Neuropilin-1–Dependent Regulation of EGF-Receptor Signaling

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Abstract

Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2) form a small family of conserved and widely expressed transmembrane proteins, originally implicated in the regulation of axon guidance and vascular development (1, 2). The extracellular portion of neuropilins mediates protein–protein interactions; in particular, “a” and “b” domains are known to interact with secreted class 3 semaphorins and VEGFs (3, 4), whereas the “c” (MAM) domain mediates neuropilin homo- and heterodimerization (5; see Fig. 1A). The role of the short cytoplasmic tail of neuropilins is poorly understood, and its relevance is still controversial (6–8).

Beside embryonal development, neuropilins are widely distributed in the adult tissues, and their levels are often significantly increased in cancer cells and tumor biopsies of various origin, compared with normal counterparts [(9, 10); reviewed in ref. (11)]. In addition, high levels of Nrp1 were significantly associated with poor outcome in patients with colon cancer (9), breast cancer (12), and non–small lung cancer (13), and correlated with invasive behavior and metastatic potential in gastrointestinal carcinoma, glioma, and prostate carcinoma (11). Notably, EGF stimulation has been found to induce Nrp1 expression in tumor cells (9, 14, 15). Moreover, Nrp1 expression was upregulated in epithelial cells upon neoplastic transformation driven by constitutive activation of the Ras pathway (16). Nrp1 overexpression in advanced tumors may suggest a link with the acquisition of a functional advantage at the cellular level. Yet, experimental data on the role of Nrp1 in cancer cells are contradictory, and the implicated molecular mechanisms have not been elucidated. For example, knocking down Nrp1 expression in carcinoma cells inhibits proliferation, cell survival, and extracellular matrix invasion in vitro (13, 17); consistently, other studies indicated that Nrp1 overexpression can inhibit cancer cell apoptosis (18, 19). These effects have been often explained by the role of Nrp1 in supporting VEGF signaling (17, 19, 20). In contrast to these findings, in other studies, an elevated expression of Nrp1 was associated with more favorable prognosis of patients with colon cancer (21) and reduced tumor growth in experimental models in mice (22); moreover, a VEGF/Nrp1–dependent pathway suppressing cell viability has been proposed in 1 specific case (23). These discrepancies are currently unresolved and they might reflect cell-specific responses and/or the involvement of different signaling pathways.

Antibodies and short peptides interfering with Nrp1 function have been shown to inhibit tumor angiogenesis and tumor growth in vivo in mice (24–26); these data have validated Nrp1 as a significant target for antiangiogenic and antitumor agents. Intriguingly, by applying a computational model, it was predicted that a more potent VEGF inhibition may be achieved by using an anti-Nrp1 antibody that does not block ligand binding, but rather interferes with Nrp1 oligomerization (27). This is consistent with previous evidence that deleting the “c”/MAM oligomerization domain impairs neuropilin function (5). Moreover, a synthetic peptide derived from the transmembrane ...
segment of Nrp1 prevents Nrp1 dimerization and oligomerization and blocks glioma growth in mice (28).

In this study, we identified a novel molecular mechanism to account for the acquisition of selective advantage coupled with Nrp1 overexpression in cancer cells. We found that Nrp1 is in complex with EGFR on the cell surface, where it mediates ligand-induced EGFR clustering and endocytosis, leading to intracellular activation of AKT signaling cascade. Notably, upon Nrp1 depletion, EGFR signaling is significantly affected in cancer cells. Our work identifies a new function of Nrp1 in association with a growth factor receptor, and envisages a putative role of Nrp1 in sustaining EGFR activation in a large fraction of human tumors.

Materials and Methods

Materials and Methods have also been described in Supplementary Material.

Cell lines

American Type Culture Collection provided tested and authenticated cell lines used in our study, which were passaged in our laboratory for fewer than 6 months after resuscitation. Cells were grown in standard medium supplemented with 1% L-glutamine (2 mmol/L) and 10% FBS (Sigma).

Gene expression knockdown by RNA-interference

Neuropilin expression was silenced by transfecting the following targeted siRNA sequences (chemically synthesized) with Lipofectamine 2000 (Invitrogen) or by using the Amaxa Nucleofector Kit T (program X-01, Lonza); #1: agatgcagcttagcttcaa; #2: aacacctagtggagtgata; #3: CAATCACGTGCAGGCTCAA (where not specified, siRNA #2 was used). To achieve stable gene knockdown for long-term experiments, we transduced cells with lentiviral constructs expressing targeted short hairpin RNA (shRNA). The sequence targeting Nrp1 was previously published (17). Control shRNAs (shC) were generated by introducing 4 base substitutions in Nrp1 targeting sequence (GATAGGTCTATGACTCCCA). These shRNA sequences were inserted in the lentiviral transfer plasmid pCCLsin.PPFPshPGK.GFP.Wpre under control of the H1 promoter, as previously reported (29). We similarly designed shRNA-expressing lentiviral constructs targeting the transcript of Nrp2.

Figure 1. Nrp-1 expression levels regulate tumor cell growth and viability. A, schematic drawing depicting Nrp1 structure and the conserved domains comprised in the different constructs analyzed in this study (including point mutations in Nrp1_3mut). B, the viability of multiple different Nrp1-depleted tumor cells (expression data shown in Supplementary Fig. S1A) was assessed by MTT assay (see Supplementary Methods). Values shown are the mean ± SD of 3 independent experiments carried out in triplicate, normalized to controls for each cell line; *, P < 0.01; **, P < 0.001; ***, P < 0.0001. C, the growth of control-EV, Nrp1-depleted (shNrp1), and Nrp1-overexpressing A549 carcinoma cells was compared in cell proliferation assays (see Supplementary Methods). Plotted values represent the mean ± SD of 3 independent experiments carried out in triplicate; *, P < 0.01; **, P < 0.001; ***, P < 0.0001. D, wild-type or point-mutated Nrp1 unable to bind VEGF (Nrp1_3mut; see A and Supplementary Fig. S3B) was overexpressed in U87MG tumor cells, and cell growth was assessed in culture (compared with controls) as in C. Data shown are the mean ± SD of 3 independent experiments carried out in triplicate; *, P < 0.01; **, P < 0.001; ***, P < 0.0001. E, U87MG cells engineered as described earlier were transplanted subcutaneously in immunodeficient mice. Tumor growth was assessed by periodical volumetric measurements (left), and tumor weight was eventually measured after excision (right). Values reported in graphs represent the average ± SD of 6 mice per each experimental condition; *, P < 0.01; **, P < 0.001, *** P < 0.0001.
(TTCAAAAGATGCTGCTAT), and EGFR (GCAGTCTTATC- TAACTATGAT; kindly provided by L. Trusolino, IRCC, Cardiolo, Italy). PTEN expression was silenced by using Sigma Mission shRNA-expressing lentiviral vector TRCN0000002749. Control empty vector pLKO was from Sigma.

Results

Nrp-1 expression regulates tumor cell viability and proliferation

We knocked down the expression of either Nrp1 or Nrp2 by RNA interference (RNAi) in multiple different human cancer cells (validation data shown in Supplementary Fig. S1A and S1B). Proliferation and viability of Nrp2-depleted tumor cells were comparable with control cells treated with a nontargeting shRNA (see Supplementary Fig. S1C and S1D). In contrast, Nrp1 knockdown resulted in a significant impairment of viability and proliferation in a variety of cancer cell models (Fig. 1B and Supplementary Fig. S1E). The specificity of this effect was validated by applying 3 distinct siRNA sequences directed against Nrp1 (Supplementary Fig. S1F). Flow cytometry–based detection of the early apoptotic marker Annexin-V revealed a significantly increased number of apoptotic cells following Nrp1 knockdown (Supplementary Fig. S2A); moreover, the prosurvival PI3K-AKT signaling pathway was strikingly attenuated in these cells compared with controls (Supplementary Fig. S2B). Notably, upon forcing the constitutive activation of AKT by means of PTEN silencing we could partly rescue cell viability in Nrp1-depleted cells (Supplementary Fig. S2C and S2D); this implicated AKT signaling in a putative Nrp1-regulated pathway supporting cancer cell survival.

Complementary to gene knockdown experiments, we found that Nrp1 overexpression was sufficient to confer a proliferative advantage to tumor cells and to enhance their viability (Fig. 1C and Supplementary Fig. S3A). Notably, Nrp1 is known to bind VEGF-A, a growth factor especially active in endothelial cells via the tyrosine kinase receptor KDR/VEGF-R2. Although VEGF-R2 is not expressed in most cancer cells (including those used in our experiments), a previous report proposed that VEGF binding to Nrp1 could promote tumor cell survival via poorly defined KDR-independent pathways (19). We have therefore generated and expressed in cancer cells a mutated Nrp1 construct unable to bind VEGF- A_166 (Nrp1_3mut, see Fig. 1A and Supplementary Fig. S3B). In analogy to the wild-type counterpart, Nrp1_3mut promoted cancer cell growth and viability (Fig. 1D and Supplementary Fig. S3C), indicating that VEGF-binding is not implicated in this function. Moreover, cancer cells overexpressing either wild-type Nrp1 or its Nrp1-3mut variant formed larger tumors in mice compared with controls (Fig. 1E). Unlike what reported in a previous study (20), Nrp1-overexpressing tumors (either wt or mutant forms) were not characterized by increased vessel density (Supplementary Fig. S3D), indicating that Nrp1 can sustain cancer cell growth in vivo independently of tumor angiogenesis.

Notably, the cytoplasmic domain of Nrp1 is very small, and its functional relevance in cancer cells is unclear. To experimentally address this issue, we overexpressed in tumor cells a truncated secretable form of Nrp1, lacking both transmembrane and cytoplasmic domains (Nrp1ec; Fig. 2A). The isolated extracellular domain of Nrp1 was sufficient to promote tumor cell proliferation and viability (Fig. 2B and C), consistent with the effects mediated by full-length Nrp1. Moreover, Np1ec expression was sufficient to rescue the effect of Nrp1 depletion in cancer cells (Fig. 2D). In keeping with our findings in vitro, we observed a growth advantage of cancer cells overexpressing Nrp1ec transplanted in mice (Supplementary Fig.S4A), and this was not accompanied by an increased tumor vessel density (Supplementary Fig. S4B). Altogether, these data suggested that Nrp1 activity promoting cancer cell survival and proliferation is not due to a signaling cascade elicited by its intracellular tail, but rather implicate a function of the extracellular domain of Nrp1, independent from VEGF binding.

Nrp-1 and EGFR form a signaling complex on the cell surface

Because Nrp1ec expression was sufficient to induce proliferation and rescue cell viability, we asked about the implicated mechanisms. Interestingly, we found that treating cancer cells with affinity-purified Nrp1ec activated AKT and mitogen-activated protein kinase (MAPK) signaling cascades (Fig. 3A and B). These effectors have a crucial role in controlling tumor cell survival and proliferation, and are often activated downstream to receptor tyrosine kinases (RTK) expressed on the cell surface. By applying an unbiased screening approach, we found that the extracellular domain of Nrp1 could elicit the phosphorylation of EGFR receptor (EGFR; Fig. 3C). This was further confirmed by Western blot analysis using specific antibodies directed against the major EGFR auto-phosphorylation site PTyr_1068 (Fig. 3D). Pretreating cells with cetuximab (an EGFR-blocking monoclonal antibody) impaired Nrp1ec-induced MAPK and AKT activation (Fig. 3E), indicating pathway specificity; similar results were obtained by applying the small molecule EGFR inhibitor gefitinib (not shown). These data suggested that the Nrp1-dependent signaling cascade controlling cell growth and survival is largely mediated by EGFR activation. Moreover, upon analyzing tissue sections of Nrp1-overexpressing tumors grown in mice (described in Fig. 1E), we found that EGFR tyrosine phosphorylation was significantly increased compared with controls (Supplementary Fig. S5).

Coimmunoprecipitation experiments indicated that the extracellular domain of Nrp1 interacts with EGFR upon overexpression (Fig. 4A). Furthermore, endogenous Nrp1 and EGFR were basally associated in A549 non–small lung cancer cells, and the complex was induced upon stimulation with either EGF or TGF-α, 2 major EGFR ligands found in tumors (Fig. 4B). By confocal microscopy analysis, we found that endogenous Nrp1 and EGFR largely colocalized on the surface of A549 cells (Supplementary Fig. S6A). Moreover, in response to EGF stimulation, a large fraction of Nrp1 was found in intracellular vesicles colocalizing with EGFR (Supplementary Fig. S6A). On the basis of the colocalization with the early-endosome antigen-1 [EEA1 (30; Supplementary Fig. S6B)], these vesicles were identified as early endosomes, a vesicular compartment that typically traffics internalized EGFR (31).
Nrp1 ectodomain regulates EGFR clustering and endocytosis

To understand the functional relevance of Nrp1 in EGFR signaling, we first checked EGFR levels on the plasma membrane in control and Nrp1-depleted cells by cell surface biotinylation experiments, and found them to be comparable (Supplementary Fig. S7A). Moreover, cell surface incubation with fluorescently labeled EGF revealed that receptor binding was not affected upon Nrp1 knockdown (Supplementary Fig. S7B). Ligand-induced activation of EGFR is thought to elicit receptor oligomerization and clustering on the cell surface, followed by internalization by endocytosis (32, 33). We therefore applied total internal reflection fluorescence (TIRF) microscopy to study EGFR distribution at the cell surface. This revealed that, at the steady state, EGFR-signal was dispersed on the plasma membrane and rarely aggregated in small spots (Fig. 4C). In contrast, upon stimulation with physiologic concentrations of EGF (2 ng/mL) the receptors clustered in many distinct aggregates at the cell surface (Fig. 4C). Importantly, this ligand-induced effect was barely detectable in Nrp1-depleted cells, which behaved similarly to non-stimulated cells (Fig. 4C), strongly suggesting that Nrp1 plays a role in ligand-engaged EGFR clustering on the cell surface.

Consistent results were furthermore obtained assessing EGFR- or TGF-α-induced EGFR clustering by conventional confocal microscopy (Fig. 4D). Notably, the treatment with soluble extracellular domain of Nrp1 (Nrp1ec) promoted EGFR clustering in tumor cells and was sufficient to rescue the functional defect resulting from endogenous Nrp1 knockdown (Fig. 4D). Altogether, these data indicate that the extracellular domain of Nrp1 is required and sufficient to induce EGFR oligomerization and clustering on the cell surface.

Ligand-induced EGFR clustering is followed by its internalization in endocytic vesicles, from where EGFR can sustain prolonged intracellular signals (34–36). Intriguingly, we noticed that ligand-induced EGFR trafficking into EEA1-positive endosomes was impaired in Nrp1-silenced cells, potentially suggesting the requirement of Nrp1 for EGFR endocytosis (Supplementary Fig. S8). To track the internalization of activated EGFR, A549 carcinoma cells were ligated with EGF at +4°C (to put on hold the endocytosis of activated receptors), and then surface biotin labeled either before or after allowing ligand-induced endocytosis to occur (by shifting the cells at 37°C). Whereas in control cells activated EGFR was efficiently internalized and its levels on the cell surface dramatically decreased, in Nrp1-deficient cells ligand-engaged EGFR largely
remained at the cell surface, indicating that it was inefficiently internalized (Fig. 5A). By immunofluorescence experiments, we tracked EGFR trafficking in response to EGF or TGF-α; unlike in control cells, in Nrp1-depleted cells ligated receptors remain on the cell surface and their internalization is impaired (Fig. 5B and Supplementary Fig. S9A and S9C). Further experiments applying fluorescent-labeled EGF to track the internalization of receptor complexes (Fig. 5C) showed the requirement of Nrp1 expression for this function. Notably, Nrp1-deficient cells did not show any defect in the uptake mediated by transferrin receptor (TfR), a cargo receptor that is continuously internalized from the cell surface (Supplementary Fig. S10); this indicated that Nrp1 is not part of the basic molecular machinery mediating receptor endocytosis, but it specifically affects the endocytosis of selected molecules, such as EGFR.

We found that the differential internalization of EGF–EGFR complexes in control and Nrp1-depleted cells was unchanged upon treatment with the catalytic inhibitor erlotinib (selectively blocking EGFR autophosphorylation; Supplementary Fig. S11A), or with the tyrosine phosphatase inhibitor sodium orthovanadate (preventing phosphorylation turnover; Supplementary Fig. S11B), strongly suggesting that Nrp1 can control ligand-induced EGFR internalization even independently from its tyrosine phosphorylation. Thus, to elucidate the functional role of specific Nrp1 domains for EGFR internalization, we reconstituted its expression in silenced cells by ectopically transfecting either nontargetable full-length Nrp1 or 2 different mutants of the intracellular domain, lacking either the C-terminal–SEAcooh sequence (Nrp1_ASEA) or the entire cytoplasmic domain (Nrp1_Accto). Reexpression of full-length Nrp1, as well as of either of the above mutants (shown in Supplementary Fig. S12A), restored ligand-induced EGFR internalization to normal rates, indicating that the cytoplasmic domain of Nrp1 is not implicated for this function.

We then focused our attention on the extracellular domain of Nrp1, based on our data supporting its crucial role in eliciting EGFR clustering on the cell surface. A Nrp1 mutant lacking the extracellular MAM domain, required for receptor oligomerization (ΔMAM; ref. 5; expression shown in Supplementary Fig. S12B), could not rescue EGFR endocytosis (Fig. 5E). Unlike wild-type Nrp1, the monomeric ΔMAM mutant was furthermore unable to reinstall ligand-induced EGFR clustering in Nrp1-depleted cells (Supplementary Fig. S13A), consistent with that Nrp1 oligomerization is required to mediate the clustering of associated EGFR. We then tested a complementarily truncated mutant of Nrp1 including the oligomerization MAM domain only, anchored to the plasma membrane (Nrp1-MAM-TM). This protein strongly associated with endogenous Nrp1 (Supplementary Fig. S13B) but was unable to interact with EGFR (not shown). Notably, not only Nrp1-MAM-TM could not rescue the effect of Nrp1 knockdown, but it even
acted as dominant negative molecule, by blocking the function of endogenous Nrp1 and leading to impaired EGFR clustering on the cell surface (Supplementary Fig. S13C), as well as defective AKT activation (Supplementary Fig. S13D) and reduced tumor cell viability (Supplementary Fig. S13E). Taken together, these data strongly suggest that the oligomerization of the extracellular domain of Nrp1 associated with EGFR is responsible for ligand-induced EGFR clustering on the cell surface and subsequent internalization.

**Nrp1 expression controls ligand-induced EGFR activation and signaling in tumor cells**

In keeping with its impact on EGFR oligomerization, Nrp1 knockdown also inhibited ligand-induced EGFR phosphorylation and the activation of intracellular AKT effector pathway in cancer cells (Fig. 6A and B). Notably, AKT activation in response to the ErbB3/ErbB4-ligand heregulin-β1 or the unrelated growth factor insulin was unaffected by Nrp1 knockdown in the same cells (Fig. 6C). We further assayed the functional effects of Nrp1 knockdown on ligand-induced EGFR clustering on the cell surface (Fig. 6D).
relevance of Nrp1 in EGFR signaling by treating 2 different cancer cell lines with a Nrp1-blocking antibody (3). In treated cells, ligand-induced phosphorylation of both EGFR and the downstream effector AKT was impaired (Fig. 6D). These data suggest that Nrp1 is specifically required for EGFR signaling in cancer cells.

EGFR pathway is frequently activated in human tumors, and pivotaly implicated in sustaining cell proliferation (37). This may be associated with receptor gene amplification and overexpression (38), or ligand overexpression and autocrine signaling in cancer cells (39, 40). Interestingly, upon forced EGFR overexpression and constitutive activation in A549, Nrp1 expression became dispensable for cell viability (Fig. 7A). On the other hand, constitutive EGFR activation sustained by autocrine TGF-α signaling was strikingly dependent on Nrp1 expression in cancer cells (Fig. 7B, left). Consistently, cells carrying TGF-α overexpression displayed a modest but significant increment of cell viability in culture, which was completely lost upon Nrp1 silencing (Fig. 7B, right).

Discussion
Accumulating evidence seems to associate neuropilins expression in cancer cells with tumor progression. In particular,
Nrp1 overexpression is often found in human cancers of different origin, and correlates with tumor aggressiveness and poor clinical outcome. Tumor models, in vivo and in vitro, have been used to try elucidating the functional role of Nrp1 in cancer progression; however, experimental data were partly contradictory and did not identify the implicated molecular mechanisms. The results of the present study indicate that Nrp1 expression plays a pivotal role in cancer cell survival and proliferation. In fact, while knocking down Nrp1 expression severely impaired growth and viability of different cancer cells, its overexpression conversely promoted cell proliferation in vitro and tumor growth in mouse models.

By investigating the molecular mechanisms underlying this activity, we found that the extracellular domain of Nrp1 (Nrp1ec) could elicit the phosphorylation of both AKT and MAPK intracellular effectors and promote cell proliferation, recapitulating the effects of overexpressing the full-length molecule; moreover, Nrp1ec was sufficient to restore viability of cancer cells deprived of the endogenous transmembrane molecule. These results suggested that the small cytoplasmic domain of Nrp1 is not specifically required for the regulation of cancer cell viability and proliferation. Moreover, by experiments in vitro and in vivo, we showed that the VEGF-binding site in the extracellular domain of Nrp1 is not required to mediate these effects, thereby implicating a major alternative signaling pathway.

Initially, we ruled out 2 candidate effectors previously associated with Nrp1: VEGF-R2 and c-Met (data not shown). Then, by applying an unbiased screening for RTK activation in cancer cells, we found out that Nrp1ec selectively triggered the phosphorylation of EGFR (but not other ErbB family members). Notably, Nrp1ec did not interfere with EGF-induced receptor activation, or even slightly improved it in costimulation experiments (data not shown), thus suggesting that Nrp1ec does not engage EGFR ligand-binding site or EGF itself. We then showed the physical association between endogenous Nrp1 and EGFR in cancer cells and found it to be induced by EGF and TGF-α. Moreover, while ligand stimulation induced EGFR clustering in large complexes on the cell surface, this was impaired upon Nrp1 silencing. We found that this process is mediated by the oligomerization (MAM) domain of Nrp1.

Ligand-induced EGFR internalization into endocytic vesicles was furthermore impaired in the absence of Nrp1. Importantly, EGFR internalization was recovered in Nrp1-silenced cells upon reexpression of mutated Nrp1 isoforms lacking the cytoplasmic domain, or by the treatment with Nrp1ec alone. On the other hand, EGFR internalization could not be
recovered upon reexpression of mutated Nrp1 lacking the oligomerization MAM domain, responsible for Nrp1 clustering on the cell surface. It is known from literature that EGFR oligomerization is intimately connected to receptor activation (41). However, whether the kinase activity is required for EGFR internalization is debated (33, 42–43), and it cannot be ruled out that tyrosine phosphorylation and receptor internalization may be partly independent consequences of receptor ligation on the cell surface. This issue started to be mechanistically addressed, and receptor oligomerization per se is emerging as a driving force for EGFR internalization independent of kinase activity (44). In this framework, our study points at Nrp1 as an additional player in the regulation of EGFR trafficking. Indeed, in our experimental model, we found that Nrp1 controls EGFR endocytosis even independently from tyrosine autophosphorylation, by mediating receptor oligomerization and clustering on the cell surface.

It is well known that receptor endocytosis is a major regulatory mechanism controlling receptor signaling in space and time (45). This is particularly important for EGFR, because the signaling cascade elicited by receptor activation is not limited to the plasma membrane, and crucially continues during receptor trafficking through endosomal compartments, especially for mediating AKT activation (36). Because ligand-induced internalization in tumor cells was severely reduced in the absence of Nrp1, EGFR-bound receptors remained at the cell surface, where they are hardly capable to activate AKT (46). This mechanism is potentially implicated in the pathway leading to tumor cell growth and survival mediated by EGF. Indeed, we found that the extracellular domain of Nrp1 is sufficient to promote AKT activation, cancer cell viability and tumor growth in vivo, independent from VEGF binding. Moreover, our data suggest that Nrp1-dependent AKT activation is mediated by EGFR signaling, thereby suggesting a molecular mechanism to account for increased Nrp1 expression in human tumors. Complementary to this, and relevant for cancer treatment, the treatment with a Nrp1-blocking antibody (or knocking down Nrp1 expression by RNAi) abated EGFR auto-phosphorylation and downstream AKT signaling induced by EGF or TGF-α, which sustains tumor cell survival and proliferation.

EGFR is in fact a major driver of progression in several human cancers. Even tumors carrying wild-type nonamplified EGFR have been found to depend on the signaling activity of this proto-oncogene for progression, and they can be successfully targeted with EGFR-blocking drugs (47–49). Intriguingly, our data suggest that Nrp1 function is dispensable in EGFR-overexpressing cancer cells. Instead, we found that constitutive EGFR activation and prosurvival signaling due to an autocrine loop of TGF-α, which is also frequently seen in human tumors, is dependent on Nrp1 expression.

Preclinical trials in mice with 2 different anti-Nrp1 antibodies showed a strong tumor suppressor effect that could not be explained by interference with VEGF binding or VEGFR2 signaling (24), although the implicated molecular mechanisms remained unclear. On the other hand, one anti-Nrp1 MoAb, selected for targeting the VEGF-binding domain to block tumor angiogenesis (MNRIP1685A), was tested in the clinics revealing moderate toxicity. Therefore, our data could prompt future studies focusing on antibodies or therapeutic active...
molecules targeting Nrp1-EGFR interaction in tumor cells, independent of its function as coreceptor for VEGF in endothelium.

In conclusion, Nrp1, thanks to its ability to regulate EGFR activation, internalization and signaling, could represent a new and relevant target for interfering with the EGF/TGF-α-dependent survival and proliferation of cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. Rizzolio, G. Serini, J. Norman, L. Tamagnone
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Rizzolio, N. Rahinowicz, E. Rainero, J. Norman, L. Tamagnone

References
EGFR Regulation by Neuropilin-1
