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NRP-1 interacts with GIPC1 and $\alpha 6/\beta 4$ -integrins to increase YAP1/ Np63 α -dependent epidermal cancer stem cell survival

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Abstract

We have identified an epidermal cancer stem (ECS) cell population that drives formation of rapidly growing and highly invasive and vascularized tumors. VEGF-A and neuropilin-1 (NRP-1) are highly expressed in ECS cell tumors and VEGF-A/NRP-1 interaction is required for ECS cell survival and tumor vascularization. We now identify a novel signaling cascade that is triggered by VEGF-A/NRP-1. We show that NRP-1 forms a complex with GIPC1 and $\alpha 6/\beta 4$ -integrin to activate FAK/Src signaling, which leads to stabilization of a YAP1/ Np63 α to enhance ECS cell survival, invasion, and angiogenesis. Loss of NRP-1, GIPC1, $\alpha 6/\beta 4$ -integrins, YAP1, or Np63 α reduces these responses. Moreover, restoration of constitutively active YAP1 or Np63 α in NRP-1 null cells restores the ECS cell phenotype. Tumor xenograft experiments show that NRP-1 knockout ECS cells form small tumors characterized by reduced vascularization as compared to wild-type cells. The NRP-1 knockout tumors display signaling changes consistent with a role for the proposed signaling cascade. These studies suggest that VEGF-A interacts with NRP-1 and GIPC1 to regulate $\alpha 6/\beta 4$ -integrin, FAK, Src, PI3K/PDK1, LATS1 signaling to increase YAP1/ Np63 α accumulation to drive ECS cell survival, angiogenesis, and tumor formation.

Introduction

Non-melanoma skin cancer, which develops in response to exposure to ultraviolet light, chemicals, and chronic wounding [1], is among the most commonly diagnosed cancers [1–3]. Squamous cell carcinoma, which comprises 16% of these cancers [3], is an aggressive cancer that has a high risk of metastasis [3]. Expansion of these tumors requires

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

vascularization [4], which requires vascular endothelial growth factor (VEGF) and a host of other proteins to stimulate blood vessel formation [5, 6]. VEGF receptors (VEGFRs) are present in keratinocytes [4, 7–11] and knockout mouse studies indicate a role for VEGF in tumor formation [12, 13]. VEGF also has a role in cancer stem cell survival [7, 14–16] and regulates the stem cell niche [4, 17, 18]. Blocking VEGF signaling reduces ESC cell function and number [7]. We described a subpopulation of highly aggressive stem cell marker-expressing cells (0.15% of cells per tumor) in epidermal squamous cell carcinoma [19]. These cells grow as spheroids in non-attached conditions, display enhanced invasion and migration, undergo epithelial to mesenchymal transition [20–22], and form highly vascularized and aggressive tumors following injection of as few as 100 cells in immunocompromised mice [19].

VEGF-A is required for maintenance of this phenotype, as knockdown of VEGF-A expression or treatment with VEGF-A-inactivating antibody reduces these responses and tumor formation [23]. This study also demonstrated that ECS cell lack VEGFR1 and VEGFR2 and that VEGF-A acts to stimulate ECS cell survival via the neuropilin-1 (NRP-1) co-receptor [23]. Yoshida et al. [24] identified a similar role for NRP-1 in DJM-1 skin cancer cells. These studies show that VEGF-A acts via interaction with NRP-1 to trigger intracellular events leading to formation of aggressive, invasive, and highly vascularized skin tumors. In addition, we recently demonstrated that a novel $\alpha 6/\beta 4$ -integrin, FAK, Src, PI3K, PDK1 (3-phosphoinositide dependent protein kinase-1, PDPK1), Hippo signaling cascade regulates YAP1/ Np63 α to enhance ECS cell survival, spheroid formation, invasion, and migration [20].

The mechanism whereby VEGF-A/NRP-1 regulates downstream signaling to enhance ECS cell survival is not well understood. In the present study we test the hypothesis that NRP-1 interacts with GIPC1 (GAIP-interacting protein) to stimulate the $\alpha 6/\beta 4$ -integrin/YAP1/ Np63 α signaling cascade and that NRP-1 stimulation of this cascade is required to maintain ECS cell survival, spheroid formation, invasion, migration, and tumor formation.

Results

NRP-1 knockdown impacts YAP1/ Np63 α signaling

We have shown that VEGF-A and NRP-1 are highly expressed in ECS cell tumors and that VEGF-A interacts with NRP-1 to stimulate ECS cell-associated tumor vascularization and growth [23]. However, the downstream signaling events in this process are not well understood. In the present study, we identify proteins that interact with NRP-1 to mediate VEGF-A/NRP-1 signaling and also identify downstream signaling events. To initiate these studies, we created NRP-1 knockdown cells and monitored the impact of NRP-1 loss on the ECS cell phenotype, which is characterized by enhanced spheroid formation and matrigel invasion [20, 21]. We monitored spheroid formation as a measure of cancer stem cell status. Figure 1a, b confirms NRP-1 knockdown and shows that NRP-1 loss reduces spheroid size. Figure 1c, d shows that NRP-1 loss also reduces spheroid number and matrigel invasion. We also examined the ability of extracts prepared from NRP-1 intact and knockdown cells to stimulate human umbilical vein endothelial cell (HUVEC) tube formation as a measure of angiogenic potential. Figure 1e shows that cell extract from SCC-13-NRP-1-shRNA1 cells

display reduced activity to stimulate HUVEC tube formation as compared to wild-type cell extract. These findings confirm that NRP-1 is involved in maintenance of the ECS cell phenotype and drives angiogenic potential.

A novel $\alpha 6/\beta 4$ -integrin, FAK, Src, PI3K/PDK1, LATS1, YAP1 signaling cascade has been implicated in maintaining ECS cell survival [20]. We therefore tested if NRP-1 can activate events in this cascade. Figure 1f shows that NRP-1 knockdown reduces $\alpha 6$ -integrin level and integrin-regulated FAK and Src signaling, and this is associated with increased LATS1 activity and YAP1 phosphorylation, and reduced YAP1 and Np63 α level. LATS1 kinase is part of the Hippo signaling cascade and increased LATS1 activity is associated with increased YAP1 phosphorylation and degradation [25], and reduced YAP1 activity is associated with reduced ECS cell survival [20]. Figure 1g, h confirm that loss of $\alpha 6/\beta 4$ -integrin or YAP1 reduces spheroid number and size, and Fig. 1i, j shows that loss of Np63 α reduces ECS cell spheroid number and matrigel invasion. Figure 1k confirms the siRNA-dependent knockdown of $\alpha 6/\beta 4$ -integrin, YAP1, and Np63 α .

YAP1 and Np63 α are required for NRP-1 action

If $\alpha 6/\beta 4$ -integrin, LATS1, YAP1/ Np63 α signaling is required for NRP-1-dependent signaling, overexpression of key downstream targets, YAP1 and Np63 α , should restore the ECS cell phenotype in NRP-1 knockdown cells. Figure 2a shows that NRP-1 loss reduces YAP1 level and increases YAP1-*P*, responses which are consistent with reduced YAP1 function. This is also associated with loss of Np63 α . It is of interest that overexpression of YAP (S127A) (constitutively active YAP1) or Np63 α reduces YAP1-*P* and partially restores YAP1 level. Figure 2b–d shows that expression of YAP(S127A) or Np63 α in NRP-1 knockdown cells restores spheroid formation, matrigel invasion, and migration, suggesting that YAP1 and Np63 α are NRP-1 downstream targets. In addition to effects on these aspects of ECS cell function, NRP-1 also influences angiogenesis [26]. We therefore measured the ability of extracts derived from control-, YAP1-, or Np63 α -deficient ECS cells to stimulate tube formation in a HUVEC angiogenesis assay. Figure 2e shows that cell extracts prepared from YAP1 or Np63 α knockdown ECS cells are less able to stimulate HUVEC tube formation. In addition, expression of YAP1 or Np63 α in NRP-1 knockdown cells restores cell extract stimulation of tube formation (Fig. 2f). Thus, YAP1 and Np63 α are NRP-1 downstream targets that are required for NRP-1 regulation of ECS cell spheroid formation, invasion, migration, and vessel formation.

Role of the GIPC1 PDZ domain protein

We next asked how NRP-1 is linked to the $\alpha 6/\beta 4$ -integrin, LATS1, YAP1/ Np63 α signaling cascade [20]. GIPC1 is a PDZ domain scaffold protein involved in receptor trafficking and stabilization [27] that has been reported to interact with NRP-1 [24, 27–29]. We therefore assessed the role of GIPC1 in ECS cells. A comparison of monolayer (non-stem cancer cells) versus spheroid (ECS cells) reveals a marked increase in GIPC1 level. NRP-1 level, in contrast, was not increased (Fig. 3a). We next monitored for GIPC1 interaction with NRP-1. Figure 3b shows that NRP-1 and GIPC1 co-immunoprecipitate. In addition, GIPC1 has been reported to interact with alpha integrins [30, 31] suggesting that it may interact with $\alpha 6$ -integrin in ECS cells. Indeed, Fig. 3c shows that immunoprecipitation of $\alpha 6$ -integrin results

in co-precipitation of NRP-1 and GIPC1, suggesting the presence of a NRP-1/GIPC1/ α 6-integrin regulatory complex. These findings suggest that GIPC1 is part of the NRP-1-triggered signaling cascade that regulates YAP1/ Np63 α function to enhance ECS cell survival and ECS cell-dependent angiogenesis. This model predicts that GIPC1 is required for NRP-1 maintenance of the ECS cell phenotype. To assess this, we treated ECS cells with GIPC1 siRNA and monitored the effect on ECS cell spheroid formation and matrigel invasion. Figure 3d, e shows that GIPC1 knockdown reduces spheroid formation and matrigel invasion.

A regulatory role for GIPC1 is supported by biochemical data showing that GIPC1 knockdown reduces β 4-integrin level and FAK/Src activity, increases LATS1 activity, reduces YAP1/increases YAP1-*P*, and reduces Np63 α level (Fig. 3f), findings that mirror those observed following NRP-1 knockdown (Fig. 1f). Thus, these findings suggest that a NRP-1/GIPC1/ α 6/ β 4-integrin complex drives downstream changes in FAK, Src, LATS1, YAP1, and Np63 α to maintain the ECS cell phenotype.

NRP-1 and GIPC1 signaling in HaCaT cells

To determine whether NRP-1 and GIPC1 signaling is essential for ECS cell survival in other epidermis-derived cell lines, we studied HaCaT cells [32]. Figure 4a, b shows that NRP-1 knockdown reduces spheroid number and size, and also cell migration. As shown in Fig. 4c, NRP-1 knockdown is associated with reduced expression of α 6-integrin, reduced FAK/Src signaling, increased LATS1-*P* and YAP1-*P*, and reduced YAP1 and Np63 α level. Figure 4d, e shows that forced expression of YAP(S127A) or Np63 α restores spheroid formation and invasion in NRP-1 knockdown HaCaT cells. Figure 4f confirms the presence of overexpressed YAP(S127A) and Np63 α .

We next assessed the role of GIPC1 in HaCaT cells. Figure 4g shows that GIPC1 level is elevated in HaCaT-derived ECS cells and Fig. 4h, i shows that GIPC1 knockdown reduces spheroid formation and matrigel invasion. Figure 4j shows that NRP-1 and GIPC1 co-precipitate with α 6-integrin, suggesting the presence of a complex, including these three proteins. These findings are consistent with those observed for SCC-13 cell-derived ECS cells.

Role of NRP-1 in tumor formation

Since NRP-1 could be a therapy target in squamous cell carcinoma, we determined if compromising NRP-1 function reduces tumor formation. We have previously shown that treatment with EG00229, an agent that inhibits VEGF-A/NRP-1 interaction, reduces tumor formation [23]. However, NRP-1 knockout cells are far superior for testing the role of NRP-1 in the context of the present experiment and so we created NRP-1 knockout cells using CRISPR/Cas9 (Fig. 5a). We injected wild-type and NRP-1 null cells into NSG immune-compromised mice and determined that tumor formation is markedly reduced for NRP-1 knockout as compared to wild-type cells (Fig. 5b). In addition, consistent with a role for NRP-1 in vascularization, visual examination suggests that NRP-1 knockout tumors are less vascularized (Fig. 5c). To quantitatively assess the impact of NRP-1 loss on vascularization, we monitored CD31 as a vascularization marker. Figure 5d, e shows that

CD31 staining is reduced in tumors and that this is associated with reduced CD31 protein level by immunostaining and immunoblot of tumor extract. A key issue is the status of the $\alpha 6/\beta 4$ -integrin/YAP1/ Np63 α signaling cascade. Tumor extracts were prepared for assay of proteins in this cascade. Figure 5f shows that NRP-1 loss is associated with reduced FAK and Src activity, increased LATS1 activity, and increased YAP1 phosphorylation, and that these changes are associated with reduced levels of YAP1 and Np63 α .

However, we were surprised to observe NRP-1 in extracts from NRP-1 knockout cell-derived tumors. This could be contamination from surrounding cell types or NRP-1 re-expression in the knockout tumor cells. We therefore cultured cells from NRP-1 wild-type and NRP-1 knockout tumors and monitored NRP-1 level. We compared cells isolated from four tumors in each group. Figure 5g shows that cells from the knockout tumors lack NRP-1 expression, thereby confirming the knockout status of the tumor cells. To monitor biological status, we isolated cells from wild-type and NRP-1 knockout tumors and assessed the ability to form spheroids. Figure 5h, i shows that NRP-1 knockout cells form smaller spheroids as compared to the NRP-1 intact cells. These findings suggest that NRP-1 loss, in cultured cells and tumors, produces similar signaling changes and biological and biochemical outcomes.

Discussion

We recently characterized cells derived from squamous cell carcinoma that display properties of ECS cells [19]. These cells are enriched for expression of epidermal and embryonic stem cell markers, and injection of as few as 100 ECS cells in immune-compromised mice yields large, rapidly growing, and invasive tumors [19]. We also observed that ECS cell-derived tumors are hypervascularized compared to tumors formed by non-stem cancer cells [19, 33]. Because of the important positive correlation between tumor vascularization and tumor growth, we examined the mechanism of vascularization in greater detail.

Role of VEGF-A and NRP-1 in ECS cells

VEGF is an important regulator of angiogenesis [5, 15]. Cancer cells produce VEGF-A, which produces autocrine and paracrine effects on cancer cells and on the surrounding endothelial cells [34]. Our studies show that VEGF-A production is elevated in ECS cells and ECS cell-derived tumors and that it stimulates the ECS cell phenotype and tumor vascularization [23]. Moreover, treatment of mice with bevacizumab, which sequesters and prevents VEGF-A action, reduces ECS cell xenograft tumor vascularization and growth [23]. The classical mechanism of VEGF-A action is interaction with VEGFRs; however, the striking absence of VEGFR1 and VEGFR2 in ECS cells [23] suggested an alternate mechanism of VEGF-A action. Our finding of a lack of VEGFR in epidermis-derived cancer cells agrees with a previous report [24], although others have identified VEGFR as being present [10].

NRP-1 is a 922-amino-acid protein that includes a large (860-amino-acid) extracellular glycoprotein domain, a 22-amino-acid transmembrane segment, and a 40-amino-acid intracellular region; however, NRP-1 does not have intrinsic enzymatic activity. Instead, it has been reported that NRP-1 binds VEGF-A to facilitate its interaction with VEGFR

leading to more efficient stimulation of angiogenesis [35–37]. However, in a limited number of systems, NRP-1 mediates VEGF-A action independent of VEGFRs [23, 24, 38–41]. For example, pancreatic carcinoma cells express VEGF and NRP-1, but not VEGFRs, and VEGF action requires NRP-1 [38, 41]. VEGF also acts via NRP-1 to stimulate malignant progression in renal cell carcinoma [26, 42]. Our studies in ECS cells demonstrate that VEGF-A interacts with NRP-1 and that this interaction is required for cell survival, spheroid formation, invasion, and migration [23].

However, because NRP-1 lacks kinase activity, less is known about how it activates intracellular signaling. One possible mediator is GIPC1 [27]. GIPC1 contains an N-terminal dimerization domain and a centrally located PDZ domain. PDZ domains interact with other proteins that harbor a PDZ-binding domain to facilitate assembly of protein complexes [27]. Studies in some models suggest that NRP-1 interacts with GIPC1 [24, 27–29]. Our present study provides evidence for assembly of an NRP-1/GIPC1 complex in ECS cells. We further show that $\alpha 6$ -integrin is part of this complex. The finding of integrin/GIPC1 interaction is consistent with existing reports describing GIPC1 association with integrins, including $\alpha 6$ -integrin [28, 30, 31]. Our studies suggest that GIPC1 mediates NRP-1 action by facilitating VEGF-A/NRP-1 interaction with $\alpha 6/\beta 4$ -integrins (Fig. 5j).

Identifying downstream NRP-1-controlled signaling events

We have previously shown that $\alpha 6/\beta 4$ -integrin drives a signaling pathway that is required for ECS cell survival and tumor formation [20]. Triggering this cascade activates $\alpha 6/\beta 4$ -integrin-dependent FAK and Src signaling, which activates PI3K/PDK1 signaling. PDK1 then binds to and inhibits LATS1 kinase activity leading to accumulation of nuclear YAP1, which stabilizes Np63 α [20]. YAP1 and Np63 α then drive enhanced ECS cell survival and tumor formation. The present study suggests that NRP-1 is linked to this $\alpha 6/\beta 4$ -integrin driven cascade by GIPC1. In NRP-1 competent cells, FAK and Src activity are high leading to reduced LATS1 and YAP1 phosphorylation, and this is associated with increased levels of YAP1 and Np63 α [20]. In contrast, knockdown of NRP-1 reduces activity in this cascade leading to increased levels of YAP1-*P* and reduced level of YAP1 and Np63 α , and this response is phenocopied by GIPC1 knockdown. In addition, the reduction in spheroid formation, invasion and migration in NRP-1 knockdown cells is reversed by expression of

Np63 α or constitutively active YAP1. These studies suggest that the VEGF-A/NRP-1/ $\alpha 6/\beta 4$ -integrin/GIPC1 complex triggers downstream changes in Hippo signaling to drive

Np63 α -dependent ECS cell survival (Fig. 5j). Previous interesting studies suggest that NRP-1/GIPC1 can activate Rho [24] and RAS/ERK [29] to enhance cancer cell survival. Moreover, VEGF-A can activate YAP1 [43, 44] and this involves src activation in squamous cell carcinoma [45], but our study is the first to indicate that FAK/Src, PI3K/PDK1, LATS1, YAP1, and Np63 α are downstream targets of NRP-1/GIPC1 signaling and that GIPC1 couples NRP-1/ $\alpha 6/\beta 4$ -integrin to the downstream signaling events. In this context, Np63 α is a particularly interesting protein target, as it is a key regulatory of stem cell and cancer stem cell status in epidermis [46].

Impact of NRP-1 on tumor formation

We also examined the role of NRP-1 in tumor formation. Tumor xenograft studies show that NRP-1 knockout cells form markedly smaller tumors that have reduced vascularity as evidenced by reduced levels of CD31. A key issue is the impact of NRP-1 knockout on activity of the integrin/YAP1/ Np63 α signaling cascade in tumors. This analysis shows that NRP-1 knockdown is associated with a reduced FAK and Src activity, increased LATS1 activity, increased YAP1 phosphorylation, and reduced levels of YAP1 and Np63 α . Thus, the signaling changes observed in NRP-1 knockout tumors mimic the signaling changes observed in NRP-1-deficient ECS cell spheroids in culture, suggesting that this regulation is operational in tumors in vivo. A subtle feature of this pathway is that NRP-1 and GIPC1 can differentially regulate α 6 versus β 4-integrin level depending upon cancer cell environment (compare Figs. 1f, 3f, 4c, and 5f). We suspect that this is due to differences in the cancer cell signaling environment.

NRP-1/YAP1/ Np63 α signaling pathway

Based on the findings presented in this manuscript, we propose that VEGF-A binds to NRP-1 and that this complex is coupled to α 6/ β 4-integrin by a GIPC1 homodimer as indicated in Fig. 5j. This complex is held together because the PDZ-binding domains of NRP-1 and α 6-integrin interact with the PDZ domain of individual GIPC1 monomers of the homodimer. This interaction activates integrin signaling (FAK and Src), which activates PI3K/PDK1. PDK1 then binds to and inhibits LATS1 kinase, which leads to accumulation of non-phosphorylated YAP1, which interacts with and stabilizes Np63 α to drive ECS cell survival, spheroid formation, invasion, migration, and tumor formation [20]. Transglutaminase 2 is also a key activator of this pathway that binds to β 4-integrin [20]. Further studies will be required to fully elucidate the in vivo role of these signaling relationships; however, recent studies demonstrate a key role for VEGF-A, NRP-1, and Np63 α in human squamous cell carcinoma tumors [47–51].

Materials and methods

Chemicals and reagents

Trypsin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Frederick, MD). β -actin antibody (A5441) was obtained from Sigma (St. Louis, MO, USA). Antibodies to NRP-1 (ab81321), Src (ab47411), CD31 (ab28364), and Src-*P* (ab321012) were obtained from Abcam (Cambridge, MA, USA). Antibodies to LATS1 (9153), LATS1-*P*(9157), FAK-*P*(3283), YAP1 (4912), and YAP1-*P*(13008) were obtained from Cell Signaling Technologies (Danvers, MA, USA). Antibodies to GIPC1 (sc-9648), β 4-integrin (sc-9090), α 6-integrin (sc-374057), and p63 (sc-8431) were obtained from Santa Cruz (Dallas, TX). FAK (610087) antibody was purchased from BD Transduction Laboratories. Horseradish peroxidase-conjugated sheep anti-mouse IgG (NXA931), donkey anti-goat IgG (sc-2033), and donkey anti-rabbit IgG (NA934V) were obtained from GE Healthcare (Buckinghamshire, UK) and used at a 1:5000 dilution. Matrigel (354234) and BD Biocoat cell inserts (353097) were from BD Biosciences (Valencia, CA). The constitutively active YAP1(S127A) mutant plasmid was kindly provided by John Lamar and Richard Hynes [52]. The Np63 α plasmids were from Dr. Berry Trink. The Np63 α insert

was cloned into pENTR/D-TOTO and then combined with pLenti-CMV-Puro-DEST plus LR clonase to produce pLenti-CMV- Np63 α -FLAG-Puro expression vector (ThermoFisher, Waltham, MA). Immunoblot analysis was performed as previously described [23].

Cell culture

SCC-13 and HaCaT cells were originally obtained from American Type Culture Collection [32, 53]. Monolayer cultures of SCC-13 squamous cell carcinoma cells, and cells derived from this line, were maintained in a DMEM containing 5% fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate and appropriate antibiotics [19]. HaCaT cells were maintained in the same medium. ECS cell spheroids were grown by plating 40 000 cells/well in ultra-low attachment six-well cluster dishes and growing for 0–10 days in spheroid medium [DMEM/F12 (1:1) (DMT-10–090-CV, Mediatech Inc., Manassa, VA) containing 2% B27 serum-free supplement (17504–044, Invitrogen), 20 ng/ml epidermal growth factor (E4269, Sigma, St. Louis), 0.4% bovine serum albumin (B4287, Sigma), and 4 μ g/ml insulin (#19278, Sigma Chemical, St. Louis, MO) [19].

Electroporation

For electroporation, 1 million cells were double electroporated with siRNA (3 μ g) using the VPD-1002 nucleofection kit and Amaxa electroporator (Morristown, New Jersey) [54]. After 48 h, the cells were used for experiments. NRP-1 (sc-36038), α 6-integrin (sc-43129), β 4-integrin (sc-35678), and p63 (sc-36161) siRNA were from Santa Cruz. Control (D-001206–13-05) and GIPC1 (M-019997–02-005) siRNAs were obtained from Dharmacon (Lafayette, CO). YAP1 siRNA (S102662954) was from Qiagen (Valencia, CA).

Invasion and migration assays

Matrigel (BD Biosciences) was diluted in 0.01 M Tris-HCl containing 0.7% NaCl, filter sterilized, and 0.15 ml was added per BioCoat cell insert (353097, 8 μ m pore size, BD Biosciences, San Jose, CA) well and permitted to solidify. Cells were seeded at 20 000/well in 100 μ l of growth media containing 1% FCS. The lower chamber contained growth medium containing 10% FCS. After 24 h at 37 °C, residuals cells were removed from the top and the membrane was rinsed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 10 min, and stained with 1 μ g/ml 4',6-diamidino-2-phenylindole for 10 min. Cell number per $\times$ 10 magnification field was recorded using an inverted fluorescent microscope. The migration (wound closure) assay was as previously described [55].

HUVEC angiogenesis assay

HUVEC assays were performed as previously described [23]. To prepare extract, cells were sonicated in EBM Endothelial Basal Medium (CC-3121, Lonza, Walkersville, MD) supplemented with protease inhibitor cocktail (Millipore/Sigma, 539134). We routinely use cell extracts in the HUVEC assay, instead of conditioned medium. This permits comparison, as activity/mg protein, to tumor cell extracts.

Knockdown and knockout cell lines

NRP-1 shRNA-encoding lentivirus was produced using HEK-293T cells maintained in DMEM containing 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate. The cells were harvested and plated in 100 mm dishes at 50% confluence and then washed and transferred to serum-free medium. The HEK-293T cells were transfected with 1 µg pCMV-VSVG, 5 µg pCMV-dr8.91, and 5 µg of pLKO.1-NRP-1-shRNA vector (SHCLNG-NM_003873) using Fugene 6 (Promega, WI) and plated. The MISSION System Vectors were obtained from Sigma. At 3 h, 10% FCS was added, and at 72 h the virus-containing medium was collected, centrifuged for 15 min at 1500 rpm, sterile filtered (22 micron), and stored as -80 °C in aliquots. SCC-13 cells (4×10^6) were treated with 1 ml of virus-containing medium in serum-free growth media containing 8 µg/ml polybrene at 37 °C for 5 h. The media was then changed to growth media containing 5% FCS. Cells were plated in 100 mm dishes and grown in the presence of 0.25 µg/ml puromycin for 2 weeks. NRP-1 knockdown was confirmed by anti-NRP-1 immunoblot. These cells are referred to as SCC-13-NRP-1-shRNA1. A parallel line was generated using pLKO.1-Puro-NT-shRNA (SHC016-1EA, Sigma) using the protocol described above and called SCC-13-Control-shRNA. SCC-13-NRP-1-KOc8 are NRP-1 knockout cells created using CRISPR/Cas9 vectors purchased from Biocytogen (Worcester, MA).

Tumor growth assay

ECS cells, selected by growth as spheroids, were prepared as single-cell suspensions and injected subcutaneously into each front flank in NOD/*scid*/IL2 receptor gamma chain knockout mice (NSG mice; five mice/treatment group) [19]. Statistics were performed using the *t*-test. Tumor experiments were repeated three separate times. The studies were approved by the University of Maryland-Baltimore Animal Care and Use Committee and meet all national and international standards.

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Fig. 1.

NRP-1 is required for ECS cell survival. **a–c** Control and NRP-1 knockdown SCC-13 cells were seeded and grown as spheroids in ultra-low attachment plates. Spheroid diameter and number were measured at 5 days and extracts were prepared to confirm NRP-1 knockdown. **d** Control or SCC-13-NRP-1-shRNA1 ECS cells were plated at 25 000 cells/transwell chamber atop matrigel, and cell migration to the lower chamber was monitored at 24 h. **e** Cell lysates were prepared from 5-day spheroids and 300 μg protein was tested for ability to enhanced HUVEC tube formation. The plot shows the number of junctions, segments, and nodes measured using ImageJ analysis [23]. Bars = 100 μm. **f** Spheroids were grown for 5 days and extracts were prepared for immunoblot detection of the indicated proteins. **g–k** Cells were electroporated with the indicated siRNA and spheroid formation and size were monitored at 5 days, and matrigel invasion at 24 h. Immunoblots were prepared from 5-day spheroid cultures to confirm knockdown of the siRNA targets

Fig. 2.

YAP1 and Np63α are required for NRP-1 action. **a, b** YAP1 and Np63α can restore the cancer stem cell phenotype in NRP-1 knockdown cells. SCC-13-control-shRNA and SCC-13-NRP-1-shRNA1 cells were electroporated with 3 μg of the indicated plasmids and plated for spheroid formation. Spheroid numbers were determined at 5 days and extracts were prepared to monitor the levels of the indicated proteins. EV empty vector. **c, d** Cells were electroporated with the indicated plasmids, and seeded for 24 h invasion assay and 0–18 h migration assay. Bars = 100 μm. The wound was completely closed on all treatment groups at 18 h, except for the NRP-1-shRNA1 + EV group, which had an open 120 ± 20 μm wound measured in 10 fields. Similar results were observed in each of three separate experiments. **e, f** Cells were electroporated with siRNA or plasmid as indicated and grown as spheroids for 5 days. ECS cell lysates, derived from 5-day spheroids, were prepared and 300 μg was used in a HUVEC tube formation angiogenesis assay. All graphical values are mean

$\pm$ SEM and the asterisks indicate a significant change compared to control, $n = 3$, $p < 0.005$. Bars = 100 μm . The number of junctions, segments, and nodes was assessed using ImageJ [23]

Fig. 3.

GIPC1 is required for NRP-1 coupling to downstream signaling events. **a** GIPC1 is elevated in ECS cells. Extracts were prepared from SCC-13 non-stem cancer cells (monolayer) and ECS cells (5-day spheroids) for detection of GIPC1. **b, c** NRP-1/α6-integrin/GIPC1 interaction. Extracts were prepared from SCC-13-derived ECS cells and 200 μg of extract was immunoprecipitated with anti-GIPC1 or anti-α6-Integrin followed by immunoblot to detect the indicated epitopes. **d, e** SCC-13 cells were treated with control or GIPC1 siRNA and then assayed for ability to form spheroids and migrate through matrigel. **f** SCC-13 cells were electroporated with the indicated siRNA and grown as spheroids for 5 days before extracts were prepared for immunoblot detection of the indicated proteins

Fig. 4.

Role of NRP-1 and GIPC1 in HaCaT cells. **a, b** HaCaT cells were electroporated with 3 μg control- or NRP-1-siRNA and tested for spheroid formation and migration potential. **c** HaCaT cells were electroporated with 3 μg of siRNA as indicated and grown as spheroids for 3 days before extracts were prepared for immunoblot. **d–f** HaCaT cells were electroporated with 3 μg of siRNA and/or 3 μg of expression plasmid as indicated (EV empty vector) and spheroid formation and matrigel invasion was measured. Expression of YAP (S127A) and Np63α was monitored by immunoblot at 24 h post electroporation. **g**

Extracts were prepared from monolayer and spheroid grown HaCaT cells, and GIPC1 level was monitored by immunoblot. **h, i** ECS cells were electroporated with control or GIPC1 siRNA and then plated to monitor spheroid formation and matrigel invasion. **j** Extracts prepared from HaCaT cell spheroids were immunoprecipitated with anti-integrin- $\alpha 6$ followed by immunoblot detection of GIPC1, NRP-1, and integrin- $\alpha 6$

Fig. 5.

NRP-1 is required for optimal tumor formation. **a** Immunoblot of spheroid-derived ECS cells confirms NRP-1 knockout in SCC-13-NRP-1-KOc8 cells. **b–e** Spheroid-derived SCC-13 and SCC-13-NRP-1-KOc8 cells were injected (100 000 per each front flank) into NSG mice and tumor formation was monitored from 0 to 4 weeks, and tumors were harvested at 4 weeks, photographed, and assayed for CD31 by immunostain and immunoblot. **f** Tumors were harvested at 4 weeks and assayed for cell signaling proteins by immunoblot. Similar results were observed in each of three experiments. **g–i** NRP-1 knockout cells retain properties after tumor growth. SCC-13 and SCC-13-NRP-1-KOc8 tumors were harvested at 4 weeks. The tumor cells were isolated and cultured, and NRP-1 level and spheroid formation were monitored. Bars = 100 μ m. **j** Proposed VEGF-A/NRP-1/

GIPC1 signal transduction pathway. The events in this cascade are described in the Discussion

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