Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment

Usman Yaqoob1*, Sheng Cao1*, Uday Shergill1, Kumaravelu Jagavelu1, Zhimin Geng1, Meng Yin3, Thiago M de Assuncao1, Ying Cao2, Anna Szabolcs2, Snorri Thorgeirsson4, Martin Schwartz5, Ju Dong Yang1, Richard Ehman3, Lewis Roberts1, Debabrata Mukhopadhyay2, Vijay H. Shah1

1Gastroenterology Research Unit and Department of Internal Medicine, Mayo Clinic, Rochester, MN-55905; 2Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN-55905; 3Department of Radiology, Mayo Clinic, Rochester, MN-55905; 4National Cancer Institute, Bethesda, MD-20892; 5Division of Cardiology and Department of Cell Biology, Yale University, New Haven CT-06511

* indicates those authors contributed equally

Address correspondence to:
Vijay Shah, MD, Mayo Clinic, 200 First ST SW, Rochester, MN 55905.
Fax: (507) 255-6318 Ph: (507) 255-6028 e-mail: shah.vijay@mayo.edu

Sheng Cao, MD, Mayo Clinic, 200 First ST SW, Rochester, MN 55905.
Fax: (507) 255-6318 Ph: (507) 538-7641 e-mail: cao.sheng@mayo.edu

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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 (FN) that promote $\alpha_5\beta_1$ integrin-dependent FN fibril assembly, matrix stiffness, and tumor growth. Tumor growth and FN fibril assembly was reduced by genetic depletion or antibody neutralization of NRP-1 from stromal myofibroblasts in vivo. Mechanistically, the increase in FN 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-Abl, that augmented $\alpha_5\beta_1$ FN 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 FN fibril assembly but also have important implications for therapeutic targeting of the myofibroblast in the tumor microenvironment.
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 tumorogenic 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, transforming growth factor beta (TGFβ) 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 non-fibrillar 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, FN which is secreted in a TGFβ dependent manner as a dimer in a soluble form, which 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, FN fibril assembly may represent a critical process that is distinct from FN production, by which matrix regulates tumor growth. While 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 FN into an insoluble matrix bound FN, a number of gaps remain in our understanding of this process especially pertaining to the cellular mechanisms which 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 FN fibril assembly in endothelial cells and cell attachment (13). Based on these concepts, we hypothesized that NRP-1 might influence myofibroblast interactions with tumor matrix molecules such as FN 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 FN and initiate signals that promote α5β1 mediated FN fibril assembly. NRP-1 achieves this function through complementary utilization of extracellular and intracellular
protein domains that allow binding between FN 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 Material and Methods are provided in the Supplement)

Animal studies: All animal experiments were approved by IACUC and performed in accordance with institutional guidelines. Tumor xenografts were established from Lewis Lung Carcinoma (LLC) cell lines by subcutaneous implantation of $1 \times 10^6$ cells diluted in PBS. Tumor volumes were measured as previously described (16). Intraperitoneal administration of NRP-1 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 utilized in the present studies. In a second model, tumor co-implantation studies were performed with LLC in combination with mouse embryonic fibroblasts (MEFs). MEFs isolated from NRP-1$^{fl/fl}$ mice were transduced with AdCre or AdLacZ to genetically delete NRP-1 and these cells were co-implanted with LLC in 1:1 ratio (total number $1 \times 10^6$) subcutaneously in NRP-1$^{fl/fl}$ mice/SM22$^{cre}$ 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 O.C.T for further analysis.

Cell lines and cell culture: Human HSC (hHSC; ScienCell Research Laboratories), LX2, a well-characterized cell line derived from human HSC, or MEF isolated from NRP-1 floxed mice (10) or c-Abl/Arg-/-mice (courtesy of Tony Koleske and Ed Leaf) were used in these studies as indicated in individual figure.
legends. The tumor cell lines LLC and HepG2 were purchased from American Type Culture Collection (ATCC) 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% fetal bovine serum, 1 mmol/L L-glutamine, and 100 IU/mL streptomycin/penicillin.

Confocal immunofluorescence microscopy: 5-micron sections of harvested tumor tissues were fixed and stained using antibodies indicated in the figure legends. Confocal microscopy of tissues and cells was performed as described previously (10).

Western Blot analysis and c-Abl kinase assays: Briefly cells were stimulated with FN and used for Western blotting using antibodies as described in Supplement. c-Abl kinase assays were performed using conditions that we previously described (10).

Integrin activation assay: LX2 cells with retroviral overexpression of NRP-1 or RFP were plated on FN 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 GST-FN III 9-11, a previously-validated reagent used to determine integrin activity (18). Briefly, after stimulation with 1 mM 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 mM 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 Co-Immunoprecipitation (Co-IP) assay:** Cells were lysed in 1 ml lysis buffer with 2 μg bFN. Streptavidin conjugated agarose (10-20 μl, Sigma) was added for 2-4 hrs at 4°C. After spinning out beads, the beads were washed, eluted and bound proteins were analyzed by SDS-PAGE and Western blot analysis. IP experiments were performed as previously described (10).

**DOC solubility assay:** Cells were lysed in 0.5 ml DOC extraction buffer (1% sodium deoxycholate, 20mM Tris-Cl, pH8.5, 2mM N-ethylmaleimide, 2mM iodoacetic acid, 2mM EDTA, 1X Complete proteinase inhibitor, 1mM PMSF, 1mM 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 and stained for antibody against Ki67 to
study proliferation. For MR elastographic assessment of *in vitro* stiffness, image acquisitions were performed on a 3.0-Tesla MRI scanner (Signa Excite, GE Health Care, Milwaukee, WI) and analysed as described (21).

*Analysis of human liver cancer samples:* RNA extracted from HCC 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 myofibroblasts within the tumor microenvironment. We thus tested our model in the in vivo setting using an NRP-1 neutralizing antibody (NRP-1Ab) that has been previously demonstrated to block NRP-1 function in vivo (10, 16). We performed xenotopic 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 BSA control intraperitoneally and tumor growth was followed for 10 days. Mice treated with NRP-1Ab had less tumor burden compared to mice receiving a vehicle control (Figure 1A). Immunofluorescence analysis revealed a concurrent reduction in tumor stromal FN and collagen in the NRP-1 antibody treated mice (Figure 1B and Supplementary Figure 1A). Additional markers for activated stromal cells (PDGFR and SMA) and angiogenic endothelial cells (PECAM) were also diminished in NRP-1 antibody treated mice (Supplementary Figure 1B and 1C).

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 with Ad-
Cre or Ad-LacZ to genetically delete NRP-1 in vitro. These cells were co-implanted with syngeneic and genetically compatible LLC cells in NRP-1^fl/fl^/SM22cre mice that lack NRP-1 in SM22 expressing tumor myofibroblasts. Reduced tumor growth was observed in mice with co-implantation of LLC and NRP-1^fl/fl^ MEF transduced with Ad-Cre compared to NRP-1^fl/fl^ MEF transduced with Ad-LacZ (Figure 1C). Corroborative immunofluorescent staining for the key matrix protein, FN and additional markers for stromal and endothelial cells showed prominent reductions in tumors from mice with genetic deletion of NRP-1 from myofibroblasts (Figure 1D and Supplementary Figure 2A). In parallel experiments, MEF were isolated from NRP-1^fl/fl^ mice and transduced with Ad-Cre or Ad-LacZ. Subsequently, these cells were co-implanted with LLC in wild type 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 FN (Supplementary Figure 2B).

**NRP-1 promotes FN fibril assembly in myofibroblasts.**

Based on these in vivo data and previous studies (13), we next examined mechanisms by which NRP-1 could promote FN assembly and matrix activation in the tumor microenvironment. We focused our initial studies on potential effects of NRP-1 on myofibroblast based FN 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 is less explored than in endothelial cells (13). First, to test the hypothesis that NRP-1 promotes FN fibril assembly, we quantified FN fibril assembly from cells overexpressing NRP-1. We distinguished \textit{de novo} FN production from fibrillation of existing FN by performing studies with biotinylated exogenous FN (b-FN). We utilized 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 FN fibril assembly (Figure 2A). Furthermore, NRP-1 knockdown in these cells revealed reduced fibrillation of b-FN (Figure 2B), similar to knockdown of \( \beta_1 \) integrin, a requisite molecule for FN fibril assembly. Similar results were also observed in MEF isolated from mice containing a floxed NRP-1 allele that were transduced with AdCre \textit{in vitro} (Figure 2C), and in experiments using a FN antibody (Supplementary Figure 3A and B). 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 non-fibrillated from fibrillated FN. Indeed, DOC insoluble FN was increased in lysates prepared from NRP-1 overexpressing cells as was the total amount of cell bound b-FN (Figure 2D). Conversely, DOC insoluble FN was diminished in lysates prepared from NRP-1 siRNA transfected cells (Figure 2B). Importantly, while TGF\( \beta \) stimulates FN production from these cells based on Western blot and RT-PCR analysis (Supplementary Figure 3C), it does not influence FN fibril assembly.
(Supplementary Figure 3D). Since recent studies investigated the role of NRP-2 in TGFβ mediated EMT, we also studied the role of NRP-2 in FN fibril assembly. However, knockdown of NRP-2 did not induce a reduction of FN fibril assembly in these cells and experimental conditions (Supplementary Figure 4A). NRP-1 has also been shown to associate with other receptors such as plexins and 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 Figure 4B and C). These studies indicate that NRP-1 promotes FN 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 co-receptor 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 constructions, 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 O linked glycosylation. Interestingly, overexpression of NRP-1 S612A in cells led to reduced FN fibrillation as compared to overexpression of wild-type NRP-1 (Figure 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 FN fibril assembly as compared to the wild type NRP-1 overexpression (Figure 2D). Thus, these studies indicate that NRP-1 regulates FN fibril assembly through mechanisms that utilize NRP-1 intracellular signal transduction as well as its extracellular glycosylation.

**NRP-1 binds with FN.**

We next examined in greater biochemical detail, whether NRP-1 may promote FN fibril assembly by directly binding FN. First, to assess FN 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 (Figure 3A). Similarly, immunoprecipitation of NRP-1 coprecipitates b-FN and co-precipitation is increased in response to NRP-1 overexpression (Figure 3B). However, overexpression of the S612A NRP-1 mutant as well as the SEA deletion construct yields less FN binding as compared to overexpression of the wild-type NRP-1 (Figure 3B). Furthermore, overexpressed NRP-1 partially
colocalized with activated integrin (as assessed by HUTS-4 immunostain), on the cell plasma membrane (Figure 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 (Figure 3C). While the SEA deletion construct did target to the plasma membrane, the S612A mutant was largely retained intracellularly with limited targeting to the plasma membrane (Figure 3C). Ultimately, HUTS-4 staining intensity was diminished in cells expressing either of the mutant constructs as compared to cells expressing the wild-type NRP-1 (Figure 3C). These studies support the role of NRP-1 as a molecule which engages with soluble FN, a step that subsequently promotes FN 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 FN fibril assembly through integrin activation.

We next determined if NRP-1 promotes α5β1 activation which is thought to be responsible for FN fibril assembly. Indeed, loss of function achieved by a β1 neutralizing antibody, MAB13, resulted in a reduction in FN fibril assembly as assessed by addition of b-FN to cells (Figure 4A). A similar effect was also observed on FN fibril assembly in conditions focusing on endogenously secreted FN from cells rather than addition of exogenous b-FN (Supplementary Figure 5A). Overexpression of NRP-1 was also associated with increased
immunostaining of HUTS-4 mAb, an antibody which specifically recognizes the active conformation of β1 (Figure 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 FN that comprise the canonical RGD integrin binding domain (GST-FN; Figure 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 to the wild-type NRP-1 (Figure 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. FACS analysis of LX2 cells with NRP-1 overexpression revealed increased HUTS21 positive cells as compared to control IgG (Supplementary Figure 5B). In summary, these studies indicate that NRP-1 increases FN fibril assembly by binding FN and activating integrin α5β1. 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 α5β1.

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 FN fibril assembly. MEF genetically deficient in c-Abl -/- revealed diminished FN fibrillogenesis in experiments conducted with b-FN (Figure 5A) as well as with endogenous FN (Supplementary Figure 6A).
Overexpression of NRP-1 could not rescue FN fibril assembly in c-Abl -/- MEF (Supplementary Figure 6B) indicating a requisite role of c-Abl for NRP-1 stimulation of FN fibril assembly. DOC insoluble FN was also diminished in lysates prepared from c-Abl -/- MEF as well as in MEF from mice genetically deficient in NRP-1 (Figure 5B). Furthermore, overexpression of NRP-1 in cells incubated on FN was associated with increased c-Abl activity (Figure 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 FN (Figure 5D). However, both the SEA deleted NRP-1 as well as S612A mutant failed to bind and activate c-Abl in contradistinction to the wild type (Figure 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 FN fibrillation and bound NRP-1 (Figures 5E and 5F). 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 FN fibril assembly by NRP-1. GIPC knockdown by shRNA in LX2 cells revealed diminished FN fibril assembly as compared to control shRNA and DOC solubility assay revealed reduction in matrix bound FN from GIPC knockdown LX2 cell lysates (Supplementary Figure 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 FN (Supplementary Figure 7C). To further confirm that this protein assembly requires GIPC binding with the intracellular domain of NRP-1, we generated a purified intracellular (IC) domain of NRP-1 GST fusion protein for pull-down studies. GST-NRP-1-IC pulldown 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 Figure 7D). Finally, since α5β1 is required for FN 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 Figure 7E). These results confirm and extend other studies showing that c-Abl, GIPC, and specific integrin proteins can co-precipitate. 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 FN fibril assembly.

NRP-1 promotes tumor cell proliferation by increasing matrix stiffness.

We next determined if enhanced NRP-1 induced FN 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 towards matrix stiffness by using Magnetic resonance elastography (MRE) to measure stiffness (30). Cells were
seeded inside fibrin gels and scans were taken every 48 hours. Compared to 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 (Figure 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 (Figure 6A). Over time they developed an elongated phenotype with interconnecting tubules that were more prominently observed in cells overexpressing NRP-1 (Figure 6A).

Next, we examined the effects of 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 to gels of lesser stiffness (Figure 6C). Similar results were obtained with LLC as well (Figure 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 (Figure 6D). Parallel studies were also performed 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 (Figure 6E). In total, this set of in vitro reconstitution studies demonstrates that myofibroblast derived NRP-1
promotes matrix stiffness and that matrix stiffness in turn, promotes a more aggressive tumor cell phenotype.

*NRP-1 levels in human tumors correlate with patient prognosis.*

Finally, we sought to determine whether NRP-1 expression levels may have clinical prognostic relevance in patients with liver cancer as previously shown in other human tumors (14, 15). NRP-1 mRNA levels were ascertained by PCR array in a well validated cohort of patients with hepatocellular cancer of varying stages (22). When we compared the survival of patients with tumor NRP-1 in the lower quartile compared to the upper quartile, patients with lower tumor NRP-1 expression had significantly higher survival (Figure 7A; p=0.001), compatible with a prior study performed in lung cancer (32). Thus, NRP-1 expression correlates with patient survival in human cancer.
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, FN 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 FN binding and assembly. The conversion of soluble FN into matrix-bound FN is initiated by FN binding with cells by virtue of the RGD domain of FN which binds a cognate site generated by proximity of the α and β integrin chains. FN binding increases cell tension that further conformationally activates the integrin, stretches FN and promotes FN polymerization and conversion into stable insoluble FN fibrillar matrix (7, 8). Thus integrins not only bind FN but also provide the “molecular engine” that convert FN 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 demonstrate 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 FN and also by activating the intracellular kinase c-Abl. Indeed, prior work has demonstrated that c-Abl 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 resulting increase in FN binding and assembly (35). In endothelial cells, NRP-1 regulates β1 mediated cell adhesion and binds specific integrin subunits (13, 36-38). Moreover, c-Abl 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 (CUB) 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 FN in the subcellular vicinity of α5β1, thereby enhancing engagement of FN with α5β1. Indeed, we observe that NRP-1 co-distributes with α5β1 and mutation of S612 in NRP-1 markedly impairs FN fibril assembly and disrupts FN 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 appears to be essential for proper NRP-1 subcellular targeting to the plasma membrane, an effect which also could account for the impaired FN 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 “co-receptor” function (43). While 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, though important for vascular patterning, is dispensible for angiogenesis (26). Here we demonstrate that deletion of this domain negates the ability of NRP-1 to generate FN dependent signals that trigger FN 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 FN 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 FN 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 FN 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 epithelial-mesenchymal transition (48). Our in vivo studies were also complemented with strategic utilization of MEF (10)
that were co-implanted 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 crosslinking, a process that occurs subsequent to FN fibril assembly although some recent studies have challenged this prevailing concept (50). Importantly, NRP-1 not
only promotes FN 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 FN into a stable matrix bound insoluble FN. We anticipate that this may be an important and early step to target for cancer therapeutics.

Acknowledgments

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Figure Legends

Figure 1. Inhibition of NRP-1 function reduces tumor growth and desmoplasia in vivo

(A) NRP-1b neutralizing antibody or control was administered IP to C57BL6 mice having similar size of subcutaneously implanted Lewis lung cancer (LLC) tumors. Tumor nodules were measured on Day 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 FN and shows reduction in the FN staining in NRP-1 antibody treated mouse tumors. Representative graph shows the quantitative reduction in the fluorescent intensity in NRP-1 antibody treated group (lower panel on the right). The panels are representative of multiple fields of tumor sections. Scale bar = 100 µm. (C) Mouse embryonic fibroblasts (MEF) were isolated from NRP-1fl/fl mice and transduced with Ad-Cre or Ad-LacZ to genetically delete NRP-1. These cells were co-implanted with LLC in NRP-1fl/fl/SM22cre mice that lack NRP-1 in tumor myofibroblasts. Reduced tumor growth was observed in mice with co-implantation of LLC and NRP-1fl/fl MEF transduced with Ad-Cre compared to NRP-1fl/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 FN; 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 µm.
Figure 2. NRP-1 promotes FN fibril assembly

(A) Confocal images of HSC are depicted in presence and absence of overexpression of NRP-1 plated on the collagen matrix and incubated with b-FN for 3 hours. Cells were fixed and stained for FITC-labeled streptavidin to demonstrate b-FN. Representative images and graphic quantitation of fluorescent intensity are shown in the panel. Toto-3 staining shows cell nuclei in blue. Scale bar = 50 µm. (B) β1 integrin and NRP-1 knockdown in cells was carried out using siRNA. Cells were plated on a collagen matrix incubated with b-FN for 3 hours, fixed and then stained with FITC labeled streptavidin to visualize the assembly of exogenously added FN. Representative images and quantitative graphs are shown. Lower left panel shows immunoblots from the samples depicting knockdown of β1 integrin and NRP-1 with actin as a loading control. Lower right panel depicts DOC solubility assay showing the reduction in matrix bound DOC insoluble b-FN with LX2 cells transfected with NRP-1 siRNA. Scale bar = 100 µm. (C) Confocal images of WT and NRP-1 deleted mouse embryonic fibroblasts (MEFs) were plated on collagen matrix and incubated with b-FN for 3 hours. Cells were fixed and stained for FITC-labeled streptavidin to demonstrate b-FN. Representative images and quantitative graph is shown. Scale bar = 100 µm. (D) The left panel Western blot depicts overexpression of NRP-1 with actin as a loading control. Right panel depicts DOC solubility assay showing the increased matrix bound DOC insoluble b-FN with LX2 cells overexpressing NRP-1 WT, but not with NRP-1 mutant constructs. Images and blots are representative of three independent experiments. Graphs are
generated from compiled data from three independent experiments (*p<0.05. Error bars indicate SEM).

Figure 3. NRP-1 binds FN

(A) Left panel, 30 \( \mu l \) streptavidin conjugated agarose was added to LX2 cell lysates collected from the LacZ, NRP-1 WT, NRP-1 S612A mutant and NRP-1 SEA domain deletion mutant overexpressing groups which had been pre-treated with b-FN. Increased binding of b-FN with NRP-1 is depicted in cells overexpressing NRP-1 WT but not in cells expressing mutant constructs. Right panel shows Western blots from lysates with equal expression levels of NRP-1. Note that the upper glycosylated band of NRP-1 is reduced in S612A mutant lane. The numbers represent the NRP-1 to bFN ratio by densitometric analysis.

(B) LX2 cells transfected with RFP, wt NRP-1, NRP-1 S612A and NRP-1 delSEA were harvested after 24 hours incubation and were coprecipitated with NRP-1 antibody and immunoblotted with FN, actin and NRP-1 antibodies. The numbers at the bottom of the blot represent the ratio of FN to NRP-1 through densitometric analysis. (C) Confocal images of LX2 cells overexpressing NRP-1 WT, NRP-1 S612A and NRP-1 SEA domain deletion mutant were immunostained using HUTS-4 and NRP-1 antibody to study colocalization of activated integrin and NRP-1. Prominent co-localization of HUTS-4 was observed with WT NRP-1 but not with NRP-1 S612A or NRP-1 delSEA. Graphic analysis represents colocalization from the overlay images for HUTS-4 and NRP-
1. White broken arrow over the overlay image represents the area selected for colocalization analysis. Zoom image from NRP-1 WT is also depicted.

Figure 4. NRP-1 promotes FN fibril assembly through integrin activation

(A) Confocal images were taken of LX2 cells in presence and absence of overexpression of NRP-1 in presence and absence of β1 integrin neutralizing antibody MAB-13 after incubation with b-FN for 3 hours. Cells were fixed and stained for FITC-labeled streptavidin to demonstrate b-FN. Representative images and quantitation graph are shown in the figure from three independent experiments depicting that MAB-13 reverses NRP-1 induced increase in FN fibril assembly. Toto-3 staining depicts nuclei in blue. Scale bar = 100 µm. (B) LX2 cells retrovirally transduced with RFP or NRP-1 were plated on FN matrix and stained for HUTS-4 (activated form of β1 integrin). Confocal images were acquired which depict enhanced HUTS-4 stain in NRP-1 overexpressing cells. (C) GST-FNIII pulldown assay from LX2 cell lysates with retroviral overexpression of RFP, NRP-1 WT, NRP-1 S612A, and NRP-1 delSEA, in presence and absence of MnCl₂, depicts increased integrin activity in response to NRP-1 overexpression. Lower panels are showing actin as a loading control and equal NRP-1 overexpression (n=3; *p<0.05. Error bars indicate SEM). Data from multiple experiments is depicted graphically based on densitometric analysis.
Figure 5. NRP-1 utilizes c-Abl to achieve integrin activation

(A) Confocal images of MEFs (wild type and c-Abl/Arg<sup>−/−</sup>) plated on collagen and incubated with b-FN for 3 hours are depicted. Cells were fixed and stained for FITC-labeled streptavidin and demonstrate attenuated FN fibril assembly in c-Abl/Arg<sup>−/−</sup> MEF. Representative images and quantitation graph are shown from three independent experiments. Toto-3 staining highlights cell nuclei. Scale bar = 50µm.

(B) DOC solubility assay was done on cell lysates from WT and c-Abl<sup>−/−</sup> MEF. Cells were incubated with b-FN or from endogenously produced FN. FN fibril assembly was attenuated in c-Abl<sup>−/−</sup> MEF as assessed by reduced DOC insoluble FN.

(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 FN.

(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 FN. Upper panel shows immunoblot probed with c-Abl antibody, middle panel shows NRP-1 immunoprecipitates by Western blot and lower panel shows 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 (upper panel), c-Abl and actin (n=3). Blots depict enhanced FN 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. Upper and lower panels are immunoblots probed with c-Abl antibody and NRP-1 antibody respectively. Error bars indicate SEM.

Figure 6. Myofibroblast derived NRP-1 promotes matrix stiffness and contributes to tumor cell proliferation

(A) Mean stiffness of fibrin gels containing HSC with or without retroviral overexpression of NRP-1 was quantified using MR elastography; representative images and quantitation are depicted graphically. Representative elastograms are depicted. Graph shows stiffness of fibrin gels in kPa over days (lower panel). Phase contrast images of HSC within the fibrin gel are depicted and show increased extensions and spreading of cells in 3-D culture which was more prominent in cells overexpressing NRP-1 (upper panel). (B and C) LLC and HepG2 cells were cultured on polyacrylamide hydrogels of low and high stiffness (3.15 and 40.4 kPa, respectively). Cells were stained using Ki67 antibody and images were acquired and analyzed with Metamorph imaging software. Graph depicts the decreased proliferation of LLC and HepG2 cells on soft (3.15 kPa) vs hard (40.4 kPa) hydrogels (*p < 0.05; n=3). (C) HepG2 cells serum starved overnight were plated onto a conditioned matrix derived from HSC with retroviral overexpression of NRP-1 or RFP for 24 hours. HepG2 cells were then fixed and stained for Ki67 to assess proliferation and with nuclear DAPI stain. Images were acquired through epifluorescence microscopy and analyzed through
Metamorph imaging software. Graphs were plotted and show the increased ratio of total number of Ki67 positive cells and total number of nuclei amongst HepG2 cells plated on matrices from HSC with overexpression of NRP-1 (n=3, *p < 0.05). (D) LLC were serum starved overnight then plated onto conditioned matrix derived from WT and c-Abl/Arg -/- MEF for 24 hours and then stained for Ki67 and DAPI. Graph shows the increased ratio of total number of Ki67 positive cells and total number of nuclei in matrix from WT MEF (n=3, *p<0.01).

Figure 7. 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 was divided into upper and lower quartile and graph was plotted over time period in months. Survival of patients with high (upper 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 FN assembly is depicted in a model. In the model, NRP-1 promotes α5β1 integrin dependent FN fibril assembly. A mechanism is proposed whereby NRP-1 binds with FN and activates the nonreceptor tyrosine kinase c-Abl through association of its cytoplasmic domain with GIPC and c-Abl. C-Abl 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.
Figure 3

A

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NRP-1/6FN

0.43 1.99 0.24 0.25

B

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Intensity (AU)

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Intensity (AU)

0 50 100 150 200

μm

1 4 7 10 13 16 19 22 25 28 31 34
Figure 7

A

Time (months)

- lower 25% N=24 (10 death)
- upper 25% N=33 (26 death)

P<0.01

B

Soluble fibronectin

Insoluble fibronectin

Desmoplasia/ stiffness

Tumor growth

NRP-1

s612

NRP S612A mutant

SEA

NRP/SEA

mutant

PM

Golgi
Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment

Usman Yaqoob, Sheng Cao, Uday Shergill, et al.

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