A activity in breast CSCs formation, we further investigated the inhibition of the VEGF-A/NRP-1 axis on breast CSCs formation. Based on the physical interaction between VEGF-A and NRP-1, we assessed whether this interaction indeed exist in breast CSCs. Fig. 5A results showed that VEGF-A and NRP-1 could co-precipitate with each other in breast CSCs. Next, we used an inhibitor of NRP-1 called EG00229, which could inhibit the VEGF-A-NRP-1 interaction. Firstly, we found that treatment with EG00229 reduced spheroid size and numbers of MDA-MB-231 cells (Fig. 5B, upper panel, and Fig. 5C). The clone formation ability was suppressed in cells treated with EG00229 (Fig. 5B, down panel). Additionally, spheres with EG00229 treatment prohibited breast CSCs invasion ability (Fig. 5D). Furthermore, the effects of EG00229 on tumor formation was examined. EG00229 acted at a dosage-dependent over a wide range

Fig. 1. VEGF-A and NRP-1 expression in breast cancer cells and spheres. (A and B) Cell sphere formation assay was performed to examine the ability of tumor sphere formation in MCF-7 and MDA-MB-231 cells. (C and D) The mRNA levels of CD44 and ALDH1 were measured in breast cancer cell spheres and wide-type cells. (E and F) The mRNA levels of VEGF-A and NRP-1 were detected in breast cancer cell sphere and wide-type cells. (G) The protein levels of VEGF-A and NRP-1 were detected in breast cancer cell sphere and wide-type cells. Data were presented as the mean $\pm$ s.d.; ** $P < 0.01$ vs. control.

Fig. 2. Breast cancer spheres enhance the ability of tumor formation and tumor angiogenesis. (A and B) Co-culturing HUVECs with extracts from breast cancer cells or spheres. Extracts from breast cancer spheres enhanced the formation of HUVECs, which was attenuated by anti-VEGF-A treatment. (C and D) Breast cancer cells and spheres were inoculated into nu/nu mice subcutaneously, the tumor size was measured at 10 days. (E and F) CD34 expression was evaluated in tumors derived from MCF-7 cells or spheres. (G and H) NRP-1, VEGFR1 and VEGFR2 mRNA levels were detected in tumors derived from MCF-7 cells and spheres. VEGFR1 and VEGFR2 were not detected. Data were presented as the mean $\pm$ s.d.; ** $P < 0.01$ vs. control.

concentrations (Fig. 5E). Importantly, EG00229 was injected i.p at 15 mg/kg body weight on alternate days each week. We found that EG00229 substantially reduced tumor growth (Fig. 5F). Furthermore, EG00229 treatment reduced vascularization characterized as a reduction in CD34 staining (Fig. 5G), and the NRP-1 expression was unaffected (Fig. 5H).

The VEGF-A/NRP-1 axis promotes CSC phenotype via activating Wnt/ β -catenin pathway

Previous studies have shown that Wnt/ β -catenin pathway was engaged in CSCs formation [11, 13]. To reveal the molecular mechanisms of VEGF-A/NRP-1 axis in breast CSCs formation, the relative expressions of Wnt3a and β -catenin were detected by qRT-PCR and western blot analyses. As shown in Fig. 6A-C, MCF-7 with NRP-1 overexpression increased the expressions of Wnt3a and β -catenin, while knockdown of NRP-1 exerted an opposite effects in MDA-MB-231 cells. Furthermore, the inhibitor of Wnt/ β -catenin pathway, Wnt-C59 was

Fig. 3. VEGF-A/NRP-1 promotes tumor spheres formation. (A and B) The infection efficiency of LV-NRP-1 was examined in MCF-7 cells by qRT-PCR and western blot assays. (C and D) The infection efficiency of LV-NRP-1-shRNA was detected in MDA-MB-231 cells by qRT-PCR and western blot analyses. (E-G) The tumor spheres formation ability of breast cancer cells were evaluated with NRP-1 overexpression or knockdown. Specifically, knockdown of VEGFR1 or VEGFR2 could not affect tumor spheres formation ability in MDA-MB-231 cells. (G) The knockdown efficiency of VEGFR1 and VEGFR2 was examined in MDA-MB-231 cells. Data were presented as the mean $\pm$ s.d.; ** $P < 0.01$ vs. Control.

Fig. 4. NRP-1 increases expressions of stemness markers and sub-population of CD44⁺/CD24⁻ cells. (A-C) Overexpression or knockdown of NRP-1 increased Oct4 and Nanog, or decreased Oct4 and Nanog expressions in MCF-7 and MDA-MB-231 cells, respectively. (D) Overexpression of NRP-1 increased the sub-population of CD44⁺/CD24⁻ cells in MCF-7 cells. Data were presented as the mean $\pm$ s.d.; ** $P < 0.01$ vs. Control.

As expected, Wnt-C59 significantly inhibited Wnt3a and β -catenin expressions in MCF-7 and MDA-MB-231 spheres (Fig. 6D). We found that Wnt-C59 attenuated the tube formation induced by spheres-derived extract (Fig. 6E). Additionally, Wnt-C59 suppressed the invasion of breast CSCs (Fig. 6F). Furthermore, Wnt-C59 attenuated the NRP-1 overexpression-induced breast CSCs formation characterized as the decreased spheroid formation and the percentage of CD44⁺/CD24⁻ cells (Fig. 6G - I). Thus, our results indicate that VEGF-A/NRP-1 axis promotes CSCs phenotype via activating Wnt/ β -catenin pathway.

Knockdown of NRP-1 inhibits breast cancer progression in vivo

Data presented so far indicate that the raised level of NRP-1 is instrumental for several phenotypic traits associated with the behavior of prospective CSC populations *in vitro*. *In vivo* CSCs could be defined by their ability to seed new tumors at limited dilutions, we further determined whether NRP-1 could affect the tumor-initiation capacity. As shown in

Fig. 5. The VEGF-A-NRP-1 interaction is essential for CSCs formation. (A) Evidence for NRP-1/ VEGF-A interaction. Extracts were prepared from breast cancer cell spheres-derived CSCs in lysis buffer and 200 μ g of extract was immunoprecipitated with anti-NRP-1 or anti-VEGF-A followed by immunoblot to detect the indicated epitopes. Total extract (25 μ g) was electrophoresed as a control. (B and C) The inhibitor of NRP-1 called EG00229 decreased sphere and clone formation of MDA-MB-231 cells. (D) EG00229 decreased the spheres invasion ability of MDA-MB-231 cells. (E and F) EG00229 suppresses tumor formation and vascularization. Breast CSCs were enriched by growth factor 10% conditioned medium, and 10^5 cells were inoculated into nu/nu mice in each front flank. The mice were treated with various doses of EG00229 for 4 week (E) or 0 or 15 mg EG00229/kg body weight for various doses (F) on three times per week by IP injection. Tumor formation was monitored using calipers and tumor was photographed on week 4 at the time of harvest. n = 6. (G and H) Tumor sections were stained for CD31 (vascularization marker) (G) and NRP-1 (H) expressions. Data were presented as the mean $\pm$ s.d.; **p < 0.01 vs. Control.

Fig. 7A, palpable tumor mass developed at a higher frequency in 10^5 NRP-1-overexpressing MCF-7 cells. Spheres from only NRP-1 overexpressed cells formed tumors when as few as 10^4 cells were injected. Additionally, the frequency of tumor formation was decreased in MDA-MB-231 cells with NRP-1 knockdown. Importantly, NRP-1-overexpressing tumors increased blood vessel formation as indicated by CD34 staining, while knockdown of NRP-1 exerted an opposite effect (Fig. 7B).

Overexpression of NRP-1 reduces sensitivity of doxorubicin in breast cancer cells

It is well known that CSCs seem able to preferentially resist drugs-induced DNA damage [22]. We continued exploring whether NRP-1 is involved in doxorubicin sensitivity via conferring CSCs formation in breast cancer cells. Firstly, we found that NRP-1 expression was significantly higher in MCF-7-Adr cells than MCF-7 cells (Fig. 8A). Notably, MCF-7-Adr cells formed larger sphere and exhibited higher percentage of CD44⁺/CD24⁻ cells than MCF-7 cells, (Fig. 8B - D). The doxorubicin-induced pro-apoptotic effect was attenuated by NRP-1 overexpression (Fig. 8E and 8F). Doxorubicin-induced upregulation of cleaved-caspase3 and downregulation of Bcl-2 were reduced by NRP-1 overexpression (Fig. 8G). Additionally, NRP-1 knockdown weakened doxorubicin resistance in MCF-7-Adr cells (Fig. 8H - J). Furthermore, the inhibitor of Wnt/ β -catenin pathway, Wnt-C59 attenuated NRP-1-mediated doxorubicin resistance (Fig. 8H). Importantly, knockdown of NRP-1 reduced the percentage of CD44⁺/CD24⁻ cells in MCF-7Adr cells (Fig. 8K). Therefore, our results demonstrate that NRP-1 could confer doxorubicin resistance in MCF-7 cells.

Fig. 6. The VEGF-A/NRP-1 axis promotes CSC phenotype via activation of Wnt/β-catenin pathway. (A-C) The expression levels of Wnt3a and β-catenin were detected in MCF-7 cells with NRP-1 overexpression or not, and MDA-MB-231 cells with NRP-1 knockdown or not via RT-PCR and Western blot analyses. (D) The protein levels of Wnt3a and β-catenin were examined in MCF-7 spheres and MDA-MB-231 spheres with Wnt-C59 treatment or not via Western blot analysis. (E and F) The inhibitor of Wnt/β-catenin, Wnt-C59 inhibited spheres-induced tube formation (E) and CSCs invasion (F) via tube formation and cell invasion assays. (G-I) Wnt-C59 attenuated NRP-1 overexpression-induced sphere formation (G and H) and sub-population of CD44⁺/CD24⁻ cells (I) in MCF-7 cells via mammosphere formation and flow cytometry analysis. Data were presented as the mean ± s.d.; **P<0.01 vs. Control.

Fig. 7. Knockdown of NRP-1 decreases breast cancer progression in vivo. (A) Dissociated MCF-7 and MDA-MB-231 xenograft cells were injected into the fat pad of nude mice at indicated numbers. After 4 weeks, xenografts were collected for incidence analysis. (B) Tumor sections were prepared from tumors derived from MCF-7 cells with NRP-1 overexpression or not, and MDA-MB-231 cells with NRP-1 knockdown or not, and stained to detect CD34 and NRP-1 expressions.

		Control		LV-NRP-1	
		1×10 ⁶		1×10 ⁵	
		1×10 ⁴		1×10 ³	
		Control		LV-NRP-1-shRNA	
MDA-MB-231	1×10 ⁶	6/6	6/6	4/6	4/6
	1×10 ⁵	5/6	5/6	3/6	3/6
	1×10 ⁴	4/6	4/6	1/6	1/6
B	MCF-7	CD34		NRP-1	
		Control	LV-NRP-1-shRNA	Control	LV-NRP-shRNA
	MDA-MB-231	Control	LV-NRP-1	Control	LV-NRP-1
		Control	LV-NRP-1	Control	LV-NRP-1

Discussion

Breast cancer is the most frequent cancer in women [23]. Although endocrine therapy, targeting therapy and chemotherapeutics have greatly improved the overall survival of

Fig. 8. Overexpression of NRP-1 reduces sensitivity of doxorubicin in breast cancer cells. (A) The mRNA level of NRP-1 was detected in MCF-7 and MCF-7-Adr cells. (B) The sub-population of CD44⁺/CD24⁻ cells was evaluated in MCF-7 and MCF-7-Adr cells. (C) Sphere formation ability was examined in MCF-7 and MCF-7-Adr cells. (D) Sphere formation ability was examined in MCF-7 and MCF-7-Adr cells. (E - G) Overexpression of NRP-1 attenuated doxorubicin-induced apoptosis characterized as decreased apoptotic rate (E and F) and increased cleaved-caspase-3, and increased Bcl-2 expression (G). (H and J) Knockdown of NRP-1 enhanced doxorubicin sensitivity in MCF-7-Adr cells characterized as decreased cell viability (H) and increased cell apoptosis (I and J). (K) Knockdown of NRP-1 with doxorubicin treatment decreased the sub-population of CD44⁺/CD24⁻ cells in MCF-7-Adr cells. Data were presented as the mean $\pm$ s.d.; **P<0.01 vs. control.

breast cancer cells. The drug resistance is the main clinical problem [24]. Recent studies have demonstrated that CSCs are the major factor contributing to drug resistance because of their relatively quiescent or dormant state [25]. Since many chemotherapeutic drugs prefer to kill target cells that grow fast, the relatively dormant CSCs would be more resistant to chemotherapy. Thus, it is important to reveal the mechanisms contributing to breast CSCs formation or development.

Although NRP-1 could regulate tumor progression [27, 28], our study uncovered a novel role and signaling cascade mediated by NRP-1 in breast cancer stemness. Importantly, this work comprehensively links NRP-1 to both upstream regulator (VEGF-A) and downstream effectors (Wnt/ β -catenin), and highlights the importance of VEGF-A/NRP-1 axis dynamics and related Wnt/ β -catenin activity in regulating breast cancer stemness, demonstrating that NRP-1 is central to VEGF-A functions in breast cancer progression. Thus our work suggests that NRP-1 is a potential novel target for breast cancer therapeutics.

Here, we found that breast CSCs-derived tumors were hypervascularized compared with tumors formed by non-stem cancer cells. Since angiogenesis contributes to an aggressive tumor, the reasons why breast CSCs facilitate angiogenesis more effectively than non-stem cancer cells should be further detected. Additionally, our results indicated that more VEGF-A was produced by breast CSCs-derived tumors, which is consistent with the role of VEGF-A in facilitating CSCs phenotype. We further confirmed that treatment with anti-

VEGF-A attenuated breast cancer cells spheroid formation and reduced breast CSCs invasion. Moreover, extracts from breast CSCs enhanced HUVECs tube formation more efficiently than extracts from non-stem cancer cells, again highlighting the driving role of VEGF-A in tumor formation. Therefore, our results demonstrate that VEGF-A directly interacts with breast CSCs to maintain and facilitate breast CSCs phenotype. Importantly, the classical functions of VEGF-A are via its interaction with VEGFR2 to trigger downstream signaling. However, such signaling is inactivated in breast CSCs, as the cells lack both VEGFR1 and VEGFR2 expressions. Therefore, VEGF-A exerts its functions not via the classical signaling pathway. As NRP-1 overexpression promotes a more malignant phenotype, our results demonstrate that VEGF-A acted through NRP-1 in breast CSCs formation and breast CSCs-induced tube formation. Notably, our results showed that Wnt/ β -catenin pathway is the downstream effector of VEGF-A/NRP-1. As VEGF-A acts via NRP-1 to enhance epidermal cancer stem cell survival and formation of aggressive and highly vascularized tumors, future studies should focus on the universality of VEGF-A/NRP-1 axis in other tumors.

Thus, our study proposes that VEGF-A-NRP-1 interaction promotes breast CSCs formation, which does not depend on VEGF-A receptors. This novel signaling could provide theoretical basis for using VEGF-A/NRP-1 inhibitor, such as VEGFR2 inhibitor, in clinical experiments.

Disclosure Statement

The authors declare that they have no conflicts of interests.

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