Neuropilin-1 Stimulates Tumor Growth by Increasing Fibronectin Fibril Assembly in the Tumor Microenvironment

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Abstract

The tumor microenvironment, including stromal myofibroblasts and associated matrix proteins, regulates cancer cell invasion and proliferation. Here, we report that neuropilin-1 (NRP-1) orchestrates communications between myofibroblasts and soluble fibronectin that promote α5β1 integrin–dependent fibronectin fibril assembly, matrix stiffness, and tumor growth. Tumor growth and fibronectin fibril assembly were reduced by genetic depletion or antibody neutralization of NRP-1 from stromal myofibroblasts in vivo. Mechanistically, the increase in fibronectin fibril assembly required glycosylation of serine 612 of the extracellular domain of NRP-1, an intact intracellular NRP-1 SEA domain, and intracellular associations between NRP-1, the scaffold protein GIPC, and the nonreceptor tyrosine kinase c-Ab1 that augmented α5β1 fibronectin fibril assembly activity. Analysis of human cancer specimens established an association between tumoral NRP-1 levels and clinical outcome. Our findings indicate that NRP-1 activates the tumor microenvironment, thereby promoting tumor growth. These results not only identify new molecular mechanisms of fibronectin fibril assembly but also have important implications for therapeutic targeting of the myofibroblast in the tumor microenvironment. Cancer Res; 72(16); 4047–59. ©2012 AACR.

Introduction

Tumor growth is influenced not only by tumor cell phenotype but also by the interaction between tumor cells and their surrounding microenvironment (1). This microenvironment includes endothelial cells, immune cells, and mesenchymal cells such as fibroblasts, the latter, which in turn may activate into myofibroblasts under tumorigenic conditions (2). Tumor myofibroblasts influence cancer cell growth and metastasis through multiple mechanisms that include growth factor production, angiogenesis regulation, and matrix deposition (3). In turn, TGF-β-dependent matrix deposition increases stiffness and tensile strength, which activate mechanosignaling pathways that promote tumor cell proliferation and tumor progression (1, 4). Although important growth factor signaling pathways in the tumor microenvironment have been identified (5), additional molecular switches in myofibroblasts, which convert a dormant tumor microenvironment into an active one that promotes tumor growth and metastasis, are not fully defined.

Tumor matrix consists of a combination of fibrillar and nonfibrillar components, some of which are nonfunction moieties that are later assembled into a meshwork that provides tensile support (4). One of the key molecular components of the tumor matrix is the provisional matrix protein, fibronectin, which is secreted in a TGF-β-dependent manner as a dimer in a soluble form, and can assemble into insoluble fibrils occurs upon interaction with specific integrin proteins (6). These fibrils can bind collagen and promote collagen fiber assembly that increases tensile strength of the tumor. Thus, fibronectin fibril assembly may represent a critical process that is distinct from fibronectin production, by which matrix regulates tumor growth. Although recent studies have helped us to conceptualize the key roles of cell contractility (7) and specific integrins (8), especially α5β1 in the conversion of soluble fibronectin into an insoluble matrix-bound fibronectin, a number of gaps remain in our understanding of this process, especially pertaining to the cellular mechanisms that regulate this process.

Neuropilin-1 (NRP-1) is an axonal guidance molecule, which was subsequently revealed to play important roles in vascular wall cells as well (9). For example, recent studies have revealed that NRP-1 in pericyte-derived myofibroblasts contributes to cirrhosis, a preneoplastic fibrotic condition in liver (10). This occurs through NRP-1 stimulation of TGFβ and platelet-derived growth factor (PDGF) signaling pathways (11, 12).
Recent studies have suggested that NRP-1 regulates integrin-mediated fibronectin fibril assembly in endothelial cells and cell attachment (13). On the basis of these concepts, we hypothesized that NRP-1 might influence myofibroblast interactions with tumor matrix molecules such as fibronectin that could promote an active tumor microenvironment and thereby lead to tumor growth beyond direct effects that may be achieved by NRP-1 within the tumor cell itself (14, 15). Indeed, in this study, we reveal an important role of NRP-1 as an amplifier of the process whereby myofibroblasts engage with soluble fibronectin and initiate signals that promote α5β1-mediated fibronectin fibril assembly. NRP-1 achieves this function through complementary utilization of extracellular and intracellular protein domains that allow binding between fibronectin and NRP-1 and activation of the nonreceptor tyrosine kinase c-Abl. Importantly, these novel molecular pathways also promote tumor growth in rodent models of cancer and correlate with cancer disease severity in human liver cancer.

Materials and Methods

Detailed Materials and Methods are provided in the Supplementary data.

Animal studies

All animal experiments were approved by IACUC and carried out in accordance with institutional guidelines. Tumor xenografts were established from Lewis lung carcinoma (LLC) cell lines by subcutaneous implantation of 1 × 10⁶ cells diluted in PBS. Tumor volumes were measured as previously described (16). Intraperitoneal administration of NRP-1 monoclonal antibody (mAb) or vehicle was commenced 4 days after implantation in mice with similar size tumors in each group. NRP-1-b antibody that recognizes NRP-1-b extracellular domain (10, 16) was used in the present studies. In a second model, tumor coimplantation studies were conducted with LLC in combination with mouse embryonic fibroblasts (MEF). MEFs isolated from NRP-1–/– mice were transduced with Ad-Cre or Ad-LacZ to genetically delete NRP-1 and these cells were coimplanted with LLC in 1:1 ratio (total number 1 × 10⁶) subcutaneously in NRP-1–/– mice/SM22cre mice that are genetically deficient of NRP-1 in myofibroblasts. Tumor growth was monitored by measurements of tumor with caliper. After 15 days, mice were sacrificed and tumors were dissected from the mice and frozen in optimum cutting temperature for further analysis.

Cell lines and cell culture

Human hepatic stellate cell (hHSC; ScienCell Research Laboratories), LX2, a well-characterized cell line derived from human HSC, or MEF isolated from NRP-1–/– mice (10) or c-Abl/Arg⁻/⁻ mice (courtesy of Tony Koleske and Ed Leof) were used in these studies as indicated in individual figure legends. But the tumor cell lines LLC and HepG2 were purchased from American Type Culture Collection that carries out cell line characterizations and are passaged in our laboratory many times. Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 1 mmol/L L-glutamine, and 100 IU/mL streptomycin/penicillin.

Confocal immunofluorescence microscopy

Five-micrometer sections of harvested tumor tissues were fixed and stained using antibodies indicated in the figure legends. Confocal microscopy of tissues and cells was conducted as described previously (10).

Western blot analysis and c-Abl kinase assays

Briefly, cells were stimulated with fibronectin and used for Western blotting using antibodies as described in Supplementary data. c-Abl kinase assays were conducted using conditions that we previously described (10).

Integrin activation assay

LX2 cells with retroviral overexpression of NRP-1 or red fluorescent protein (RFP) were plated on fibronectin-coated surface and then stained for HUTS-4 antibody, which recognizes the active form of integrin (17) and confocal images were acquired. Integrin activation in adherent cells was determined using glutathione S-transferase (GST)-FN III 9–11, a previously validated reagent used to determine integrin activity (18). Briefly, after stimulation with 1 mmol/L MnCl₂ or vehicle for 16 hours, LX2 cells with retroviral expression of RFP, wild-type (wt) NRP-1, or mutant constructs were incubated with GST-FNIII 9–11 in PBS containing 1 mmol/L of MgCl₂ for 30 minutes. Cells were washed and lysed and bound GST-FN III 9–11 was assessed by Western blotting.

Agarose–streptavidin pull-down assay and coimmunoprecipitation assay

Cells were lysed in 1 mL lysis buffer with 2 μg biotinylated exogenous fibronectin (b-FN). Streptavidin-conjugated agarose (10–20 μL, Sigma) was added for 2 to 4 hours at 4°C. After spinning out beads, the beads were washed, eluted, and bound proteins were analyzed by SDS-PAGE and Western blot analysis. Immunoprecipitation (IP) experiments were carried out as previously described (10).

DOC solubility assay

Cells were lysed in 0.5 mL DOC extraction buffer (1% sodium deoxycholate, 20 mmol/L Tris–Cl, pH 8.5, 2 mmol/L N-ethylmaleimide, 2 mmol/L iodoacetic acid, 2 mmol/L EDTA, 1× Complete protease inhibitor, 1 mmol/L phenylmethylsulfonylfluoride, and 1 mmol/L sodium vanadate). After centrifugation at 20,000 × g, 100 μL of the supernatant was taken as DOC soluble aliquot. The remaining pellet was resuspended in 100 μL of DOC extraction buffer, and centrifuged at 20,000 × g at 4°C. The subsequent pellet was dissolved in 50 μL sample buffer and used as the DOC insoluble aliquot.

In vitro biogels and stiffness measurements

Fibrin gels were constructed as described and cells were cultured within the gel (19). For construction of hydrogels, polyacrylamide substrates were generated on the glass coverslips and used for cell culture as described (20). Hep G2 and LLC cells were cultured on the gels for 5 to 7 days and then fixed.
Ad-Cre or Ad-LacZ to genetically delete NRP-1. These cells were coimplanted with LLC in NRP-1
Reduced tumor growth was observed in mice with coimplantation of LLC and NRP-1
transduced with Ad-LacZ over days as indicated in the graph (fi
image staining of tumor sections was done for
were analyzed as described previously (22).
benign tissue from surgical resection specimens of patients
Analysis of human liver cancer samples
RNA extracted from hepatocellular carcinoma and adjacent
benign tissue from surgical resection specimens of patients
was analyzed as described previously (22).

Results
Inhibition of NRP-1 function reduces tumor growth and desmoplasia in vivo
We began our studies by determining if inhibition of NRP-1 function could inhibit tumor growth through effects on myo-
and stained for antibody against Ki67 to study proliferation.
For magnetic resonance elastography (MRE) assessment of in vitro stiffness, image acquisitions were conducted on a 3.0-
Tesla MRI scanner (Signa Excite, GE Health Care) and analyzed
as described (21).

Figure 1. Inhibition of NRP-1 function reduces tumor growth and desmoplasia in vivo. A, NRP-1b neutralizing antibody or control was administered intraperitoneal to C57BL6 mice having similar size of subcutaneously implanted LLC tumors. Tumor nodules were measured on days 1, 4, 8, 11, and 15 and growth curves are displayed and depict attenuated tumor growth in mice receiving NRP-1Ab (\( P < 0.05; n = 5 \) mice per group). B, immunofluorescence staining of tumor sections was done for fibronectin and shows reduction in the fibronectin staining in NRP-1 antibody-treated mouse tumors. Representative graph shows the quantitative reduction in the fluorescent intensity in NRP-1 antibody-treated group (bottom on the right). The panels are representative of multiple fields of tumor sections. Scale bar, 100 \( \mu \)m. C, mouse embryonic fibroblasts (MEF) were isolated from NRP-1\(^{fl/fl}\) mice and transduced with Ad-Cre or Ad-LacZ to genetically delete NRP-1. These cells were coimplanted with LLC in NRP-1\(^{fl/fl}\)/SM22\(^{cre}\) mice that lack NRP-1 in tumor myofibroblasts. Reduced tumor growth was observed in mice with coimplantation of LLC and NRP-1\(^{fl/fl}\) MEF transduced with Ad-Cre compared with NRP-1\(^{fl/fl}\) MEF transduced with Ad-LacZ over days as indicated in the graph (\( P < 0.05; n = 5 \) mice per group). D, immunofluorescent staining was done for fibronectin; representative graph depicts the quantitative differences in fluorescent intensity between the groups (\( P < 0.05 \)). The panels are representative of multiple image fields from at least five tumors per group. Scale bar, 100 \( \mu \)m.

topic tumor studies with LLC cells, which do not express NRP-1 (data not shown) and form tumors with local aggregation of host fibroblast/myofibroblast and their associated stroma. Four days after implantation, mice with similar baseline tumor size were randomized to receive injection of NRP-1 antibody (10) or bovine serum albumin control intraperitoneally and tumor growth was followed for 10 days. Mice treated with NRP-1Ab had less tumor burden compared with mice receiving a vehicle control (Fig. 1A). Immunofluorescence analysis revealed a concurrent reduction in tumor stromal fibronectin and collagen in the NRP-1 antibody–treated mice (Fig. 1B and Supplementary Fig. S1A). Additional markers for activated stromal cells (PDGFR and smooth muscle actin antibody) and angiogenic endothelial cells (platelet/endothelial cell adhesion molecule) were also diminished in NRP-1 antibody–treated mice (Supplementary Fig. S1B and S1C). However, administering antibody could influence diverse NRP-1 expressing cell types and so we pursued a second myofibroblast specific genetic tumor model. In this model, MEFs, which have functional similarities to liver HSC/myofibroblasts, were isolated from NRP-1\(^{fl/fl}\) mice and transduced

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with Ad-Cre or Ad-LacZ to genetically delete NRP-1 in vitro. These cells were coimplanted with syngeneic and genetically compatible LLC cells in NRP-1fl/−/SM22cre mice that lack NRP-1 in SM22 expressing tumor myofibroblasts. Reduced tumor growth was observed in mice with coimplantation of LLC and NRP-1fl/−/ MEF transduced with Ad-Cre compared with NRP-1fl/− MEF transduced with Ad-LacZ (Fig. 1C). Corroborative immunofluorescent staining for the key matrix protein, fibronectin, and additional markers for stromal and endothelial cells showed prominent reductions in tumors from mice with genetic deletion of NRP-1 from myofibroblasts (Fig. 1D and Supplementary Fig. S2A). In parallel experiments, MEFs were isolated from NRP-1fl/− mice and transduced with Ad-Cre or Ad-LacZ. Subsequently, these cells were coimplanted with LLC in wt littermate control mice. This analysis did not reveal differences in tumor size between the groups, thus highlighting the importance of host myofibroblasts that contribute importantly to the tumor microenvironment in this experiment through regulation of matrix proteins such as fibronectin (Supplementary Fig. S2B).

**NRP-1 promotes fibronectin fibril assembly in myofibroblasts**

On the basis of these in vivo data and previous studies (13), we next examined mechanisms by which NRP-1 could promote fibronectin assembly and matrix activation in the tumor microenvironment. We focused our initial studies on potential effects of NRP-1 on myofibroblast–based fibronectin fibril assembly because this is a key early step in the eventual progression of desmoplasia and stiffness, which are emerging as important regulators of tumor growth in vivo (23). Additionally, the effects of NRP-1 in myofibroblasts are less explored than in endothelial cells (13). First, to test the hypothesis that NRP-1 promotes fibronectin fibril assembly, we quantified fibronectin fibril assembly from cells overexpressing NRP-1. We distinguished de novo fibronectin production from fibrillation of existing fibronectin by conducting studies with b-FN. We used the LX2 liver myofibroblast cell line for these studies owing to their well-validated role in matrix regulation (10). Analysis of cells in presence and absence of NRP-1 overexpression and incubated with b-FN for 3 hours revealed that NRP-1 overexpression increased fibronectin fibril assembly (Fig. 2A). Furthermore, NRP-1 knockdown in these cells revealed reduced fibrillation of b-FN (Fig. 2B), similar to knockdown of β1 integrin, a requisite molecule for fibronectin fibril assembly. Similar results were also observed in MEF isolated from mice containing a floxed NRP-1 allele that were transduced with Ad-Cre in vitro (Fig. 2C), and in experiments using a fibronectin antibody (Supplementary Fig. S3A and S3B). Lastly, to corroborate these findings using a biochemical approach, we fractionated lysates from cells transduced with NRP-1 adenovirus, NRP-1 siRNA, or relevant controls, into DOC soluble and DOC insoluble fractions since DOC solubility distinguishes nonfibrillated from fibrillated fibronectin. Indeed, DOC insoluble fibronectin was increased in lysates prepared from NRP-1–overexpressing cells as was the total amount of cell-bound b-FN (Fig. 2D). Conversely, DOC insoluble fibronectin was diminished in lysates prepared from NRP-1 siRNA transfected cells (Fig. 2B). Importantly, although TGFβ stimulates fibronectin production from these cells based on Western blot and real-time PCR (RT-PCR) analysis (Supplementary Fig. S3C), it does not influence fibronectin fibril assembly (Supplementary Fig. S3D). Since recent studies investigated the role of NRP-2 in TGFβ-mediated epithelial–mesenchymal transition (EMT), we also studied the role of NRP-2 in fibronectin fibril assembly. However, knockdown of NRP-2 did not induce a reduction of fibronectin fibril assembly in these cells and experimental conditions (Supplementary Fig. S4A). NRP-1 has also been shown to associate with other receptors such as plexins and VEGF receptor (VEGFR2) but in our prior studies we could not detect PlexinA1 or VEGFR2 in these cells (10), nor in human cancer associated fibroblasts by Western blot analysis (Supplementary Fig. S4B and S4C). These studies indicate that NRP-1 promotes fibronectin fibril assembly in myofibroblasts and the mechanism by which this effect was achieved was subsequently pursued in greater molecular detail.

Controversy exists as to which NRP-1 functions are mediated through extracellular coreceptor function of NRP-1 as opposed to NRP-1 regulation of intracellular signaling (24). Prior studies showed that NRP-1-mediated angiogenesis requires involvement of both extracellular and intracellular domains (25) although more recent work suggests that effects of the intracellular domain may be subtle and influence only vascular patterning (26). To explore these concepts in tumor myofibroblast biology, we generated two mutant constructs, one with a substitution of a key functional residue of the extracellular domain that regulates NRP-1 glycosylation (10, 27) and another with a deletion of an intracellular putative signaling domain termed the SEA domain (25). In terms of the former, since NRP-1 is a glycoprotein (10, 27), and its glycosylated side chains may facilitate ligand binding (27), we generated a mutant construct, S612A, which lacks a serine residue, which allows for 0 linked glycosylation. Interestingly, overexpression of NRP-1 S612A in cells led to reduced fibronectin fibrillation as compared with overexpression of wt NRP-1 (Fig. 2D). We also analyzed a NRP-1 mutant, which lacks the intracellular SEA domain a putative intracellular signaling module of NRP-1. Interestingly, overexpression of the SEA domain mutant in cells also revealed reduced fibronectin fibril assembly as compared with the wt NRP-1 overexpression (Fig. 2D). Thus, these studies indicate that NRP-1 regulates fibronectin fibril assembly through mechanisms that use NRP-1 intracellular signal transduction as well as its extracellular glycosylation.

**NRP-1 binds with fibronectin**

We next examined in greater biochemical detail whether NRP-1 may promote fibronectin fibril assembly by directly binding fibronectin. First, to assess fibronectin binding with NRP-1, b-FN was precipitated from LX2 cell lysates collected from RFP- and NRP-1–overexpressing cells incubated with b-FN and loaded into SDS-PAGE for Western blot analysis of NRP-1 and b-FN. Indeed, b-FN coprecipitates with NRP-1 and this coprecipitation is increased in NRP-1–overexpressing cells (Fig. 3A). Similarly, IP of NRP-1 coprecipitates b-FN and coprecipitation is increased in response to NRP-1
overexpression (Fig. 3B). However, overexpression of the S612A NRP-1 mutant as well as the SEA deletion construct yields less fibronectin binding as compared with overexpression of the wt NRP-1 (Fig. 3B). Furthermore, overexpressed NRP-1 partially colocalized with activated integrin (as assessed by HUTS-4 immunostain) on the cell plasma membrane (Fig. 3C). Colocalization of the S612A NRP-1 mutant and the SEA deletion construct with HUTS-4 was also examined and revealed interesting observations. Both the S612A NRP-1 mutant and the SEA deletion construct showed little colocalization with HUTS-4 (Fig. 3C). Although the SEA deletion construct did target the plasma membrane, the S612A mutant was largely retained intracellularly with limited targeting to the plasma membrane (Fig. 3C). Ultimately, HUTS-4 staining intensity was diminished in cells expressing either of the mutant constructs as compared with cells expressing the wt NRP-1 (Fig. 3C). These studies support the role of NRP-1 as a molecule that engages with soluble fibronectin, a step that subsequently promotes fibronectin fibril assembly through cooperative regulation between intracellular and extracellular domains of NRP-1, whereby disruption of either domain impairs NRP-1 function.

NRP-1 promotes fibronectin fibril assembly through integrin activation

We next determined if NRP-1 promotes α5β1 activation, which is thought to be responsible for fibronectin fibril assembly. Indeed, loss of function achieved by a β1 neutralizing antibody, MAB13, resulted in a reduction in fibronectin fibril assembly as assessed by addition of b-FN to cells (Fig. 4A). A similar effect was also observed on fibronectin fibril assembly...
in conditions focusing on endogenously secreted fibronectin from cells rather than addition of exogenous b-FN (Supplementary Fig. S5A). Overexpression of NRP-1 was also associated with increased immunostaining of HUTS-4 mAb, an antibody that specifically recognizes the active conformation of \( \beta_1 \) (Fig. 4B). Furthermore, NRP-1 overexpression also increased integrin activity as assessed by cellular binding of a GST protein fused with 9–11 Type III repeats of fibronectin that comprise the canonical RGD integrin-binding domain (GST-FN; Fig. 4C) (18), confirming the microscopic analysis. However, overexpression of the S612A as well as the SEA deletion construct failed to increase integrin activity as compared with the wt NRP-1 (Fig. 4C). We also investigated the effect of NRP-1 overexpression on integrin activation by using an antibody HUTS21 that recognizes activated integrins (28) by flow cytometric analysis. Fluorescence-activated cell-sorting (FACS) analysis of LX2 cells with NRP-1 overexpression revealed increased HUTS21-positive cells as compared with control IgG (Supplementary Fig. S5B). In summary, these studies indicate that NRP-1 increases fibronectin fibril assembly by binding fibronectin and activating integrin \( \alpha_5\beta_1 \). Furthermore, this effect requires both an intact serine at residue 612 and an intact intracellular SEA domain.

**c-Abl and GIPC are both required for NRP-1 to activate \( \alpha_5\beta_1 \)**

Recent studies indicate that the nonreceptor tyrosine kinase c-Abl is important in NRP-1 function in myofibroblasts although the mechanism by which this occurs has not been elucidated (10, 29). We first sought to determine if c-Abl may mediate effects of NRP-1 on fibronectin fibril assembly. MEF genetically deficient in c-Abl \( ^{-/-} \) revealed diminished fibronectin fibrillogenesis in experiments conducted with b-FN (Fig. 5A) as well as with endogenous fibronectin (Supplementary...
Fig. S6A). Overexpression of NRP-1 could not rescue fibronectin fibril assembly in c-Abl\textsuperscript{−/−} MEF (Supplementary Fig. S6B), indicating a requisite role of c-Abl for NRP-1 stimulation of fibronectin fibril assembly. DOC insoluble fibronectin was also diminished in lysates prepared from c-Abl\textsuperscript{−/−} MEF as well as in MEF from mice genetically deficient in NRP-1 (Fig. 5B). Furthermore, overexpression of NRP-1 in cells incubated on fibronectin was associated with increased c-Abl activity (Fig. 5C). Finally, the association of NRP-1 with c-Abl as well as c-Abl activity was increased in response to NRP-1 overexpression in cells plated in the presence of fibronectin (Fig. 5D). However, both the SEA-deleted NRP-1 and S612A mutant failed to bind and activate c-Abl in contradistinction to the wt (Fig. 5C and D). To further ascertain this effect, we used a c-Abl construct, which lacks the autoinhibitory SH3 domain, thereby conferring constitutive c-Abl activity. As anticipated, overexpression of this construct in LX2 increased fibronectin fibration and bound NRP-1 (Fig. 5E and F). Since prior studies have indicated that NRP-1 can transduce intracellular signals by binding with GIPC (25), we also investigated the role of GIPC in fibronectin fibril assembly by NRP-1. GIPC knockdown by short hairpin RNA (shRNA) in LX2 cells revealed diminished fibronectin fibril assembly as compared with control shRNA and DOC solubility assay revealed reduction in matrix-bound fibronectin from GIPC knockdown LX2 cell lysates (Supplementary Fig. 7A and B). Since we could not detect direct binding between recombinant NRP-1 and recombinant c-Abl in vitro (data not shown), we hypothesized that GIPC, through its scaffolding function and known NRP-1-binding domain (25), could serve as an adaptor protein for this protein complex. Indeed, co-IP analysis from lysates of LX2 cells overexpressing Flag-tagged NRP-1 revealed that NRP-1 resides with GIPC and c-Abl within the same complex upon treatment of cells with fibronectin (Supplementary Fig. S7C). To further confirm that this protein assembly requires GIPC binding with the intracellular domain of NRP-1, we generated a purified intracellular domain of NRP-1 GST fusion protein for pull-down studies. GST-NRP-1-IC pull down with LX2 cell lysates co-precipitates c-Abl and GIPC, thus corroborating the association of c-Abl and GIPC in a complex with NRP-1 (Supplementary Fig. S7D). Finally, since α5β1 is required for fibronectin fibril assembly, we anticipated that this protein should reside within this complex as well. Indeed, IP of α5β1 coprecipitates Flag-tagged NRP-1 from LX2 cell lysates (Supplementary Fig. S7E). These results confirm and extend other studies showing that c-Abl, GIPC, and specific integrin proteins can coprecipitate. The above studies also support the role of GIPC as an adaptor protein, which promotes the assembly and activation of a protein complex consisting of NRP-1, c-Abl, and α5β1 that leads to α5β1-dependent fibronectin fibril assembly.
NRP-1 promotes tumor cell proliferation by increasing matrix stiffness

We next determined if enhanced NRP-1–induced fibronectin assembly from myofibroblasts could lead to enhanced matrix deposition, stiffness, and more aggressive tumor cell characteristics. First, we used fibrin gels to study whether myofibroblast-derived NRP-1 contributes toward matrix stiffness by using MRE to measure stiffness (30). Cells were seeded inside fibrin gels and scans were taken every 48 hours. Compared with the acellular matrix (not shown) and matrix with control HSC, increasing stiffness was observed with myofibroblasts overexpressing NRP-1 over the first 8 days based on MRE measurements, after which stiffness of all the gels decreased with degradation of the fibrin (Fig. 6A and data not shown). In parallel, we also examined HSC morphology in the fibrin gel. Initially, cells were spheroid in appearance as shown in the depicted phase contrast images (Fig. 6A). Over time, they developed an elongated phenotype with interconnecting tubules that were more prominently observed in cells overexpressing NRP-1 (Fig. 6A). Next, we examined the effects of control HSC, increasing stiffness was observed with myofibroblasts overexpressing NRP-1 over the first 8 days based on MRE measurements, after which stiffness of all the gels decreased with degradation of the fibrin (Fig. 6A and data not shown). In parallel, we also examined HSC morphology in the fibrin gel. Initially, cells were spheroid in appearance as shown in the depicted phase contrast images (Fig. 6A). Over time, they developed an elongated phenotype with interconnecting tubules that were more prominently observed in cells overexpressing NRP-1 (Fig. 6A).

Figure 5. NRP-1 uses c-Abl to achieve integrin activation. A, confocal images of MEFs (wt and c-Abl/Arg−/−) plated on collagen and incubated with b-FN for 3 hours are depicted. Cells were fixed and stained for FITC-labeled streptavidin and show attenuated fibronectin fibril assembly in c-Abl/Arg−/− MEF. Representative images and quantification graph are shown from three independent experiments. Toto-3 staining highlights cell nuclei. Scale bar, 50 μm. B, DOC solubility assay was done on cell lysates from WT and c-Abl−/− MEF. Cells were incubated with or without b-FN. Fibronectin fibril assembly was attenuated in c-Abl−/− MEF as assessed by reduced DOC insoluble fibronectin. C, c-Abl kinase activity was measured from LX2 cells with overexpression of RFP, wt NRP-1 and S612, delSEA NRP-1 mutants. c-Abl kinase activity was assessed by using GST-Crk as a substrate after cell stimulated with fibronectin. D, IP of NRP-1 from lysates of LX2 cells with adenoviral overexpression of LacZ, wt NRP-1, and NRP-1 mutants shows the association of wt NRP-1, but not NRP-1 mutant constructs, with c-Abl. Binding is enhanced in the presence of fibronectin. Top, immunoblot probed with c-Abl antibody; center, NRP-1 was immunoprecipitated and shown in Western blot; and bottom, the equal expression levels of NRP-1 wt and mutants from the lysates. E, LX2 cells were transfected with the constitutive active SH3-deleted mutant of c-Abl or pcDNA3 and then incubated with b-FN for 4 hours and harvested for DOC solubility assay. The samples were run on SDS-PAGE and blots were probed by HRP–streptavidin (top), c-Abl, and actin (n = 3). Blots depict enhanced fibronectin fibril assembly in response to c-Abl ΔSH3 overexpression. F, IP of NRP-1 from LX2 cells lysates after retroviral overexpression of RFP or NRP-1 as well as plasmid transfection of c-Abl wt, ΔSH3 c-Abl, or pcDNA3 control. Top and bottom, immunoblots probed with c-Abl antibody and NRP-1 antibody, respectively. Error bars indicate SEM.
enhanced matrix stiffness on tumor cell proliferation by using hydrogels of varying stiffness that simulate in vivo conditions ranging from normal to desmoplasia (31). Ki67 staining of HepG2 cells revealed an increase in proliferation of cells on stiffer gels compared with gels of lesser stiffness (Fig. 6C). Similar results were obtained with LLC as well (Fig. 6B).

Finally, to simulate in vivo matrix conditions, tumor cells were plated on acellularized extracellular matrices derived from myofibroblasts expressing NRP-1 or control plasmid (Fig. 6D). Parallel studies were also conducted in acellularized extracellular matrices derived from MEFs from control or c-Abl−/− mice. Analysis of tumor cell proliferation revealed a statistically significant increase in proliferation of tumor cells residing on matrices derived from NRP-1-overexpressing myofibroblasts and a conversely reduced rate of tumor cell proliferation in MEF isolated from c-Abl−/− mice (Fig. 6E). In total, this set of in vitro reconstitution studies shows that myofibroblast-derived NRP-1 promotes matrix stiffness and that
Increased NRP-1 expression in human liver tumors correlates with decreased survival. A, NRP-1 mRNA was measured from patients with hepatocellular carcinoma and depending on expression of mRNA, data were divided into top and bottom quartile and graph was plotted over time period in months. Survival of patients with high (top 25%) or low (25%) levels of NRP-1 mRNA in their tumor is shown in the graph. B, the role of NRP-1 in myofibroblast regulation of fibronectin assembly is depicted in a model. In the model, NRP-1 promotes α5β1 integrin-dependent fibronectin fibril assembly. A mechanism is proposed whereby NRP-1 binds with fibronectin and activates the nonreceptor tyrosine kinase c-Abl through association of its cytoplasmic domain with GIPC and c-Abi. c-Abi in turn promotes α5β1 integrin activity through potential direct or indirect mechanisms. This process is disrupted by deletion of either the NRP-1 intracellular SEA domain or mutation of the S612 residue. These pathways in tumor myofibroblasts ultimately promote an active tumor microenvironment and enhanced tumor growth through enhanced stiffness of the tumor matrix.

Discussion
Increasing evidence highlights an important role for tumor microenvironmental factors such as matrix stiffness that can contribute to cancer progression beyond that achieved by the dysregulation of intracellular signaling pathways within cancer cells themselves (2, 4). Although these microenvironmental factors are complex and not fully understood, fibronectin fibril assembly is a dynamic, cell-based, and highly regulated determinant of matrix stiffness (8). The present studies expand our current model of how integrin family proteins regulate fibronectin binding and assembly. The conversion of soluble fibronectin into matrix-bound fibronectin is initiated by fibronectin binding with cells by virtue of the RGD domain of fibronectin, which binds a cognate site generated by proximity of the α and β integrin chains. Fibronectin binding increases cell tension that further conformationally activates the integrin, stretches fibronectin, and promotes fibronectin polymerization and conversion into stable insoluble fibronectin fibrillar matrix (7, 8). Thus, integrins not only bind fibronectin but also provide the "molecular engine" that convert fibronectin into an insoluble matrix. It has been proposed that other cell surface proteins associate with integrins to achieve these complex molecular dynamics. Indeed, NRP-1 was recently detected in a proteomic analysis of extracellular matrix adhesion sites by mass spectrometry (33, 34). Here, we show using biotin labeling studies and integrin activation assays that NRP-1, through coordinated actions of both its intracellular and extracellular protein domains, promotes integrin function both by binding fibronectin and also by activating the intracellular kinase c-Abl. Indeed, prior work has shown that c-Abi promotes the function of integrin family members by virtue of activating small GTPases such as Rac or Rho, which can increase local mechanical tension that is transmitted to α5β1 integrins with a resultant increase in fibronectin binding and assembly (35). In endothelial cells, NRP-1 regulates β1-mediated cell adhesion and binds specific integrin subunits (13, 36–38). Moreover, c-Abi has also been recognized to function as a signal transducer for integrins (39). Thus, these studies confirm and extend prior concepts and thereby importantly add to our understanding of how integrins cooperate with other cellular proteins to enhance matrix assembly and maturity.

The NRP-1 protein structure contains a large extracellular domain with specific subdomains termed the cubulin
homology domain and coagulation factor V/VIII homology domains (40). These domains bind extracellular ligands that include but are not limited to VEGF and semaphorin (25). The NRP-1 extracellular domain also contains a residue at S612, which is susceptible to O-linked glycosylation (27, 41). O-linked glycosylation usually occurs at the Golgi stage of protein processing and is thought to facilitate interactions between the large sugar complexes of proteoglycans with extracellular ligands that can promote diverse protein functions (42). We postulated that such glycosylation modifications could confer an increase in avidity of NRP-1 for extracellular ligands such as fibronectin in the subcellular vicinity of α5β1, thereby enhancing engagement of fibronectin with α5β1. Indeed, we observe that NRP-1 codistributes with α5β1 and mutation of S612 in NRP-1 markedly impairs fibronectin fibril assembly and disrupts fibronectin binding with NRP-1. However, mutation of S612 also disrupts NRP-1–induced activation of c-Abl, suggesting that extracellular modifications of NRP-1 can coordinately regulate intracellular signaling functions of NRP-1, thus adding complexity to the simpler model of NRP-1 glycosylation functioning as a “ligand reservoir.” Furthermore, the majority of the exogenously expressed S612 mutant did not target to the plasma membrane, but rather was retained in a perinuclear region. Thus, S612 glycosylation seems to be essential for proper NRP-1 subcellular targeting to the plasma membrane, an effect that also could account for the impaired fibronectin binding observed upon overexpression of the S612 mutant construct.

In addition to its extracellular and transmembrane domains, NRP-1 also contains a small 40 amino acid intracellular domain that lacks intrinsic kinase activity but does contain a PDZ-binding signaling motif (25). However, most evidence to date has indicated that NRP-1 function is mediated in large part through the extracellular domain that facilitates a “coreceptor” function (43). Although the intracellular domain has been implicated in signal transduction through the adaptor protein GIPC (13), the importance of this short signaling motif has remained unclear especially in view of recent work showing that this domain, although important for vascular patterning, is dispensable for angiogenesis (26). Here, we show that deletion of this domain negates the ability of NRP-1 to generate fibronectin-dependent signals that trigger fibronectin fibril assembly. Indeed, upon deletion of this domain, NRP-1 can no longer activate c-Abl. SiRNA–based depletion of GIPC revealed a similar effect on fibronectin fibril assembly, an anticipated observation, since GIPC has been shown to bind with NRP-1 through a PDZ–SEA domain interaction that transduces NRP-1 signals (13). However, GIPC also interacts with sequences contained within specific integrin family proteins as well as other intracellular signaling molecules through non-PDZ domain mechanisms (44–47). Thus, the protein complex between NRP-1, GIPC, c-Abl, and α5β1 is likely to contribute to increased fibronectin fibril assembly observed in response to NRP-1 overexpression in our studies. Indeed, increasing evidence supports a requisite role of c-Abl in myofibroblast phenotype and function owing to the ability of this nonreceptor tyrosine kinase to link plasma membrane kinase and integrin–based signals with intracellular second messengers and nuclear transcription factors (10, 29). Thus, our observations, in combination with these prior studies support a model whereby NRP-1, GIPC, and c-Abl binding creates a functional signaling unit that can both bind extracellular molecules and also generate secondary intracellular signals that in this case regulate integrin α5β1.

Myofibroblasts are increasingly recognized as a cell type within the tumor microenvironment that impact tumor growth. These cells are integral for fibronectin fibril assembly and contribute importantly to matrix stiffness and tumor desmoplasia. Myofibroblasts likely populate the tumor microenvironment through diverse mechanisms including activation of resident stromal cells such as pericytes, recruitment from blood/bone marrow, and EMT (48). Our in vivo studies were also complemented with strategic utilization of MEF (10) that were coimplanted with tumor cells, thereby assuring that the studies can be broadly generalized to other mesenchymal cells.

Approaches are currently underway to try to improve cancer outcomes by targeting the tumor microenvironment. This strategy is based on increasing recognition that sequential changes in stromal cells and their surrounding matrix importantly regulate tumor development, growth, and metastasis (49). The present studies highlight the role of NRP-1 in tumor matrix maturation. We also show that NRP-1 expression levels in human tumors correlate directly with poor prognosis in a well-characterized and well-validated cohort of patients with liver cancer. Our work, especially when viewed in combination with other recent publications focused on the role of NRP-1 in angiogenesis, myofibroblast activation, and tumor cell invasiveness, highlights NRP-1 as a potentially important target for cancer therapeutics (10, 16).

Various mechanisms may explain why desmoplasia leads to a more aggressive tumor phenotype, one possibility that is receiving increasing attention is the role of tumor stiffness. Recent studies have shown that stiffness and tensile strength of the tumor matrix increases tumor invasiveness in both preclinical and human analyses, consistent with the results we show here. Current models indicate that the majority of tumor stiffness may occur through collagen cross-linking, a process that occurs subsequent to fibronectin fibril assembly although some recent studies have challenged this prevailing concept (50). Importantl, NRP-1 not only promotes fibronectin assembly by promoting integrin activation as we show here but also directly promotes collagen secretion (10), with both these effects ultimately contributing to matrix stiffness. Eventually, matrix stiffness initiates mechanical signals that shift tumor cell behavior to a more proliferative phenotype as we show here and as others have shown elsewhere (20). These observations have highlighted the need for a more informed understanding of how tumor–associated matrix is generated and how ensuing stiffness is regulated in the tumor microenvironment. The present studies inform this need by providing evidence that NRP-1 regulates assembly of soluble fibronectin into a stable matrix-bound insoluble fibronectin. We anticipate that this may be an important and early step to target for cancer therapeutics.
Disclosure of Potential Conflicts of Interest

R. Ehmans ownership interest (including patents) and intellectual property rights. No potential conflicts of interest were disclosed by the other authors.

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