Nucleolin Targeting Impairs the Progression of Pancreatic Cancer and Promotes the Normalization of Tumor Vasculature

Maud-Emmanuelle Gilles, Federica Maione, Méliadisse Cossutta, Gilles Carpentier, Laure Caruana, Silvia Di Maria, Claire Houppe, Damien Destouches, Ksenya Shchors, Christopher Prochasson, Fabien Mongelard, Simona Lamba, Alberto Bardelli, Philippe Bouvet, Anne Couvelard, José Courty, Enrico Giraudo, and Ilaria Cascone

Abstract

Pancreatic cancer is a highly aggressive tumor, mostly resistant to the standard treatments. Nucleolin is overexpressed in cancers and its inhibition impairs tumor growth. Herein, we showed that nucleolin was overexpressed in human specimens of pancreatic ductal adenocarcinoma (PDAC) and that the overall survival significantly increased in patients with low levels of nucleolin. The nucleolin antagonist N6L strongly impaired the growth of primary tumors and liver metastasis in an orthotopic mouse model of PDAC (mPDAC). Similar antitumor effect of N6L has been observed in a highly angiogenic mouse model of pancreatic neuroendocrine tumor RIP-Tag2. N6L significantly inhibited both human and mouse pancreatic cell proliferation and invasion. Notably, the analysis of tumor vasculature revealed a strong increase of pericyte coverage and vessel perfusion both in mPDAC and RIP-Tag2 tumors, in parallel to an inhibition of tumor hypoxia. Nucleolin inhibition directly affected endothelial cell (EC) activation and changed a proangiogenic signature. Among the vascular activators, nucleolin inhibition significantly decreased angiopoietin-2 (Ang-2) secretion and expression in ECs, in the tumor and in the plasma of mPDAC mice. As a consequence of the observed N6L-induced tumor vessel normalization, pre-treatment with N6L efficiently improved chemotherapeutic drug delivery and increased the antitumor properties of gemcitabine in PDAC mice. In conclusion, nucleolin inhibition is a new anti-pancreatic cancer therapeutic strategy that dually blocks tumor progression and normalizes tumor vasculature, improving the delivery and efficacy of chemotherapeutic drugs. Moreover, we unveiled Ang-2 as a potential target and suitable response biomarker for N6L treatment in pancreatic cancer.

Introduction

Pancreatic cancer, which includes pancreatic ductal adenocarcinoma (PDAC) and pancreatic neuroendocrine tumors (PNET), is the fourth most common cause of cancer-related deaths worldwide (1). PDAC is a highly aggressive cancer with a very poor prognosis and an overall 5-year survival rate less than 5%. Current therapies in PDAC and PNET, consist solely of surgery followed or not by targeted or chemotherapies (2, 3).

Nucleolin is highly expressed in several types of cancer (4), and is a cancer-specific target, being localized at the cell surface of tumor cells and activated endothelial cells (ECs; refs. 5–7). Nucleolar nucleolin principally regulates mRNA transcription and ribogenesis whereas cell surface nucleolin acts as a low-affinity receptor for specific ligands (4). Moreover, nucleolin stabilizes the mRNA of antiapoptotic proteins (8). Nucleolin is a novel target for anticancer therapy as demonstrated by the effects of several nucleolin-targeting molecules (9–11). We recently developed a multivalent synthetic pseudopeptide N6L that selectively binds to nucleolin (9). N6L strongly inhibits breast cancer growth by inducing apoptosis of tumor cells and is currently in preparation for phase II clinical trials (IIP-204106; ref. 9). Interestingly, N6L, as well as a nucleolin-blocking antibody impairs both experimental and in vivo angiogenesis by targeting ECs and tumor vessels (9, 12, 13). The mechanisms of regulation of tumor angiogenesis by nucleolin are poorly described, such as the effect of nucleolin inhibition in tumor cells and stroma of pancreatic cancer.
Several molecules that regulate tumor angiogenesis are overexpressed in pancreatic cancer. In human PDAC, VEGF expression is increased and high levels of angiopoietin-2 (Ang-2) correlate with metastatic spread and poor survival of PDAC patients (14, 15). However, blood vessels in PDAC are compressed by the fibrous stroma and PDAC is poorly perfused with a consequent aberration in local blood flow and oxygenation (16). This contributes to the promotion of cancer growth, tumor hypoxia, metastasis formation, and prevents an efficient delivery of chemotherapeutic drugs (17, 18). PNETs, differently from PDAC are highly vascularized, but share the same vascular abnormality phenotype that contributes to cancer progression and metastatic dissemination (3).

On the basis of these findings our aim was to target both cancer cells and tumor vasculature in pancreatic cancer. In this work, we studied nucleolin-targeted therapy, demonstrating that N6L, hampers pancreatic cancer growth and metastasis by dually targeting cancer cell growth and tumor vasculature, and we explored the potential mechanisms of action.

Materials and Methods

The source of antibodies (Ab) and the experimental procedures not described herein are detailed in Supplementary Data.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were authenticated by Lonza and periodically provided between 2013 and 2015, cultured in EGM-2 and used until the fourth passage. BxPC-3 cells were obtained in 2014 from ATCC and frozen in 2015, cultured in EGM-2 and used until the fourth passage. Human brain vascular pericytes (HBVP) were authenticated and provided by ScienCell in 2014, cultured in DMEM 10% FBS. Human umbilical vein endothelial cells (HUVEC) were authenticated by Lonza and provided between 2013 and 2015, cultured in DMEM 10% FBS. Human brain vascular pericytes (HBVP) were authenticated and provided by ScienCell in 2014, maintained in Pericyte Medium phenol red free (PM-prf, ScienCell) containing appropriated growth supplements and used until the fourth passage. Murine pancreatic cancer cell (mPDAC), were isolated, as described in Supplementary Methods, from tumor-bearing mice. Fresh aliquots were used for each experiment. Cells were cultured in Pericyte Medium phenol red free (PM-prf, ScienCell) containing appropriated growth supplements and used until the fourth passage. Murine pancreatic cancer cell (mPDAC), were isolated, as described in Supplementary Methods, from tumor-bearing mice. Fresh aliquots were used for each experiment.

Tumor mouse models

Cohorts of female FVB/n syngenic mice were obtained from Charles River (Calco). Eight weeks of age FVB/n mice (strain code 207, weight average of 20g) female mice. One month after cell injection, mice were treated 3 times a week for the duration of 2 weeks by intraperitoneal injections with either N6L (10 mg/kg) or vehicle (saline solution) as a control. Mice were sacrificed and total tumor burden was quantified as previously described (9). All in vivo experiments were carried out with the approval of the Institutional Ethical Committees and of the Italian and French Ministries of Health in compliance with European laws and policies.

Measurement of tumor delivery of doxorubicin and vessel perfusion

To evaluate tumor vessel perfusion, 0.05 mg FITC-labeled tomato lectin (Vector laboratories) were injected intravenously into PDAC-carrying mice, as previously described (20). After 10 minutes, the animals were euthanized, and lectin distribution was visualized by fluorescent confocal microscopy.

To measure the tumor delivery of doxorubicin mice were injected with 10 mg/kg doxorubicin hydrochloride (Sigma-Aldrich) via the lateral tail vein 4 hours before sacrifice. Tumors and kidneys as controls were collected from each mouse and weighed. Samples were reexpanded in a lysis buffer (0.25 mol/L sucrose, 5 mmol/L Tris-HCl pH 7.6, 1 mmol/L MgSO4, 1 mmol/L CaCl2) and homogenized in an ice-cold Potter homogenizer. 200 μL of each homogenate was added to 10% Triton X-100 and 1.5 mL acidified isopropanol, kept at −20 °C overnight and centrifuged 15,000 × g for 20 minutes. Doxorubicin was quantified by spectrophotometric analysis at 590 nm using TECAN Infinite M1000 plate reader (Tecan). These values were calculated as the fluorescence/weight ratio of the tumor divided by the fluorescence/weight ratio of the kidney and expressed as μg equivalents/g tissue of doxorubicin. Data are mean ± SD of triplicate aliquots from tumor homogenates.

Cell transfection and cell migration

Cell transfection by siRNA was performed by following manufacturer instructions (Hiperfect, Qiagen), for siRNA sequences see Supplementary Methods.

For pericyte motility, 9 × 10^4 HUVECs per well were seeded in 6-well plate. The day after, HUVECs were transfected with 10 nmol/L siRNA or treated with 30 μmol/L N6L. Three days later HUVECs were washed and medium was replaced by EBM-2. Cell supernatant was collected 1 hour after. A total of 20 × 10^4 HBVPs were seeded in the upper chamber (with or without 400 ng/mL recombinant Ang-2) coated with 1.5 μg/mL collagen type I and

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the EC supernatant was added in the lower chamber. For each Transwell, nuclei of cells from 5 fields were counted using Leica Aristoplan microscope equipped with a CoolSNAP CCD camera.

Statistical analysis

Unless indicated otherwise, bars represent mean ± SEM (n ≥ 3). P values have been calculated using a two-tailed or one-tailed unpaired t test using GraphPad Prism software. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.00001. P values of the Kaplan–Meier curve of survival have been calculated by using the Long-rank (Mantel-Cox) test.

Results

Nucleolin is a potential new target of tumor progression in PDAC

Nucleolin is overexpressed in tumors and its inhibition hampers breast, prostate, and melanoma cancer growth and angiogenesis (4). To evaluate nucleolin as a potential target in human PDAC, we analyzed nucleolin protein expression levels in 47 tumors included in TMA. 74.5% of the tumors showed a moderate (Fig. 1C) or high nucleolin staining (Fig. 1A and B, arrows). Nontumoral pancreas, corresponding to pancreatitis (Fig. 1D), normal peritumoral tissue or normal ducts either around or included in the tumors (Fig. 1B, arrowheads) were not or very faintly stained by anti-nucleolin antibody. Next, we checked the correlation between nucleolin expression level and overall survival (OS) in patients with PDAC. Notably, the OS significantly increased in PDAC patients with low levels of nucleolin compared with patients with high levels of this protein (Fig. 1E).

Nucleolin protein level was analyzed in four different human pancreatic cancer cell lines (hPDAC), a murine PDAC cell line (mPDAC), and ECs (Supplementary Fig. S1A). Capan-2 and BxPC-3 showed similar protein level than nontumoral cells, whereas Mia PaCa-2, Panc-1 and mPDAC cell lines displayed higher nucleolin protein levels. We sought to investigate whether nucleolin inhibition affects progression of PDAC. The multivalent pseudopeptide N6L (9) bound to nucleolin in human pancreatic cancer cells Panc-1, as well as in ECs (Supplementary Fig. S1B). N6L inhibited pancreatic cancer cell growth of all cell lines cited above (Supplementary Fig. S1C). The GI50 was in a range between 5 and 36 μmol/L and increased with nucleolin levels. Coherently, N6L significantly reduced the amount of PDAC cells in S phase after 48 hours of treatment (Supplementary Fig. S1D), and the active caspase-3 levels increased after 48 hours of treatment (Supplementary Fig. S1E). Moreover, N6L strongly inhibited the migration of Mia PaCa2 and mPDAC cell lines by 69% and 72%, respectively, compared with controls (Supplementary Fig. S1F), and the invasion of mPDAC cells through a layer of Matrigel, as compared with controls (Supplementary Fig. S1G). The implication of nucleolin in PDAC cell proliferation was tested by other experimental approaches. For instance, the viability of mPDAC cells was decreased by a nucleolin blocking antibody (MS3; Supplementary Fig. S2A). Moreover, the knock down of the nucleolin gene (NCL) in mPDAC by means of CRISP-Cas9 technology caused massive death of the cells (Supplementary Fig. S2C). Nucleolin blocking antibody pretreatment decreased the efficacy of the entry of Alexa546-N6L in PDAC cells (Supplementary Fig. S2B). In line with these results, the combination of nucleolin-blocking antibody and N6L did not show a cumulative effect on cell viability (Supplementary Fig. S2A). These results suggested that N6L and nucleolin blocking antibody competed for nucleolin targeting in PDAC cells.

N6L treatment hampers PDAC growth and liver metastasis

On the basis of the high levels of nucleolin found in human PDAC (Fig. 1), and according to the observation that N6L inhibited hPDAC and mPDAC cell proliferation (Supplementary Fig. S1), we decided to assess the antitumor effect of N6L in an orthotopic mouse model of PDAC. The model was obtained by injecting mPDAC tumor cells orthotopically into the pancreas of a cohort of FVB/n syngenic mice (from here the model will be called mPDAC model). This model recapitulated many features of the human PDAC, showing a malignant epithelial neoplasm with ductal differentiation (Supplementary Fig. S3A) or sarcomatoid carcinoma (Supplementary Fig. S3B; ref. 21). Tumor tissues in mPDAC model were highly hypoxic (Supplementary Fig. S3C) and fibrotic (Supplementary Fig. S3E, arrows), and showed a high heterogeneity of vessel density as in human patients (22), with poorly vascularized regions (arrows in Supplementary Fig. S3D) and regions with a higher vessel density (arrowheads in Supplementary Fig. S3D). Notably, carbonic anhydrase 9 (CA9) expression significantly increased in parallel with
enhanced synthesis of collagen I during cancer progression in mPDAC (Supplementary Fig. S3E, S3F, and S3G). These data suggest that, similarly to the human disease, hypoxic level was well correlated with increased fibrosis in PDAC tumors. Similarly to human samples (Fig. 1A–D), nucleolin was expressed in the nuclei of pancreatic acinar cells (Supplementary Fig. S3K) but highly expressed in tumor tissues compared with healthy pancreas (Supplementary Fig. S3H, S3I, S3K, and S3L), in particular in ducts and sarcomatoid regions of the tumor (Supplementary Fig. S3H and S3I). In addition, nucleolin was significantly expressed in the tumor vasculature of the mPDAC model (Supplementary Fig. S3J).

The treatment of mPDAC mice with 10 mg/kg of N6L significantly decreased the tumor volume by 43.4% (Fig. 2A). The rate of proliferative cells decreased in PDAC tumors treated by N6L compared with controls (Fig. 2B and C) while, N6L treatment enhanced apoptosis in tumor cells (Fig. 2B and D). Because nucleolin expression is coupled to tumor cell proliferation (6), we analyzed the effect of N6L on nucleolin protein (Fig. 2B and E) and mRNA levels in tumors (Fig. 2F). Consistently, both nucleolin mRNA and protein were decreased in N6L-treated mPDAC (Fig. 2B, E, and F).

Two ways of dissemination to the liver are described in PDAC patients, through vessels and through a peritoneal dissemination. mPDAC model developed liver metastasis prominently in the liver at close contact to the peritoneal surface (Fig. 2G, arrows). The total metastatic area was quantified (Fig. 2H). Remarkably, N6L strongly reduced liver metastasis area by 67% (Fig. 2G and H). These findings are further supported by our data describing a strong effect of N6L in blocking the motility and the invasion of mouse and tumor cell lines (Supplementary Fig. S1F and S1G).

Nucleolin targeting by N6L normalizes tumor vessels and counteracts tumor hypoxia in PDAC and PNET

Because nucleolin targeting inhibits EC growth and nucleolin is significantly expressed in the vasculature of mPDAC, we investigated the effect of N6L on tumor blood vessel density and morphology. Vessel density and vessel branching were significantly decreased in N6L-treated mPDAC by 42% and by 62%, respectively (Fig. 3A, B, and C). Therefore, the effect of N6L on tumor vessel normalization in mPDAC tumors was studied by analyzing the changes in pericyte vessel coverage, perfusion, and hypoxic levels, all hallmarks of vessel normalization in cancer (23). In mPDAC model, tumor blood vessels typically had low pericyte coverage, detected with two different markers of pericytes (24). The treatment of mPDAC tumors with N6L 10 mg/kg resulted in an increase of pericyte coverage of tumor blood vessels. NG2 pericytes increased by 71% (Fig. 3A and D) and PDGFR-B pericytes by 77% (Fig. 3A and E). The treatment with N6L 2 mg/kg resulted in an increase of NG2 pericyte coverage of tumor blood vessel by 52% (Supplementary Fig. S4A), supporting a dose effect of N6L. Then, we sought to evaluate the effect of the inhibition of stromal nucleolin versus tumoral cell nucleolin on tumor vascularization. We evaluated the tumor vascularization and pericyte coverage in an orthotopic mouse model of pancreatic tumor generated by injecting PDAC cells (Panc-02) into the pancreas of wild type (NCLfloxed/Cre−/−) mice or in animals in which nucleolin was deleted in one allele (NCLfloxed/Cre+/−; Supplementary Fig. S4B and S4C). Remarkably, we observed reduced tumor vascularization (Supplementary Fig. S4D and S4E) and increased pericyte coverage in NCL−/− (Supplementary Fig. S4F and S4G), compared with NCL+/− mice.

Blood vessel perfusion is a parameter of vessel homeostasis and correlates with pericyte coverage and oxygenation (25). Interestingly, the treatment of mPDAC with N6L enhanced the perfusion of the tumor vasculature, compared with controls (Fig. 3F and G). Next, we assessed whether the tumor oxygenation levels were affected by N6L. In line with its normalizing effect, N6L reduced the hypoxic area, detected by pimonidazole staining (Fig. 3H and I). In addition, N6L strongly inhibited the expression of carbonic anhydrase 9 (CA9), a marker of hypoxia (Fig. 3J and K; ref. 26). To better assess the effects of N6L on tumor vessel normalization and the related antitumor properties, we employed a transgenic mouse model of pancreatic neuroendocrine tumor (PNET) RIP-Tag2. This model has been widely used to assess the efficacy of several antiangiogenic compounds and to evaluate the effect of vessel normalization to block tumor growth and invasion (19, 24). To this aim, we performed a regression trial (24), treating a cohort of tumor-bearing RIP-Tag2 mice with N6L. This treatment showed an inhibition of tumor growth by 40% (Fig. 4A) and an increase of tumor cell apoptosis (Fig. 4B), compared with controls. Similarly to PDAC, N6L significantly induced tumor vessel normalization by increasing pericyte coverage (by 42%; Fig. 4C and F) and enhanced the perfusion of the tumor vasculature (Fig. 4G and H). In line with these vessel normalization effects, N6L-treated tumors showed reduced vessel number and branching (Fig. 4C, D, and E).

All together, these results demonstrate that the inhibition of nucleolin induces vessel normalization in two different mouse models of pancreatic cancer by increasing pericyte coverage, vessel perfusion, and reducing intratumoral hypoxia.

Nucleolin inhibition affects EC activation and Ang-2 secretion

The mechanisms of tumor inhibition and vessel normalization by N6L were further studied. N6L does not induce apoptosis of ECs (15), and did not change the viability value of confluent ECs (Supplementary Fig. S5A), suggesting that nucleolin inhibition specifically target proliferating and activated ECs. N6L significantly decreased the percentage of ECs in S phase when compared with control-treated cells (14% vs. 27%) and increased the percentage of cells in G1 (64% vs. 47%; Fig. S5A). The involvement of nucleolin...
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A

Control  N6L

MECA32

NG2+MECA32

PDGFRα+MECA32

B

Vessel density (% relative to the control)

Control  N6L

C

Vessel branching (% relative to the control)

Control  N6L

D

NG2 Pericyte coverage (yellow channel, %)

Control  N6L

E

PDGFRα Coverage (yellow channel, %)

Control  N6L

F

Control  N6L

LECTIN-FITC

G

Lectin-positive vessels / total vessels (%)

Control  N6L

H

Control  N6L

Pimonidazole

I

Pimonidazole area (%)

Control  N6L

J

Control  N6L

Carbonic anhydrase 9

K

CA9 Staining (% relative to the control)

Control  N6L

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in EC cycle progression was further confirmed by depletion of nucleolin in ECs. In fact, nucleolin depletion by siRNA significantly decreased the percentage of ECs in S phase, and increased the amount of cells in G1 phase when compared to control ECs (4% vs. 32% and 86% vs. 42%, respectively; Fig. 5B).

Another feature of EC activation is the secretion of pro-angiogenic molecules by ECs (27). Therefore, we evaluated the effect of N6L on the basal secretion of angiogenesis-related molecules by ECs by using a proteome angiogenesis antibody array (R&D Systems). Interestingly, among the different secreted pro-angiogenic factors, we observed that Ang-2, shown to promote tumor progression and whose inhibition induces vessel normalization (28–32), was inhibited by N6L treatment (Supplementary Table S1). On the basis of this screening, we analyzed the effect of N6L on the secretion of Ang-2 by ECs. Ang-2 level decreased in the EC supernatant upon 5 hours of N6L treatment but not in EC lysates (Fig. 5C). Ang-2 was stock in Weibel and Palade bodies (WPB) and basally secreted by WPB exocytosis in activated ECs (33). Ang-2 colocalized with the WPB protein vWF in ECs (Fig. 5D, inset). Interestingly, 5 hours of N6L treatment enhanced Ang-2 content (Fig. 5D and Supplementary Table S1), indicating that N6L interferes with the turnover of Ang-2 secretion, accumulating this protein into ECs. The importance of nucleolin in Ang-2 secretion inhibition under N6L was tested (Fig. 5F). Although significant inhibition of secreted Ang-2 has been observed in siControl ECs treated with N6L, no detectable differences in Ang-2 levels were measured in siNCL-EC supernatants, compared with their respective controls (Fig. 5F). The effect of longer N6L treatment (72 hours) on Ang-2 expression was analyzed (Fig. 5H and I). Ang-2 level decreased in EC lysates at the protein level (Fig. 5H) and at the mRNA level (Fig. 5I) under N6L treatment. Coherently, Ang-2 basal secreted levels (Fig. 5F) and Ang-2 protein level in ECs (Fig. 5G) were also decreased in nucleolin-depleted cells. These data suggest that nucleolin inhibition affects Ang-2 secretion and expression. In line with the observed pro-normalizing effect of N6L, among angiogenic-related secreted molecules in ECs, we observed that PDGFβ, a factor mediating pericyte recruitment (34), was also upregulated by N6L (Supplementary Table S1), and its mRNA was significantly increased under N6L treatment (Supplementary Fig. S5B).

Together these data demonstrate that nucleolin depletion or inhibition by N6L affects EC activation by decreasing the percentage of cells in S phase and regulating angiogenic molecules involved in pericyte recruitment.

Nucleolin inhibition decreases plasma Ang-2 level in PDAC model

In human cancers Ang-2 is highly expressed by ECs in tumor blood vessels and tumor cells (35, 36). Notably, Ang-2 is expressed mostly in tumor blood vessel of the PDAC model (Supplementary Fig. S5D). Pilot studies aiming to measure the time-course of plasmatic Ang-2 amount showed that the levels of Ang-2 in the plasma were unchanged until the second week of PDAC growth and increased then after (Supplementary Fig. S5E). On the basis of these preliminary data, we next analyzed plasmatic Ang-2 level of different mice injected by PDAC cells or saline solution after 3 weeks of inoculation (Fig. 6). As expected from our previous observation, Ang-2 was significantly increased in tumor-bearing mice (Fig. 6A). To evaluate the effect of N6L treatment on secreted Ang-2 levels in vivo, we checked the plasma of control and N6L-treated mPDAC at the end of the treatment. Remarkably, Ang-2 was significantly decreased by 68% in the plasma of N6L-treated mice compared with control (Fig. 6B), whereas PDGFβ level did not change (Supplementary Fig. S5G). In parallel, Ang-2 expression was evaluated in tumors. Because tumor vessel density was decreased in N6L-treated mPDAC (Fig. 3A and B), Ang-2 expression was normalized to the tumor vessel gene MECA32 and Fig. 6C shows that Ang-2 expression decreased under N6L treatment. Notably, VEGF signaling was not affected by N6L, because VEGF-A expression (Supplementary Fig. S5F) was unchanged in N6L-treated PDAC tumors, and VEGFR2 levels was not affected in N6L-treated ECs (Supplementary Fig. S5C).

To better assess the role of secreted Ang-2 in vessel normalization, we sought to investigate whether decreased Ang-2 secretion by Ang-2-depleted or N6L-treated ECs was sufficient to induce pericyte recruitment. Pericyte migration toward the supernatants of Ang-2-depleted ECs was strongly increased compared with control (Fig. 6D). Moreover, pericyte migration toward the supernatants of ECs treated with N6L was similarly increased (Fig. 6E). Because pericytes express the Ang-2 receptor Tie-2 and Ang-2 induces dose-dependent pericyte loss on retina vessels (37), we investigated if Ang-2 could directly affect pericyte migration. Ang-2 functions are dependent to the context of angiogenic cytokines or factors regulating pericyte response (37). Recombinant Ang-2 did not affect alone the migration of control pericytes, as previously shown (37). However, recombinant Ang-2 significantly reduced the increased pericyte migration toward the supernatants of Ang-2-depleted ECs (Fig. 6D) or N6L-treated ECs (Fig. 6E). Together these data demonstrate the crucial involvement of Ang-2 in the pro-normalizing effect induced by N6L.

N6L enhances drug delivery in PDAC treatment

It has been shown that tumor vessel normalization represents a remarkably advantageous anticancer strategy, being also able to enhance drug delivery and, consequently, chemotherapy efficacy (23). To first assess whether the enhanced perfusion induced by N6L could also increase drug delivery, doxorubicin was injected in the tail vein of control or N6L-treated mice at the end of the trial period and its fluorescence intensity was plotted as a percentage relative to the control (Fig. 7A). In control mice, fluorescence intensity was not affected by N6L (Fig. 7B), whereas in N6L-treated mice, doxorubicin fluorescence intensity was significantly increased (Fig. 7C). These results suggest that the enhanced perfusion induced by N6L can also increase drug delivery, allowing increased accumulation of doxorubicin in tumors (Fig. 7D). The increased accumulation of doxorubicin in tumors after N6L treatment was confirmed by a significant reduction of tumor growth (Fig. 7E) and in vivo survival of the mice (Fig. 7F). These results suggest that N6L represents a remarkable strategy for tumor treatment and drug delivery, being able also to enhance drug delivery and, consequently, chemotherapy efficiency (23).
Figure 4.
N6L normalizes RIP-Tag2 tumor blood vessels and blocks tumor growth. **A**, Total tumor volume in 4-weeks treatment regression trial showed that the treatment with N6L reduced tumor burden by 40% compared with controls (Student t test, \(P < 0.01\); \(n = 6\) mice). Tumor sections were immunostained by anti-active caspase 3 antibody (B) or coimmunostained by the anti-MECA32 and anti-NG2 for pericyte analysis (C); scale bars, 20 \(\mu\)m. Quantification of apoptotic staining, tumor blood vessel density, tumor vessel branching, and pericyte coverage was performed as in Fig. 3 and is shown in B, D, E, F, respectively. (Student t test; \(\ast\), \(P < 0.05\); \(\ast\ast\), \(P < 0.01\); \(\ast\ast\ast\), \(P < 0.001\); \(\ast\ast\ast\ast\), \(P < 0.0001\); \(n = 5\) mice). **H**, Vessel perfusion was assessed as in Figure 3 (\(n = 4\) mice, Student t test; \(\ast\), \(P < 0.05\); \(\ast\ast\), \(P < 0.01\)) **G**, Images are representative of lectin-FITC signal.
Figure 5.
Nucleolin inhibition blocks cell cycle in G1–S and decreases Ang-2 secretion. **A** and **B**, ECs were incubated with N6L for 24 hours or transfected with siControl or siNCL. Cell-cycle progression was analyzed by BrdUrd incorporation and the percentage of cells in each phase (G0–G1, S, G2–M) is shown in the graph. **B**, Efficiency of siNCL is shown by immunoblotting analysis. **C** and **F**, Supernatants of ECs were treated with increasing concentrations of N6L for 5 hours or from siControl- or siNCL-transfected ECs were analyzed by ELISA. The concentration of Ang-2 was normalized to the whole protein amount of the corresponding cell lysates in the different treatment groups. **D**, N6L-treated ECs for 5 hours were fixed and coimmunostained with an anti-Ang-2 and anti-vWF antibodies. The Ang-2 area of staining per cell is plotted in **E**. Insets show colocalization between the two stainings. (Student t test, †, P < 0.01; ‡, P < 0.05; ‡‡, P < 0.001; n = 3 independent experiments). **G** and **H**, ECs treated by N6L at 10 μmol/L or transfected by nucleolin siRNA were lysed and Western blotting of Ang-2 in EC lysates is shown. **I**, Ang-2 mRNA level of ECs treated by N6L were quantified by qPCR and normalized to the GAPDH mRNA level. Histograms represent the fold change relative to control cells (±SEM) of three independent experiments, calculated from the \(2^{-\Delta\Delta C_t}\) (Student t test, †, P < 0.05).
and the amount of drug present into the tumor tissues was quantified. In line with the normalized vessel phenotype, N6L treatment increased by 3.5 fold the efficacy of the doxorubicin delivery to the tumors of mPDAC, compared with controls (Fig. 7A). The resistance of PDAC tumors to chemotherapies, and consequently the extremely bad prognosis for PDAC patients is at least partly due to the extremely poor perfusion of blood vessels and drug delivery (38, 39). Increasing drug delivery represents a key strategy to treat PDAC patients. To evaluate whether the effect observed in mouse tumors was also observed in human cancers, both human BxPC-3-derived orthotopic (Fig. 7B) and subcutaneous (Supplementary Fig. S5H) xenograft tumors were treated with N6L and doxorubicin was injected and quantified at the end of the treatment. N6L-treatment significantly increased the delivery of the drug into the tumor similarly to the mPDAC (Fig. 7B; Supplementary Fig. S5H). The time course of N6L treatment necessary to increase doxorubicin delivery induced by N6L was evaluated by a treatment of 1 or 2 weeks in subcutaneous BxPC-3 (Supplementary Fig. S5H). The improvement of the doxorubicin delivery by N6L was clearly improved by kinetics between the first and the second week of treatment (Supplementary Fig. S5H). Stemming from these data, we sought to investigate whether the pretreatment of N6L was able to enhance the antitumor effect of gemcitabine, the standard of care for PDAC human patients. Our preliminary data in mPDAC showed that the dose of 2 mg/kg N6L impaired tumor growth with less efficacy compared with the dose of 10 mg/kg, but was still able to induce pericyte coverage of tumor vessels (Fig. 7C; Supplementary Fig. S4A). This suboptimal antitumor dose was therefore used to test the effects of the combination of N6L and gemcitabine in mPDAC model. Gemcitabine and N6L used as single agents had a similar effect in reducing tumor growth. Remarkably, the pretreatment of mPDAC with N6L and the subsequent treatment with the combination of N6L with gemcitabine showed a greater effect in decreasing tumor volume in mPDAC, by 75% compared with the single treatments and by 82% compared with the control (Fig. 7C).

Discussion

Nucleolin inhibition is known to reduce tumor growth, and different strategies of nucleolin-targeted therapy are in development for clinical application in renal cell cancer and breast cancer (10, 11). In this study, we described for the first time nucleolin-targeted therapy in pancreatic cancer. We used a highly aggressive and invasive orthotopic mouse PDAC model and RIP-Tag2 transgenic mouse model and demonstrated the antitumoral and antimetastatic potential of the N6L pseudopeptide on pancreatic
cancer. Besides the effect of nucleolin on tumor cell proliferation, N6L targets also the tumor microenvironment reducing blood vessel area and promoting tumor vessel normalization that, in turn, impairs hypoxia and improves drug delivery.

PDAC is one of the most lethal cancers. The analysis of PDAC from human patients reveals that 74.5% of patients have a higher nucleolin level when compared with nontumoral tissues. Importantly, low level of nucleolin in PDAC correlates with increased survival of patients and may be a good prognostic factor. N6L exerts a potent antitumor and antimetastatic effect in mPDAC, by inhibiting tumor proliferation and invasion, and inducing tumor apoptosis as previously described in breast and prostate xenograft tumors (9, 40). Because the complete knock down of nucleolin in cells and adult animals was not viable, the side effects of nucleolin inhibition induced by a target therapy have to be carefully verified. The dose of N6L used in the treatment in vivo was obtained from the results of the clinical trial phase I. The safety and tolerability of N6L tested in a range of 1 to 40 mg/kg in patients with different tumor types is described at http://www.immupharma.org/cancer-treatment. Nucleolin expression is regulated by cell proliferation (10) and the antiproliferative activity of N6L was accompanied by a decrease of 50% of nucleolin expression in PDAC tumors.

Together, these findings, along with the OS observed in patients with low nucleolin level, suggest that the downmodulation of nucleolin levels in human patients by N6L treatment could contribute to the improvement of the survival in patients with pancreatic cancer.

Importantly, in PDAC, N6L induced tumor vessel normalization improving vessel perfusion and drug delivery. In addition, N6L efficiently affected tumor growth and tumor vasculature in RIP-Tag2, a mouse model highly vascularized in which antiangiogenic therapies and vessel normalization has been demonstrated to be an efficient strategy to inhibit tumor growth (20). The strong effect of N6L on vessel normalization also in this model that displays a different angiogenic pattern compared with PDAC, further corroborates the selective effect of nucleolin inhibition on tumor stroma in pancreatic cancer. To further understand the importance of nucleolin inhibition in the tumor vessel compartment, cancer vascularization was studied in a model of orthotopic PDAC developed in NCL−/− or NCL+/− background. This approach allowed us to clearly show that the single allele deletion of the stromal nucleolin is sufficient to impact the tumor vascularization, and that the tumor vessel normalization induced by nucleolin inhibition is not a secondary effect due to a reduced tumoral cell proliferation.

Nucleolin is a marker of angiogenic vessels (7) and our data support an autocrine effect of the nucleolin inhibition on ECs. During angiogenesis, EC activation induces loss-of-quiescence of ECs (33) and the secretion of proangiogenic molecules (33). Nucleolin inhibition by N6L starts a program of EC quiescence, because Ang-2 is only expressed in remodeling and activated vessels (28). It is known that the angiopoietins/TIE2 system regulates vascular development and maturation (41). Ang-1 activates TIE2 receptor and promotes vessel stabilization, while Ang-2, produced by activated ECs, promotes angiogenesis by inducing blood vessel destabilization and sprouting (41). Ang-2 blockade induces tumor stroma.
vessel stabilization, decreases angiogenesis and slows the growth of several tumor models (28–32). On the basis of these observations, we can argue that the observed pro-normalizing effect of N6L on tumor vasculature could be in part mediated by the inhibition of Ang-2 during the treatments. Notably, we observed that the depletion of Ang-2 and the inhibition of secretion by N6L in ECs are sufficient to promote pericyte recruitment. Because recombinant Ang-2 is able alone to rescue these effects, we could argue that the regulation of Ang-2 expression and secretion by ECs is crucial for pericyte recruitment. These findings suggest that the arrest of the EC cell cycle, along with the reduction of Ang-2 level in vivo, could contribute to the anti-angiogenic and pro-normalizing effect of nucleolin inhibition observed in mPDAC and RIP-Tag2 mice.

Single-agent gemcitabine is the standard-of-care treatment for PDAC patients, but the addition of targeted therapies to chemotherapy failed to show any improvement (47). One possible novel strategy to improve the current therapy in PDAC is to enhance drug delivery by targeting tumor microenvironment (39, 48, 49). However, decrease of PDAC solid stress by Shh deletion or Smoothened inhibition, increased vascular density, which in turn accelerated tumor growth and promoted metastasis (48). Interestingly, whereas the antiangiogenesis therapies fail to improve PDAC survival, VEGFR inhibition was capable to counteract tumor angiogenesis induced by reduction of stroma stiffness (48). There is a growing body of evidences highlighting, both in preclinical and clinical settings, the importance of tumor vessel normalization, described by Jain and colleagues (23, 50). It has been demonstrated that the strong reduction of tumor hypoxia and the enhancement of vessel perfusion, accompanied by improved drug delivery, is a great advantage of using a pronormalizing agent in anticancer therapies in the clinic (23). Remarkably, N6L treatment increased tumor vessel perfusion, strongly reduced tumor hypoxia and enhanced chemotherapeutic drug delivery in vivo. Consistently with the observed improved vessel perfusion and drug delivery to the tumor, pretreatment of tumors with N6L strongly enhanced the effect of gemcitabine on tumor growth in mPDAC. Further experiments will be needed to better assess the combinational effects of N6L and chemotherapeutic treatments on metastasis.

In conclusion, this work highlights a new therapeutic strategy that selectively targets nucleolin by dually targeting both cancer cells and tumor vessels in pancreatic cancer. We uncovered, for the first time, the inhibition of Ang-2 as a pro-normalizing mechanism of nucleolin-inhibition and important biomarker of N6L treatment. N6L treatment represents a new and more efficient antitumor and antiangiogenic therapy for PDAC and insulinoma and could represent a promising drug to design combination therapies with established anticancer drugs or stroma-targeting molecules.

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

Authors’ Contributions
Conception and design: F. Maione, D. Destouches, J. Courty, E. Giraudo, I. Cascone
Development of methodology: F. Maione, M. Cossutta, G. Carpenter, C. Prochasson, S. Lamba, P. Bouvet, I. Cascone
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.-E. Gilles, F. Maione, M. Cossutta, S.D. Maria, C. Houppe, K. Shchors, F. Mongelard, P. Bouvet, A. Courvelard, E. Giraudo, I. Cascone
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-E. Gilles, F. Maione, M. Cossutta, G. Carpenter, S.D. Maria, A. Bardelli, A. Courvelard, J. Courty, E. Giraudo, I. Cascone
Writing, review, and/or revision of the manuscript: M.-E. Gilles, F. Maione, M. Cossutta, G. Carpenter, D. Destouches, K. Shchors, P. Bouvet, A. Courvelard, J. Courty, E. Giraudo, I. Cascone
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.-E. Gilles, I. Canuana, C. Houppe, F. Mongelard, S. Lamba, A. Courvelard, I. Cascone
Study supervision: E. Giraudo, I. Cascone

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References
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Maud-Emmanuelle Gilles, Federica Maione, Mélissande Cossutta, et al.

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