Cancer Research

Cancer-Associated Fibroblasts Produce Netrin-1 to Control Cancer Cell Plasticity

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Abstract

Netrin-1 is upregulated in a large fraction of human neoplasms. In multiple animal models, interference with netrin-1 is associated with inhibition of tumor growth and metastasis. Although netrin-1 upregulation was initially described in cancer cells, we report here that in the human colorectal cancer database, the expression of netrin-1 and its receptor UNC5B correlates with a cancer-associated fibroblasts (CAF) signature. Both colon and lung CAF secreted netrin-1 when cocultured with respective cancer cells, and netrin-1 upregulation in CAF was associated with increased cancer cell stemness. Pharmacologic inhibition of netrin-1 with a netrin-1–mAb (Net1–mAb) abrogated the CAF-mediated increase of cancer stemness both in coculture experiments and in mice. Net-1–mAb inhibited intercellular signaling between CAF and cancer cells by modulating CAF-mediated expression of cytokines such as IL6. Together these data demonstrate that netrin-1 is upregulated not only in cancer cells but also in cancer-associated stromal cells. In addition to its direct activity on cancer cells, inhibition of netrin-1 may reduce proneoplastic CAF–cancer cell cross-talk, thus inhibiting cancer plasticity.

Significance: Netrin-1, a navigation cue during embryonic development, is upregulated in cancer-associated fibroblasts and regulates cancer cell stemness.

Introduction

Tumor heterogeneity has over the last decade became a major theme, and functional tumor heterogeneity has been proposed to rely on a distinct subset of tumor cells that possesses the capacity to sustain tumor growth, referred to as cancer stem cells (CSC) or tumor-initiating cells (1, 2). Whether CSCs are really a distinct subset of cancer cells or whether they represent a functional state of some cancer cells remains a matter of debate, yet the general consensus is that CSCs are keys in clinical progression due to their ability to self-renew and to resist chemotherapies, thereby facilitating tumoral relapse (3, 4). CSCs are thought to express selective markers at the cell surface and are identified on the basis of their ability to propagate tumors when serially transplanted into recipient mice (5). Over the past years, it has been proposed that CSCs can be regulated by extrinsic signals provided by stromal cells of tumor microenvironment, which establishes favorable conditions for CSCs growth (6). Multiple types of stromal cells have been reported to interact with CSCs and to influence their behavior. For instance, endothelial cells, mesenchymal stem cells, and immune cells establish permissive growing conditions for CSCs (7). Cancer-associated fibroblasts (CAF), the fibroblasts that are high jacked by cancer cells, have recently turned out to be major cellular components in the stroma of several cancer types, and have been involved in many aspects of tumor progression, from *in situ* growth of primary tumors to metastatic spread of cancer cells (8). We propose here that netrin-1 is involved in this dialogue between cancer cells, CSCs, and CAFs.

Netrin-1 is a multifunctional secreted glycoprotein that plays key roles in neuronal navigation, angiogenesis, and cell survival (9), and as a consequence, netrin-1 has been implicated in numerous diseases including diabetes, cardiovascular diseases, and cancer (9). Most of its activity has been shown to occur through the regulation of the signaling pathways transduced by its main receptors, deleted in colorectal carcinoma (DCC) and UNC5-Homolog (UNC5H, i.e., UNC5A, UNC5B, UNC5C, UNC5D; ref. 10). Of interest, netrin-1 has been shown to be upregulated in many cancers, and this upregulation has been proposed to act as a selective mechanism that blocks apoptosis induced by the dependence receptors DCC and UNC5H (9). In support of this notion, netrin-1 expression in brain metastases is an independent prognostic factor for poor patient survival (11), and netrin-1 detection in blood or urine has been proposed to be a predictive marker for cancers (12, 13). Efforts to develop drugs that inhibit the interaction of netrin-1 with its receptors have therefore been initiated. Several preclinical proof-of-concept studies have shown that candidate drugs interfering with netrin-1–receptor interactions, either used alone or in combination with conventional chemotherapies or epidrugs, inhibit tumor growth

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and metastasis markedly (14–16). A humanized anti-netrin-1–mAb (Net1–mAb), also called NP137, has been developed previously (15) and is currently in a phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT02977195). While the initial view was that netrin-1 was produced by cancer cells, we show here that netrin-1 is also upregulated by CAFs when cocultured with netrin-1–negative or netrin-1–expressing cancer cells. We also demonstrate that this netrin-1 upregulation is part of the dialogue between cancer cells and CAFs allowing CAFs to promote cancer cells plasticity.

Materials and Methods

Cell lines, coculture procedures

Human non–small cell lung cancer (NSCLC) cell line A549 and colon cancer cell line HCT116 were obtained from the ATCC and cultured in DMEM (Life Technologies) containing 10% FBS. Human NSCLC cell line (CLS1), a primary cell line derived from lung cancer patients with adenocarcinoma, as described in (8), were cultured in RPMI1640 medium, supplemented with 10% FBS at 37°C under a humidified atmosphere consisting of 20% O_2 and 5% CO_2. Human NSCLC CAFs (NSCLC CAF, catalog no. CAF07) and colorectal CAFs (CRC CAF, catalog no. CAF05), derived from patients with non–small cell lung adenocarcinoma and colorectal tumor, respectively, were purchased from Vitro BioPharma Inc. Human CAFs were cultured in SC00B1 medium (Vitro BioPharma) in a hypoxic condition: 37°C under a humidified atmosphere consisting of 1% O_2 and 5% CO_2. Routine *Mycoplasma* testing was performed by MycoAlert *Mycoplasma* Detection Kit (catalog no. LT07-118). Cancer cells grown for no more than 20 passages were used in all the experiments. CAFs grown for no more than 10 passages were used in all the coculture experiments.

Standard coculture procedures. For qRT-PCR and Western blot analysis, 8×10^4 CAFs were seeded in a 10-cm petri dish 1 day prior to cancer cells (4,000 cells/10-cm petri dish). The ratio between CAFs to cancer cells ranged between 10:1 and 50:1 (depending on the experiment and cell types). Cocultures were maintained in cancer cell growth medium for 5–10 days to allow colony formation. Medium was changed every 2–3 days. To maintain cancer stemness, cancer stem–like cells that formed spheroids on CAFs, were collected by hand picking and passed on to a freshly prepared dish of CAFs.

Generation of GFP-stable cell lines and separation of cocultured cells

Experiments were carried out with A549, HCT116, and CLS1 parental cell lines infected with GFP (A549-GFP, HCT116-GFP, and CLS1-GFP) to allow us to determine the population of two subgroups of cells after 10 days of coculture by a FACS sorter. Stable cell lines A549, CLS1, and HCT116, constitutively expressing GFP were generated by lentiviral infection. Stable cell clones were selected for 4 weeks in puromycin-containing medium. The infected cells were then sorted by flow cytometry to select GFP-positive cells. Prior to FACS sorting, coculture was performed with GFP-positive cancer cells and CAFs for 5–10 days.

For antibody treatment experiments, cocultures were carried out as described above, followed by treatment of anti-netrin1 antibody (Net1–mAb, 20 μg/mL) or an isotype control antibody for 7 days. Cell growth media was refreshed every 2 days. For other treatment experiments: recombinant human IL6 protein (rIL6)—10 ng/mL (R&D Systems, catalog no. 206-IL), AG490, JAK2 inhibitor that blocks IL6-JAK2 pathway—starting concentration of 50 μmol/L for 24 hours and decreased to 10 μmol/L for another 6 days (Sigma, catalog no. T3434), IL6–mAb—10 μg/mL (Abcam, catalog no. ab6672; or siltuximab from Euromedex, catalog no. TAB-212), IL8–mAb—10 μg/mL (Abcam, catalog no. ab18672), and combination of IL6–mAb and IL8–mAb (same concentration as above) for 7 days.

Clonogenic assay

Two-hundred CLS1 cancer cells, picked from CAF-cancer cell coculture, were seeded in 6-cm petri dishes following standard coculture procedures described above. The incubation time for colony formation was 1 week. Culture media was gently removed from each dish and washed with PBS. Colonies were then fixed and stained with 0.5% crystal violet containing 4% paraformaldehyde for 1 hour at room temperature. Crystal violet was rinsed off with dH_2O and allowed to air dry. Colonies containing more than 50 individual cells were counted using a stereomicroscope. Digital images of the colonies were obtained using a scanning device. Plating efficiency = number of colonies formed/number of cells plated × 100%.

Bioinformatic analyses

Colon adenocarcinoma data were downloaded with TCGA2STAT (R package) and $\log_2$ transformed. We used CAF score signature from Isella and colleagues (17). Briefly, Isella and colleagues identified 131 genes specific to CAF. The CAF score is the expression mean of these genes. Correlation between NTN-1 or UNC5B and CAF score were analyzed with R. For the gene set enrichment analysis (GSEA), we defined two groups from COAD dataset [The Cancer Genome Atlas (TCGA)]: the high NTN1 (NTN1 expression upper to the median of NTN1 expression) and a low NTN1 group (NTN1 expression lower to the median of NTN1 expression). As geneset, we used SSM signature from Isella and colleagues (17).

ELISA

The protein levels of IL6 and IL8 in the supernatant of cocultured cells (and CAFs alone) were measured by IL6 and IL8 ELISA Kits (R&D Systems, catalog no. D6050 for IL6 ELISA kit; catalog no. D8000C for IL8 ELISA kit), according to the manufacturer's instructions.

qRT-PCR

RNA extraction and qRT-PCR were performed as described previously (15). Briefly, 1 μg of total RNA, isolated from cells using NucleoSpin RNAII Kit (Macherey-Nagel) was used for the reverse transcription with oligo-dT priming and iScript reverse transcriptase (iScript cDNA Synthesis Kit, Bio-Rad). The resulting cDNA was diluted in respective provided buffers and assessed by qRT-PCR using TaqMan qPCR Kit (Roche Applied Science), according to the manufacturer's instructions. Measurements were performed using a Roche instrument LC480. For each sample, qPCR reactions were carried out in duplicates and the entire analysis was done at least three times independently. Relative gene expression levels shown in the figures were normalized to the expression of housekeeping genes using TATA-binding protein and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) genes. Fold changes were calculated as the expression

of gene of interest in the cocultured cells normalized to the expression in the cells without coculture. The sequences of the primers are listed in Supplementary Materials and Methods (Supplementary Table S1).

Immunoblot analysis

Subconfluent cells were harvested by trypsin, washed with cold PBS, and lysed in a lysis buffer containing SDS. Lysates were incubated for an hour at 4°C and cellular debris were pelleted by centrifugation (10,000 × *g* 15 minutes at 4°C). Protein quantifications were carried out using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and 50 μg of protein extracts were loaded onto 4%–15% SDS-polyacrylamide gels (Bio-Rad) and blotted onto nitrocellulose sheets using Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were then blocked with 5% nonfat dried milk for an hour at room temperature and incubated at 4°C overnight with primary antibodies (anti-netrin1 antibody, 1:1,000, Abcam, catalog no. ab126729; anti-UNC5B antibody, 1:1,000, Cell Signaling Technology, catalog no. D9M7Z). After three washes with PBS-T, it was followed by incubation with secondary antibodies coupled with HRP (1:10,000, Jackson ImmunoResearch) for an hour at room temperature. After washing in TBST, immunoreactive antibody–antigen complexes were visualized with the enhanced chemiluminescence reagents, West Dura Chemiluminescence System (Pierce). Membranes were imaged using a ChemiDoc Touch Imaging System (Bio-Rad).

Immunofluorescence staining

To determine the localization of Net-1 protein, cocultured CAFs and CLS1 cancer cells were seeded in chamber slides (with a ratio of 10:1, 4,000 CAFs plus 400 CLS1 cells) for 8 days prior to immunofluorescence staining. Standard immunofluorescence staining was performed as described previously in ref. 18. Briefly, cells were washed with PBS and fixed in 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were permeabilized for 10 minutes and then blocked for 30 minutes. Cells were incubated with primary antibodies, anti-netrin-1 antibody (Abcam, catalog no. ab126729) at a dilution of 1:300, at 4°C overnight, then with secondary antibody (dilution 1:1,000) at room temperature for 2 hours.

Xenograft *in vivo* models

All the animals were housed and bred in a specific pathogen-free animal facility. All animal protocols were in accordance with the animal care guidelines of the European Union and French laws, and further approved by the University of Lyon local Animal Ethic Evaluation Committee (CECCAPP Authorization no CLB-2015-005; accreditation of laboratory animal care by CECCAP, ENS Lyon-PBES, France). Six-week-old female SCID mice were randomly divided into different groups receiving subcutaneous injections into the right flank with 2×10^6 or 1×10^6 cancer cells alone and cocultured cells (CAFs plus cancer cells, seeding with a ratio of 20:1 for 10 days of coculture). Tumor size was measured regularly by a caliper followed the formula $V = 0.5 \times (\text{length} \times \text{width}^2)$.

For *ex vivo* experiment, before xenografting, cocultured (8×10^4 CAFs with 4,000 cancer cells in a 10-cm petri dish) or non-cocultured cancer cells were pretreated with an isotype antibody control (as a nontreated control), and Net1–mAb antibody (20 μg/mL) for 7 days, according to *in vitro* treatment protocol described above. For intraperitoneal and intratumoral treatment experiments, cells were cocultured for 10 days before xenografting, as described above. Mice were assigned randomly to receive intraperitoneal or intratumoral treatments. To determine the effects of anti-netrin-1 antibody on tumor initiation in the presence of CAFs, 10 mg/kg of Net1–mAb or vehicle was injected intraperitoneally every 2 days, throughout the time of measurement was carried out. Intraperitoneal treatments started from 24 hours post xenografting. When tumors reached the size between 150 and 230 mm^3, 100 μg/mice of Net1–mAb or vehicle in total volume of 20 μL was injected intratumorally for 8 consecutive days (8 intratumoral injections). Mice were sacrificed either when tumors reach 1,500 mm^3 or 24 hours after the last intratumoral injection, and tumors were extracted for weight measurements. All the *in vivo* experiments were performed independently three times with 6–15 mice per each experimental group. The numbers of tumor and mice shown in the Tables 1 and 2 were the total number per each experimental group summed from three independent experiments.

Statistical analysis

The data reported are the mean ± SEM of at least three independent determinations, each performed in duplicate or triplicate. Statistical analysis was performed by unpaired Student *t* test (Figs. 2–5).

Results

In colon cancer, the CAF signature is associated with high netrin-1 expression

It has been shown that netrin-1 is upregulated in a large fraction of cancers, and most of the literature supports the view that this upregulation occurs in an autocrine manner by cancer cells as a mechanism preventing UNC5B-induced cancer cell death (9, 14). From a bioinformatic analysis of the expression of netrin-1 and UNC5B in human cancer database, high expression of netrin-1 and UNC5B was strongly associated with a stromal signature, and more specifically with a CAF signature, particularly in colon cancer. As demonstrated by Isella and colleagues, a CAF score defined by the contribution of 131 genes was extracted from TCGA data in colon cancer (17). As shown previously, along with the general hypothesis that CAFs contribute to cancer stemness, and consequently to clinical progression (19, 20), a high CAF score is associated with poor prognosis. From this TCGA data, a correlation between CAF score and αSMA expression (CAF marker) was observed, whereas a control gene (GAPDH) showed no correlation to CAF score. Of interest, as shown in Fig. 1A, when such CAF score is analyzed together with netrin-1 expression, a significant association between netrin-1 level and CAF score was detected ($R = 0.62$; $P = 2.2 \times 10^{-16}$). A similar significant association was also seen between UNC5B and CAF score ($R = 0.72$; $P = 10^{-48}$; Fig. 1B). Along the same line, we observed a strong enrichment of the stromal SSM (stem/serrated/mesenchymal) gene set signature obtained by GSEA analysis (17) when comparing colorectal tumors with high versus low netrin-1 expression level (Fig. 1C).

Coculture of cancer cells and CAFs is associated with CAF-mediated netrin-1/UNC5B upregulation

To investigate whether the association of netrin-1/UNC5B expression and CAFs could be modeled *in vitro*, coculture experiments of CAFs with cancer cells were performed. It has been

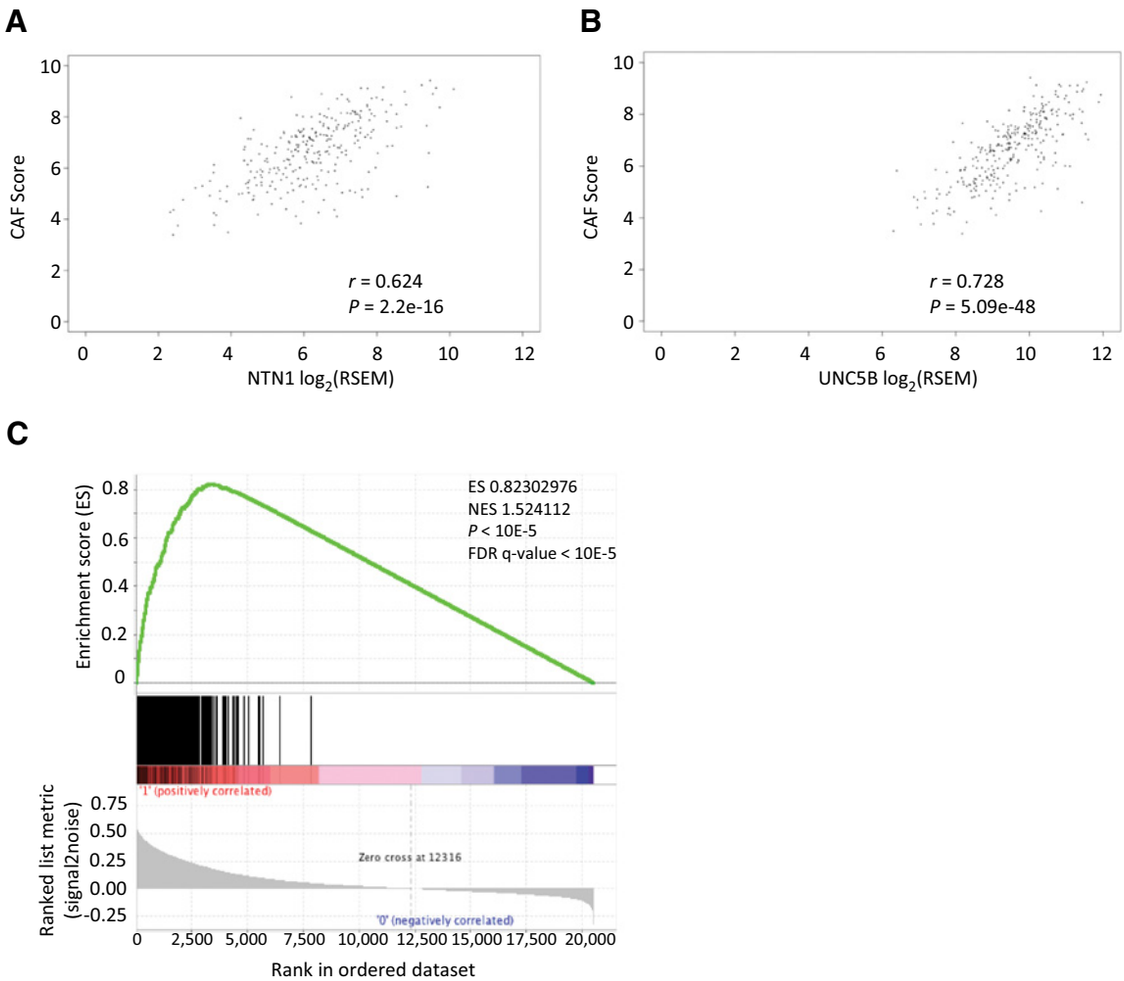

Figure 1.

In human colorectal cancer, netrin-1 and UNC5B level correlates with high CAF contribution. **A** and **B,** Scatter plot of CAF score versus NTN1 and UNC5B expression. It is shown that expression of NTN1 and UNC5B is correlated to CAF score in a colorectal cancer database. **C,** Enrichment profile from the stromal SSM (stem/serrated/mesenchymal) gene set signature obtained by GSEA analysis. Enrichment plot is used to calculate the enrichment score (ES) for this gene set when comparing colorectal tumors with high and low netrin-1 expression levels.

shown that coculturing cancer cells with CAFs leads to upregulation of several secreted factors that may contribute to increased cancer stemness (8). As schematically represented in Fig. 2A, different GFP-expressing cancer cell lines, including colorectal cancer cells (HCT116) and lung cancer cells (A549 and CLS1), were cocultured with respective CRC CAFs or NSCLC CAFs. FACS sorter was then used to separate the GFP-positive cancer cells from GFP-negative CAFs. In this setting, we first analyzed the expression levels of netrin-1 (NTN1) and its receptors DCC and UNC5A-B-C-D in the coculture system. DCC and UNC5A, C, and D mRNA were not detectable in CAFs or in cancer cells, with or without coculture. When expression of NTN1 and UNC5B was analyzed by qRT-PCR in cocultured cancer cells (sorted GFP-positive cells), no significant transcriptional difference of these genes was observed (Fig. 2B and C). However, when CAFs (sorted GFP-negative cells) were monitored, a clear and reproducible 5- to 10-fold transcriptional upregulation of NTN1 was observed compared with CAFs cultured alone in the CLS1 (Fig. 2D), A549 (Fig. 2E), and HCT116 (Fig. 2F) models. Of interest, netrin-1 upregulation was associated with a similarly robust upregulation of its receptor UNC5B (Fig. 2D–F) in these coculture models. Moreover, the netrin-1/UNC5B upregulation detected at the mRNA level was confirmed at the protein level (Fig. 2G). When immunostaining was performed directly on the coculture, we observed that the gain of netrin-1 expression in CAFs was intrinsically linked to a close cell to cell contact between CAFs and cancer cells, as netrin-1–specific signal appeared restrictedly to the border of cancer cell patches (shown in Fig. 2H). Together, these data demonstrated that in colon and lung coculture systems CAFs cocultured with cancer cells upregulated netrin-1 and its receptor UNC5B expression.

Interference with CAF-produced netrin-1 inhibits cancer stemness

During 5–10 days of coculture with respective CAFs, cancer cells (A549, CLS1, and HCT116) grew on CAFs as spheroid-like

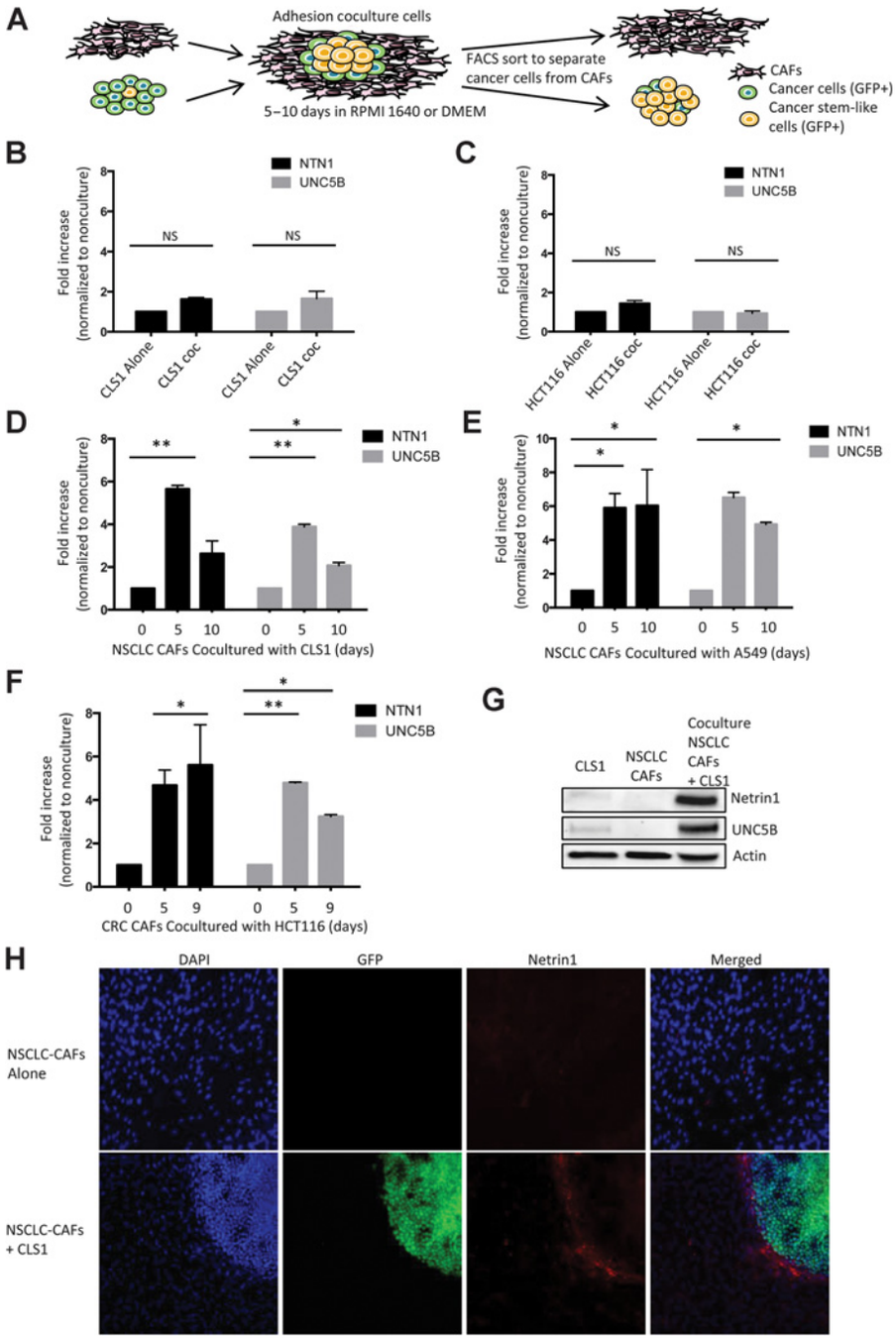

Figure 2.

CAFs upregulate netrin-1 and UNC5B when cocultured with cancer cells. **A,** A schematic illustration of coculture model. After 5–10 days of coculture maintained in RPMI 1640 (for CLS1-GFP cells) or DMEM (for A549-GFP and HCT116-GFP cells), FACS sorter was used to separate GFP-positive cancer cells from GFP-negative CAFs. **B,** Expression of netrin-1 (NTN1) and UNC5B was quantified by qRT-PCR in CLS1-GFP cancer cells cocultured with lung CAFs (coc) for 5 days, followed by FACS sorting. **C,** Expression of netrin-1 and UNC5B was quantified by qRT-PCR in HCT116-GFP cancer cells cocultured with colorectal CAFs for 5 days, followed by FACS. No significant change was observed in cocultured CLS1 cells or in cocultured HCT116 cells, compared with non-cocultured conditions (cancer cell alone). Expression of netrin-1 and UNC5B was measured by qRT-PCR, respectively, in lung CAFs (**D** and **E**) and colon CAFs (**F**) after FACS sorting. **D,** Lung CAFs cocultured with CLS1-GFP cells for 5 and 10 days. **E,** Lung CAFs cocultured with A549-GFP cells 5 and 10 days. **F,** Colorectal CAFs cocultured with HCT116-GFP cells 5 and 10 days. **G,** Netrin-1 and UNC5B expression was measured by immunoblotting after 8 days of coculture CLS1/CAFs. **H,** Netrin-1 expression was detected by immunofluorescence staining in lung CAFs cultured alone and CSL1/CAF after 8 days of coculture. Nuclei are visualized with DAPI staining (blue). Green-GFP staining, CLS1 cancer cells. Red, netrin-1 staining. Error bars, SEM; *P* values, two-tailed unpaired Student *t* test from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; NS, nonsignificant.

colonies, providing the first hint on increased cancer stemness as shown in Supplementary Fig. S1. Along this line and similarly to what was described previously, coculture of GFP-tagged lung cancer line CLS1 and GFP-tagged colon cancer line HCT116 with respective CAFs was associated with an enhanced expression of cancer stemness markers, such as Nanog and Oct4 (for lung cancer CLS1/CAFs coculture model, shown in Fig. 3A), and Nanog, Oct4, and ALDH1A1 (for colon cancer HCT116/CAFs coculture model, shown in Fig. 3B). To further associate this increase of stemness markers with a functional assay, the same number of cancer cells alone (without coculture) and cocultured cells (cancer cells plus CAFs previously cocultured for 10 days) were engrafted in mice. In agreement with the overall working model of CAFs potentiating cancer stemness, engrafting the same number of cocultured cells in immunocompromised SCID mice led to an enhancement of tumor take (Supplementary Table S2) and higher percentage of tumor formation in both lung cancer model (CLS1/CAFs) and colon cancer model (HCT116/CAFs), when comparing with engrafting cancer cells alone.

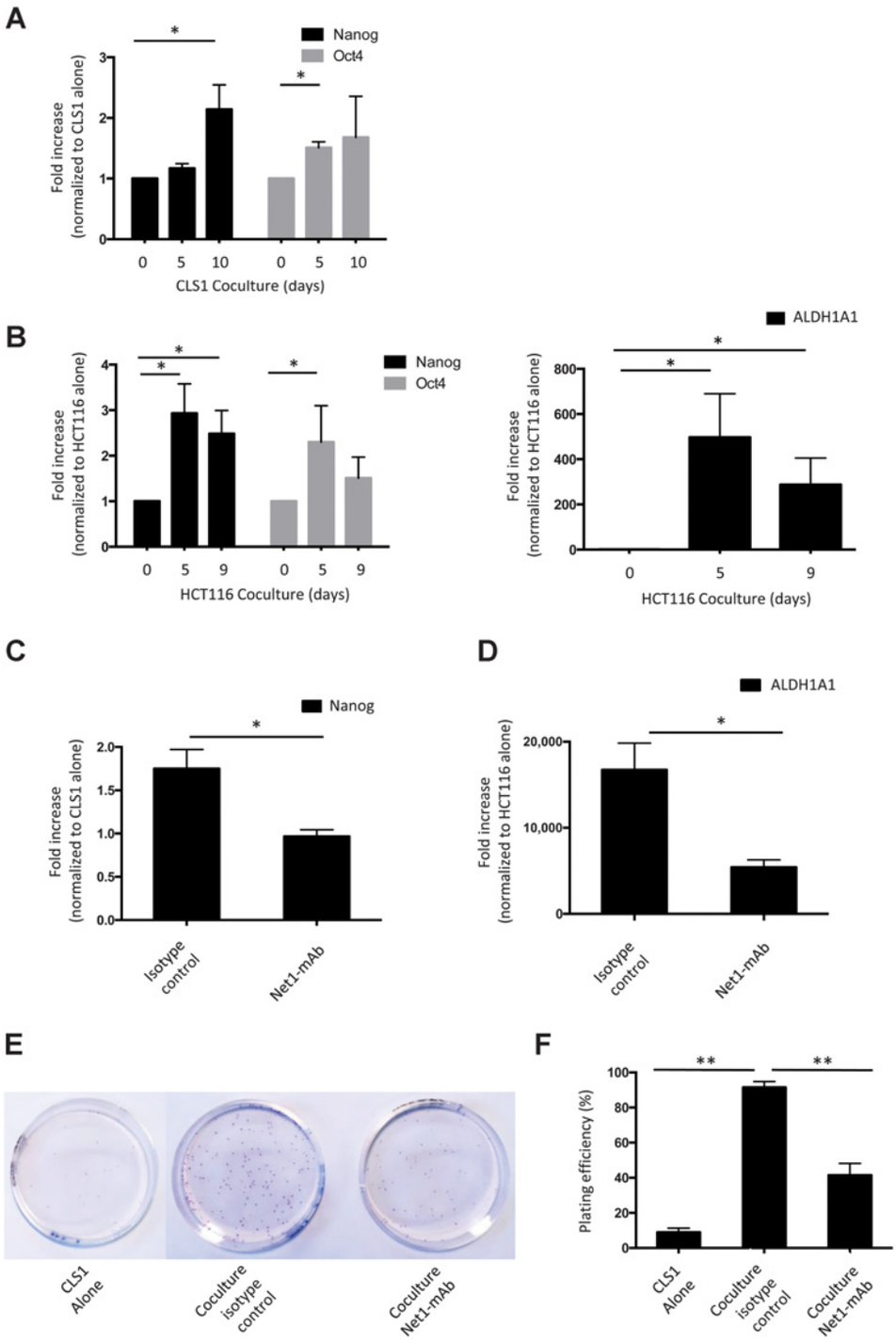

Figure 3.
Interference with CAF-produced netrin-1 inhibits cancer stemness. qRT-PCR results of various stemness markers (Nanog, Oct4, and ALDH1A1) in cocultured CLS1 or HCT116 cells. **A,** Expression of Nanog and Oct4 in CLS1 cells cocultured with lung CAFs for 5 and 10 days. **B,** Expression of Nanog, Oct4, and ALDH1A1 in HCT116 cells cocultured with colorectal CAFs for 5 and 9 days. **C,** Expression of Nanog in cocultured CLS1 cells in the absence or presence of the Net1–mAb (20 μg/mL for 7 days) compared with isotype control was decreased in Net1–mAb–treated cocultured cells. **D,** Expression of ALDH1A1 in cocultured HCT116 cells in the absence or presence of the Net1–mAb (20 μg/mL for 7 days) compared with isotype control was decreased in Net1–mAb–treated cocultured cells. **E,** Clonogenic assay. Digital images showing colonies produced by CLS1 cells alone and cocultured with NSCLC CAFs with and without Net1–mAb treatment (20 μg/mL for 7 days) following plating 200 CLS1 cells in 6-cm petri dishes and 7 days of incubation. **F,** Plating efficiency was compared among different culture conditions and treatments. Error bars, SEM; *P* values, two-tailed unpaired Student *t* test from three to four independent experiments. * $P < 0.05$; ** , $P < 0.01$.

On the basis of these results demonstrating that CAFs promoted cancer stemness in the cocultures, we then investigated whether netrin-1 produced by CAFs may be implicated in this process. We took advantage of the netrin-1/UNC5B blocking antibody that is currently used in clinical testing. Humanized Net1–mAb (NP137, Net1–mAb) specifically interacts with netrin-1 and blocks netrin-1/UNC5B interaction at nanomolar range (15). When cocultures of CLS1/CAFs and HCT116/CAFs were treated with Net1–mAb for 7 days, a significant inhibition of Nanog expression (in lung cancer CLS1 model, Fig. 3C) and ALDH1A1 expression (in colon cancer HCT116 model, Fig. 3D) in cocultured cancer cells was observed. Moreover, clonogenic assays were performed with colonies produced by CLS1 cells cultured alone and cocultured with CAFs, treated or not with Net1–mAb (Fig. 3E and F). As shown in Fig. 3E and F, cocultured CLS1 cells showed more clonogenicity than CLS1 cells cultured alone, and

Table 1. Tumor formation in response to Net1–mAb (20 μg/mL) and IL6–mAb siltuximab treatment (10 μg/mL)

Cells	No. of cells per injection	Treatment procedure	Days after injection	No. of tumor/no. of injection			% of tumor formation		
				Iso	Net1–mAb	IL6–mAb	Iso	Net1–mAb	IL6–mAb
A549 Alone	1×10^6	*Ex vivo* treatment of 20 μg/mL Net-1–mAb, 10 μg/mL IL6–mAb (or isotype control) for 7 days before injection	21	9/21	7/21	10/30	42.86	33.33	33.33
			35	16/21	12/21	15/30	76.19	57.14	50
			42	18/21	16/21	20/30	85.71	76.19	66.76
A549 + CAFs	1×10^6		21	8/20	0/20	1/16	40	0	6.25
			28	10/20	1/20	5/16	50	5	31.25
			35	17/20	6/20	12/16	85	30	75
			42	17/20	8/20	12/16	85	40	75

NOTE: Tumors with a size >50 mm^3 were considered and counted.
Abbreviation: Iso, isotype control.

treatment with Net1–mAb significantly decreased the clonogenicity of the cocultured cells.

This effect on expression of cancer stemness markers and clonogenic assay was further confirmed using engraftment in mice. Lower tumor formation rate was observed in Net1–mAb–treated groups compared with the control groups (treated with the isotype antibody) in A549/CAFs coculture models, whereas the differences between isotype control group and Net1–mAb group in mice engrafted with A549 cultured alone were much smaller (Table 1). These results further supported the view that Net1–mAb treatment on coculture decreased cancer stemness, resulting in less tumor formation. Similar results were observed in HCT116/CAF coculture models (Table 2). Less tumor uptake was observed, especially at day 10 after engraftment, in Net1–mAb–treated coculture groups compared with isotype control groups.

Complementary *in vivo* studies were then performed. As CLS1 cells do not express endogenous netrin-1, we speculated that this may represent an informative model to study *in vivo* stromal effects of netrin-1 produced by CAFs. We grafted in mouse flanks either CLS1 alone or a coculture of CLS1 with CAFs, and then systematically treated mice with either Net1–mAb or a vehicle (control). As expected, by the absence of netrin-1 expression and as described in the second part of Table 2, tumor take was not affected by Net-1–mAb treatment in mice engrafted with CLS1 cultured alone. However, when CAFs were included in the graft, not only was tumor take enhanced in the control-treated mice, but more importantly, the systemic Net1–mAb delivery clearly inhibited tumor take and tumor formation in the mice engrafted with the coculture, but not in mice engrafted with CLS1 cultured alone (Table 2). Accordingly, systemic Net1–mAb treatment did not affect tumor growth in mice grafted with CLS1 cultured alone, whereas it nicely triggered tumor growth inhibition in mice engrafted with CLS1 cocultured with CAFs (Supplementary Fig. S2).

In another set of experiments, either CLS1 cultured alone or the coculture (CLS1/CAFs) were grafted in SCID mice, followed by the treatment of Net1–mAb (or a vehicle as control) starting when tumor size reached 150–230 mm^3, and tumor growth was further measured routinely after treatment. While Net1–mAb did not affect tumor growth when CLS1 cells were grafted alone, the mice grafted with coculture of CLS1-CAFs responded significantly to Net1–mAb treatment, and this was associated with both tumor growth inhibition (Fig. 4A and B) and the reduction of tumor weight (Fig. 4C). Taken together, the *in vitro* and *in vivo* data reported here support the view that netrin-1 upregulation observed in CAFs when in closed proximity with cancer cells is implicated in CAF-mediated increase of cancer cell stemness, and that the interference of netrin-1/UNC5B interaction with anti-Net1–mAb in CAFs profoundly inhibits tumorigenicity, that is, the capacity of cancer cells to form tumors.

Netrin-1 produced by CAFs regulates secretion of plasticity-mediating cues

We thus looked for the nature of the implication of CAF-produced netrin-1 in the acquisition of cancer cell stemness. The first possible function was related to the survival activity of netrin-1. Indeed most of the literature on netrin-1 in the oncology

Table 2. Tumor formation in response to Net1–mAb treatment

Cells	No. of cells per injection	Treatment procedures	Days after injection	No. of tumor/no. of injection		% of tumor formation	
				Iso	Net1–mAb	Iso	Net1–mAb
HCT116 Alone	2×10^6	*Ex vivo* treatment of 20 μg/mL Net-1–mAb (or isotype control) for 7 days before injection	7	1/21	0/21	4.76	0
			14	6/21	9/21	28.57	42.86
			21	9/21	15/21	42.86	71.43
HCT116 + CAFs	2×10^6		7	4/33	0/33	12.12	0
			10	17/33	2/33	51.52	6.06
			14	28/33	25/33	84.85	75.76
CLS1 Alone	2×10^6	Intraperitoneal injection of Net-1–mAb (or isotype control) every 2 days on a dose of 10 mg/kg	7	0/30	0/30	0	0
			10	3/30	3/30	10	10
			14	12/30	14/30	40	46.67
CLS1 + CAFs	2×10^6		7	0/27	0/27	0	0
			10	12/27	2/27	44.44	7.41
			12	20/27	9/27	74.07	33.33
			14	23/27	16/27	85.19	59.26

NOTE: Tumors with a size >100 mm^3 were considered and counted.
Abbreviation: Iso, isotype control.

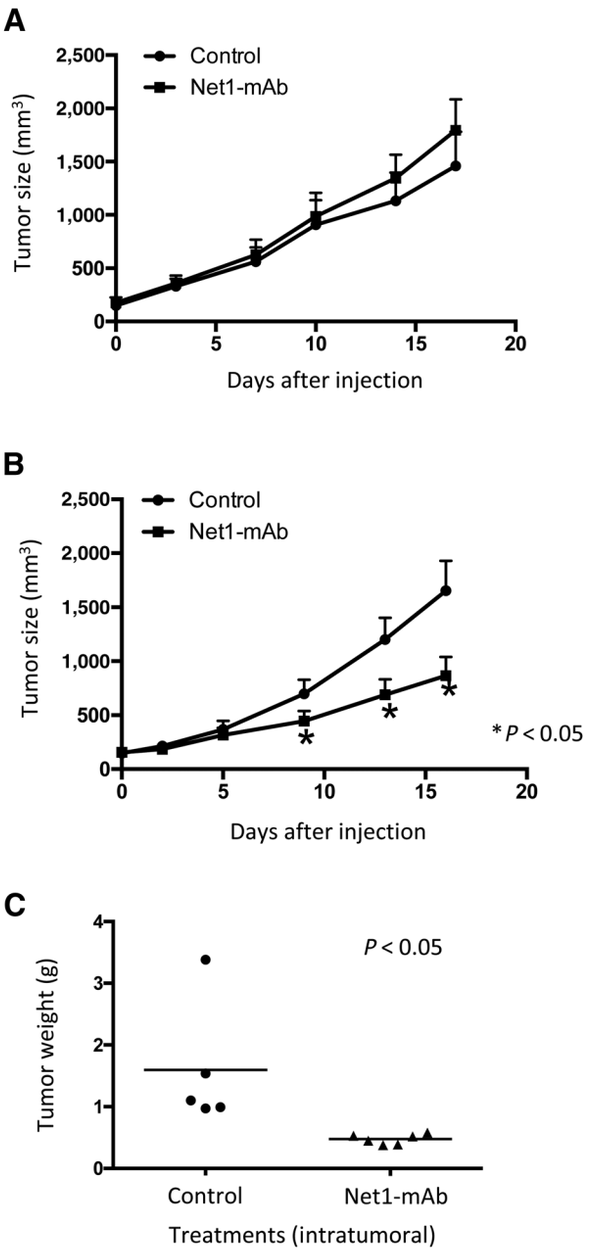

Figure 4.

Production of netrin-1 by CAFs contributes to tumor growth. **A,** Two million of CSL1 cells cultured alone were engrafted subcutaneously in SCID mice. Tumors, with a size between 150–230 mm^3, were randomly treated either with Net1–mAb (100 μg per mice) or with vehicle intratumorally for 8 consecutive days. **B,** Same procedure as described in **A**, but 2×10^6 of CLS1/CAF cocultured cells were engrafted subcutaneously in SCID mice. Significant growth inhibition caused by Net1–mAb was observed in the engraftment with CLS1/CAF, but not in CLS1 alone, indicating that netrin-1 produced by CAFs played a role in tumor growth. **C,** Tumor weight was measured 1 day after the last Net1–mAb injection. Tumors treated with Net1–mAb were significantly smaller compared with controls. Three independent *in vivo* experiments were carried out with 5–12 mice per each experimental group. Results from only one single experiment are presented here in this figure. Error bars, SEM; *P* values were determined by two-tailed unpaired Student *t* test.

field relates to the property of netrin-1 to block UNC5B/DCC-induced cell death (21). However, we did not observe any change of CAF death in the coculture conditions in the absence or presence of Net1–mAb. We thus searched by candidate approaches what could link the increase of cancer stemness and netrin-1 produced by CAFs. Of interest, at least two cytokines produced by CAFs, IL6 and IL8 have been described to be involved in the promotion of stem cell–like phenotype (22, 23). As shown in Fig. 5A and B, using coculture of CLS1 and CAFs, upon Net1–mAb treatment, changes of IL6 and IL8 at mRNA level were not significant in CAFs alone or in cancer cells alone. However, both IL6 and IL8 were found upregulated in the coculture compared with culture alone, and Net1–mAb treatment significantly decreased the expression of IL6 and IL8 in the co-coculture (Fig. 5A and B). We thus more specifically focused on the contribution of IL6 to this coculture system. Decrease of IL6 expression after netrin-1 antibody treatment was observed at the protein level, using an IL6-based ELISA assay (Fig. 5C); however, decrease of IL8 at the protein level was not observed (shown in Supplementary Fig. S3A). To determine whether IL6 was predominantly expressed in CAFs or cancer cells (CLS1), cocultures were sorted after Net1–mAb treatment. IL6 was found upregulated massively in cocultured CAFs, and significantly decreased after Net1–mAb treatment while its expression in CLS1 cocultured cells upon treatment was not changed (Fig. 5D). Similar data were observed for IL8 (Supplementary Fig. S3B).

Because of this specific change of IL6 level in CAFs upon Net1–mAb treatment, we looked at whether the inhibition of IL6 by Net1–mAb was causally implicated in the observed inhibition of cancer stemness. IL6 has been shown to mediate increased cancer stemness by activating JAK2 and STAT3 in cancer cells (23). Using treatment with a JAK2 inhibitor (AG490) or with IL6–mAb, a significant inhibition of Nanog expression in the coculture CLS1/CAFs was observed (Fig. 5E). Along this line, Table 1 shows that IL6–mAb (siltuximab) treatment of the cocultures impaired tumor intake in mice. We also performed the coculture experiment in the presence of Net1–mAb but then added recombinant human IL6 to see whether Nanog expression could be restored by adding recombinant human IL6. As shown in Fig. 5E, addition of recombinant human IL6 protein to Net1–mAb–treated coculture fully restored Nanog level to control level, thus supporting the view that netrin-1 upregulation in CAFs enhances cancer stemness by impacting the secretion of cytokines such as IL6 (Fig. 5F).

Discussion

Netrin-1 has been shown to promote cancer progression (24, 25). To date, netrin-1 has been hypothesized to do so either by promoting tumor cell survival (26, 27) or, by analogy to its role during neuronal navigation, by modulating cancer cell motility (28, 29). Although netrin-1 has been shown to be upregulated in a large fraction of human neoplasms, the prevailing assumption has been that cancer cell population upregulated netrin-1 expression in an autocrine manner (13, 30). However, using glioblastoma cell lines, Ylivinkka and colleagues recently reported that netrin-1, rather than being expressed in stem-like cancer cells, was instead expressed in the neighboring stromal cells of stem-like cells or invasive cells (such as Nestin-positive or CD133-positive cells; ref. 28). Their data suggested that netrin-1–expressing cells enhance stem-like cancer cells, potentially

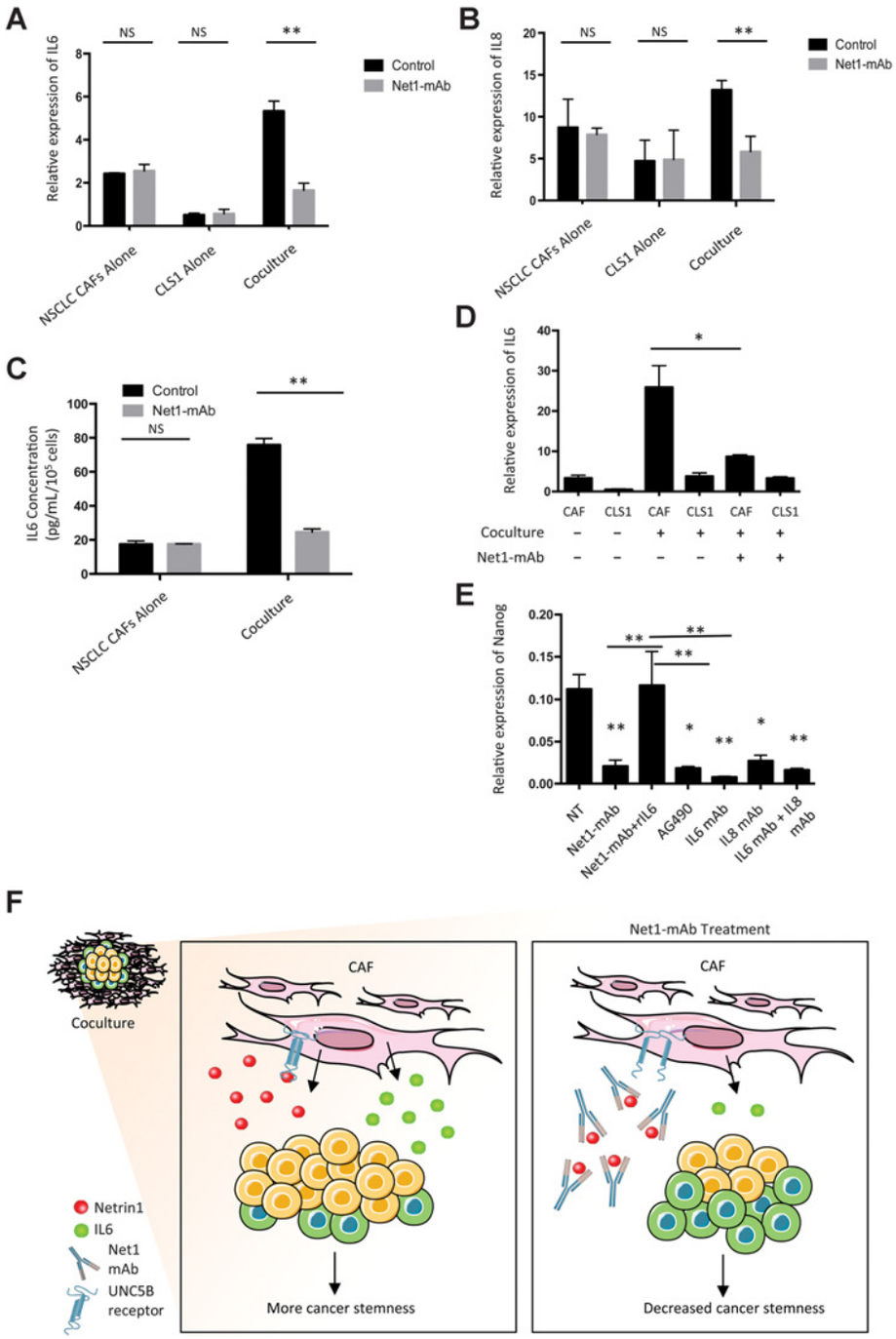

Figure 5.

Netrin-1 produced by CAFs has an impact on IL6 to promote cancer stemness. **A,** Expression of IL6 was measured by qRT-PCR in CSL1 alone, lung CAFs alone, and coculture (CLS1/CAFs) with or without treatment of Net1–mAb (20 μg/mL) for 7 days. **B,** Same as **A**, but IL8 mRNA was measured. **C,** Protein expression of IL6 was measured by ELISA in the supernatant of NSCLC CAFs alone and coculture (8 days of coculturing CLS1/CAFs) treated with and without Net1–mAb (20 μg/mL for 7 days). **D,** Same as **A** but CAFs (GFP-negative) and CSL1 cells (GFP-positive) were sorted before analysis of IL6 expression by qRT-PCR. **E,** Expression of Nanog (qRT-PCR) in CSL1/CAFs coculture treated 7 days with Net1–mAb (20 μg/mL), recombinant human IL6 protein (rIL6, 10 ng/mL), AG490 (50 μmol/L for 24 hours and decreased to 10 μmol/L for another 6 days), IL6–mAb (10 μg/mL), IL8–mAb (10 μg/mL), and combination of IL6–mAb and IL8–mAb (same concentration). Data are representative of at least three independent experiments. Error bars, SEM; *P* values were determined by two-tailed unpaired Student *t* test. **F,** Proposed scheme model showing the paracrine cross-talk between CAFs and cancer cells that may contribute to cancer stemness. Netrin-1 secreted by cocultured CAFs was required for the production of IL6 by CAFs, which in turn triggered an increase of cancer stemness. Interfering netrin-1 expression in tumor microenvironment with Net1–mAb resulted in decreased IL6 expression and consequently reduced cancer stemness. *, $P < 0.05$; **, $P < 0.01$; NS, nonsignificant.

increasing the possibility of metastasis. We demonstrated here that CAFs may also be important stromal cells expressing netrin-1. We showed, using a large colon cancer dataset and *in vitro* coculture models, that netrin-1 and its receptor (UNC5B) were upregulated in lung and colon CAFs. We observed this CAF-mediated increase of netrin-1/UNC5B expression in cocultures of non–small cell lung CAFs/non–small cell lung cancer cells and of colon CAFs/colon cancer cells but failed to see this effect in breast CAFs/cancer cells pairs (Supplementary Fig. S4A). Of interest increase of cancer stemness markers was not observed in those coculture models (Supplementary Fig. S4B). Along the same line when using a metastatic colorectal cancer cell line such as LoVo, we did not see enrichment of cancer stemness markers (i.e., expression of ALDH1A1 was already very high in LoVo cells alone, see Supplementary Fig. S5) nor upregulation of netrin-1 in CAFs, supporting the view that the netrin-1 gain is somehow intrinsically associated with an enrichment of stemness phenotype. This upregulation in CAFs does not appear to exert a paracrine effect in cancer cells, at least from the cell death/survival perspective, as we could not observe increased cancer cell survival in the coculture

nor decreased cancer cell survival in the coculture treated with Net1–mAb.

We propose here that this netrin-1/UNC5B upregulation in CAFs seems to be a mechanism triggering, probably indirectly, secretion of cytokines such as IL6/IL8, which in turn promotes cancer stemness (Fig. 5F). Inflammation has been reported to be highly associated with various types of cancer (31, 32). IL6 is a proinflammatory cytokine that is secreted by immune cells, epithelial/malignant cells, stromal cells, and fibroblasts, and plays a crucial role in promoting tumorigenesis, angiogenesis, and metastasis (32–34). IL6 has been reported to be a direct regulator of CSC self-renewal, and is involved in the induction of the epithelial–mesenchymal transition phenotype in various cancer types (35–37). More interestingly, IL6 was also reported to be expressed approximately 100-fold higher in a CAF line compared with normal fibroblasts (38). From RNA-sequencing analysis, we found that interleukin pathway–related genes were upregulated in cocultured cells. This result is consistent with the concept that interleukin family genes are highly associated with cancer stemness (33, 39–42). Furthermore, we reported that interleukin family–related genes were globally decreased in the cocultured cells treated with Net1–mAb, suggesting that inhibition of netrin-1–mediated signaling had an effect on the regulation of interleukin family–related genes, particularly IL6, consequently leading to decreased cancer stemness. Along this line, we demonstrated that Net1–mAb treatment of coculture CAF-cancer cells resulted in a decrease of stemness that was restored by adding recombinant human IL6 into the coculture. How netrin-1 impacts interleukin family expression remains to be investigated. There has been evidence showing that netrin-1 is involved in the activation of PI3/Akt and p38/MAPK via either UNC5B or DCC (43, 44) and these pathways have been shown to regulate IL6/IL8 expression in various cell culture models (45–50). Therefore, we hypothesize that CAFs, when growing in contact with cancer cells, upregulate netrin-1, which in turn enhances these signaling pathways, thus contributing to IL6 production. IL6 production is not sufficient to explain completely the effect of netrin-1 as Table 1 shows that netrin-1 interference has more pronounced effect on tumor intake in mice than IL6–mAb treatment. Even although the mechanisms linking netrin-1 upregulation and IL6 secretion remain to be elucidated, the observation that interference with netrin-1 is associated with an inhibition of this production and with an indirect effect on cancer stemness is of major interest when considering the therapeutic implications.

We provide here a series of *in vivo* models where we showed that Net1–mAb prevents tumor intake and prevent tumor growth in a CAF-dependent manner probably by impacting on cancer stemness. Clinical investigation of Net1–mAb should be explored in adjuvant settings to demonstrate that interference with netrin-1 is associated with an extension of progression-free survival. The main mode of Net1–mAb envisioned currently is the induction of cell death of tumor bulk cells. The data shown here supports the view that Net1–mAb, when considering at least in colon and NSCLC indications, has a dual effect: it may trigger cell death of the bulk tumor cells but it may also inhibit cancer stemness and thus be associated with the inhibition of cancer relapse and disease progression.

Disclosure of Potential Conflicts of Interest

S. Depil has ownership interest (including stock, patents, etc.) in Netris Pharma and is a consultant/advisory board member of Netris Pharma. P. Mehlen is founder/CEO at and has ownership interest (including stock, patents, etc.) in Netris Pharma. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.-J. Sung, S. Fiore, B. Ducarouge, D. Neves, A. Bernet

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