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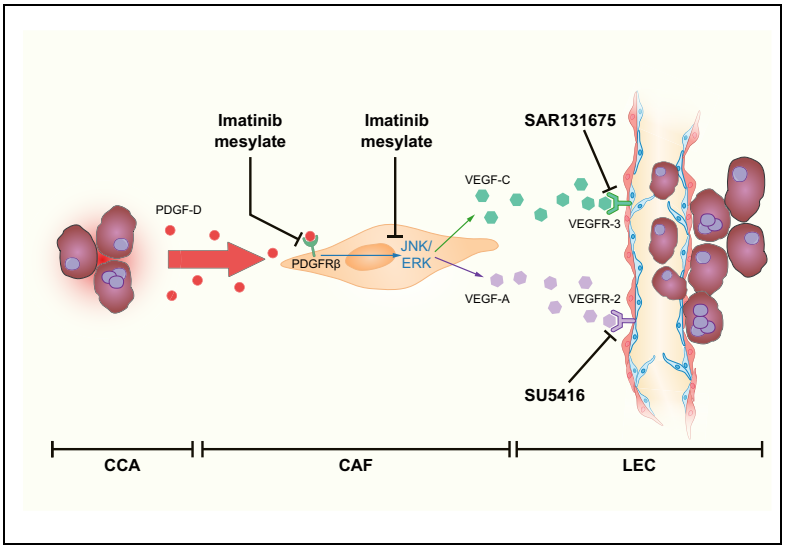
Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor lymphangiogenesis in cholangiocarcinoma

Graphical abstract



Highlights

- Cholangiocarcinomas are rich in stroma containing cancer-associated fibroblasts and lymphatic vessels.
- PDGF-D released by tumoral ducts attracts and activates liver fibroblasts to secrete VEGF-C/VEGF-A.
- Lymphangiogenesis and lymphatic invasion are driven by VEGF-A/-C released by liver myofibroblasts.
- Targeting liver myofibroblasts *in vivo* inhibits tumor-associated lymphangiogenesis and lymph node metastases.
- These studies identify new possible molecular targets for the treatment of cholangiocarcinoma.

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Lay summary

Cholangiocarcinoma is a highly malignant cancer affecting the biliary tree, which is characterized by a rich stromal reaction involving a dense population of cancer-associated fibroblasts that promote early metastatic spread. Herein, we show that cholangiocarcinoma-derived PDGF-D stimulates fibroblasts to secrete vascular growth factors. Thus, targeting fibroblasts or PDGF-D-induced signals may represent an effective tool to block tumor-associated lymphangiogenesis and reduce the invasiveness of cholangiocarcinoma.

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Platelet-derived growth factor-D enables liver myofibroblasts to promote tumor lymphangiogenesis in cholangiocarcinoma

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Background & Aims: In cholangiocarcinoma, early metastatic spread via lymphatic vessels often precludes curative therapies. Cholangiocarcinoma invasiveness is fostered by an extensive stromal reaction, enriched in cancer-associated fibroblasts (CAFs) and lymphatic endothelial cells (LECs). Cholangiocarcinoma cells recruit and activate CAFs by secreting PDGF-D. Herein, we investigated the role of PDGF-D and liver myofibroblasts in promoting lymphangiogenesis in cholangiocarcinoma.

Methods: Human cholangiocarcinoma specimens were immunostained for podoplanin (LEC marker), α-SMA (CAF marker), VEGF-A, VEGF-C, and their cognate receptors (VEGFR2, VEGFR3). VEGF-A and VEGF-C secretion was evaluated in human fibroblasts obtained from primary sclerosing cholangitis explants. Using human LECs incubated with conditioned medium from PDGF-D-stimulated fibroblasts we assessed migration, 3D vascular assembly, transendothelial electric resistance and transendothelial migration of cholangiocarcinoma cells (EGI-1). We then studied the effects of selective CAF depletion induced by the BH3 mimetic navitoclax on LEC density and lymph node metastases *in vivo*.

Results: In cholangiocarcinoma specimens, CAFs and LECs were closely adjacent. CAFs expressed VEGF-A and VEGF-C, while LECs expressed VEGFR2 and VEGFR3. Upon PDGF-D stimulation, fibroblasts secreted increased levels of VEGF-C and VEGF-A. Fibroblasts, stimulated by PDGF-D induced LEC recruitment and 3D assembly, increased LEC monolayer permeability, and promoted transendothelial EGI-1 migration. These effects were all suppressed by the PDGFRβ inhibitor, imatinib. In the rat model of cholangiocarcinoma, navitoclax-induced CAF depletion, markedly reduced lymphatic vascularization and reduced lymph node metastases.

Conclusion: PDGF-D stimulates VEGF-C and VEGF-A production by fibroblasts, resulting in expansion of the lymphatic vasculature and tumor cell intravasation. This critical process in the early metastasis of cholangiocarcinoma may be blocked by inducing CAF apoptosis or by inhibiting the PDGF-D-induced axis.

Lay summary: Cholangiocarcinoma is a highly malignant cancer affecting the biliary tree, which is characterized by a rich stromal reaction involving a dense population of cancer-associated fibroblasts that promote early metastatic spread. Herein, we show that cholangiocarcinoma-derived PDGF-D stimulates fibroblasts to secrete vascular growth factors. Thus, targeting fibroblasts or PDGF-D-induced signals may represent an effective tool to block tumor-associated lymphangiogenesis and reduce the invasiveness of cholangiocarcinoma.

Introduction

Cholangiocarcinoma (CCA) originates from the intrahepatic or extrahepatic bile ducts and carries a very poor prognosis.[1] Despite its increasing incidence, effective treatment options for CCA are scarce and limited to surgical resection or liver transplantation in few highly selected patients.[1] Less than one-third of patients are eligible for curative surgery at the time of diagnosis due to a proclivity for early lymph node metastasis.[1,2] Although mechanisms promoting CCA invasiveness are still unclear,[3] the lymphatic vessels that develop within the tumor provide an important initial route of metastatic dissemination. Indeed, several lines of evidence indicate that the expansion of the lymphatic bed correlates with both increased metastasis and poor prognosis in CCA.[4,5]

Tumor-associated lymphangiogenesis is driven by a number of soluble mediators, including vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, angiopoietin (Ang)-1 and Ang-2,

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together with their cognate receptors VEGFR2 (for VEGF-A, VEGF-C and VEGF-D), VEGFR3 (for VEGF-C and VEGF-D), and Tie2 (for angiopoietins).[6,7] In CCA, the inflammatory cells and fibroblasts of the tumor microenvironment represent the main source of VEGF.[8] In fact, as in other ductal carcinomas with pronounced invasiveness (*e.g.*, breast and pancreatic cancer),[9,10] growth of tumoral bile ducts occurs in contiguity with a rich stromal reaction, termed tumor reactive stroma, mainly composed of cancer-associated fibroblasts (CAFs), tumor-associated macrophages, and lymphatic endothelial cells (LECs).[11,12]

Within the tumor reactive stroma, a multitude of paracrine signals are exchanged between the cancer and its stromal compartment, aimed at fostering local invasiveness and metastatic spread of the epithelial counterpart.[8,13] CAFs are the most abundant cell type in the tumor stroma in CCA, and we recently demonstrated that they are locally recruited by malignant cholangiocytes that secrete platelet-derived growth factor (PDGF)-D.[14] PDGF-D is, in fact, specifically produced by CCA cells upon hypoxic stimulus, and binds its cognate receptor PDGFRβ expressed by CAFs.[14] Furthermore, the concept that CAFs are essential drivers of CCA growth has been highlighted by the observation that in a syngeneic rat model of CCA, selective CAF depletion from the tumor microenvironment by the pro-apoptotic BH3 mimetic navitoclax, a specific inhibitor of the anti-apoptotic Bcl2 proteins, suppressed tumor growth and improved animal survival.[15]

In the present study, we hypothesized that in addition to promoting CAF accumulation within the tumor stroma, PDGF-D stimulated their pro-lymphangiogenic abilities, eventually inducing the chemotaxis of LECs and their assembly in a proper vascular system favoring CCA cell intravasation. Furthermore, we tested the hypothesis that depletion of CAFs by navitoclax would reduce the lymphatic vascularization of the tumor mass and lymphatic dissemination *in vivo*.

Materials and methods

Cell lines

Human LECs (purchased from ScienCell™, US) and human male EGI-1 cells (PDGF-D expressing extrahepatic CCA cell line, purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) were employed for *in vitro* experiments. Activated stromal fibroblasts were isolated from human liver explants of primary sclerosing cholangitis (n = 3), as previously published.[14] Freshly isolated liver myofibroblasts were characterized by checking morphology and by evaluating their immunophenotype, including alpha-smooth muscle actin (α-SMA), PDGFRβ, vimentin, podoplanin, VEGFR2 and VEGFR3, and compared either with LECs or CAFs, including a primary cell line obtained from a human sample of resected intrahepatic CCA, and a commercially available cell line derived from renal carcinoma (CellBiologics, US) (Fig. S2 and Fig. S3). EGI-1 cells were characterized by expression of cytokeratin (K) 7, K19, EpCAM (clone HEA125), E-cadherin and β-catenin. Following experiments were run in cultured cells with <10 passages. Mycoplasma contamination was excluded using a specific biochemical test (Lonza, Switzerland). See Supplementary Materials for details.

Human tissue samples and immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) histological samples of surgically resected intrahepatic CCA (n = 6, 3M/3F) and hepatocellular carcinoma (HCC) (n = 6, 4M/2F) were obtained from archival tissues of Treviso Regional Hospital (Italy). Further details are given in Supplementary Methods and Tables S1 and S2.

Syngeneic rat model of CCA

To evaluate whether targeting CAFs affects tumor lymphangiogenesis and lymphatic spread *in vivo*, we used the syngeneic rat model of CCA, generated by intrahepatic injection of neoplastic, highly malignant cholangiocytes (BDEneu rat cells) into adult Fischer-344 male rats (Envigo [formerly Harlan], US). Rats were housed in a barrier facility with 12 h light–dark cycle with free access to water and standard mouse chow, in environmentally enriched cages with 2 rats for cage at a temperature of 21 °C. After allowing tumor growth for 2 weeks, animals were randomly assigned to 2 experimental groups, treated with A) navitoclax (5 mg/kg) or B) vehicle by intraperitoneum injection once daily for 10 consecutive days to selectively deplete CAFs from the tumor stroma (Fig. S4A).[15] Mice were then sacrificed. Cryosections of these rat CCA tumors with (n = 6) and without (n = 6) navitoclax treatment were stained by immunohistochemistry and dual immunofluorescence for PDGF-D, α-SMA, Lyve-1 (LEC marker) and CD31 (blood vessel marker) (same protocol as above). Slides were evaluated with respect to PDGF-D expression by CCA cells (immunohistochemistry), α-SMA density and Lyve-1$^+$ and CD31$^+$ microvascular density following CAF depletion (dual immunofluorescence). After sacrificing the rats, the abdominal cavity was opened by star-shaped incision. High-resolution photographs of the peritoneal cavity and paraaortic region were taken to assess for lymph node metastasis. Metastatic lymph nodes in both regions were counted by 2 independent observers (JM, AM). These studies were performed in accordance with and approved by the Institutional Animal Care and Use Committee (JM, AM, CF).[15]

Xenograft mouse model of CCA

To evaluate whether targeting LECs affects tumor lymphangiogenesis *in vivo*, we used the xenograft model of CCA. This was generated by intraportal injection of EGI-1 cells (500,000 cells suspended in PBS 100 μl) in male CD17/lcr-Prkdc severe combined immunodeficient (SCID) mice (6–8 weeks old; Charles River Laboratories, US), after transduction with a lentiviral vector encoding the firefly luciferase gene to enable detection of tumor engraftment by *in vivo* bioluminescence imaging before starting treatment.[14] SCID mice were housed in our Specific Pathogen Free animal facility in Allentown IVC cages (floor area 542 cm^2) with 6 mice per cage. All mice received water and food *ad libitum* and were kept under a 12 h light/dark cycle in a well-ventilated room at an approximate temperature of 22 °C. Mice were acclimatized for a minimum of 7 days and a maximum of 15 days prior to being randomly assigned to treatment or vehicle groups. Once tumor engraftment in the liver of EGI-1 cells was confirmed, mice were randomly divided into 3 experimental groups: a) controls (vehicle only, n = 6); b) SU5416 (VEGFR2 inhibitor), at the dosage of 12.5 mg/kg/day, by i.*p.* injection using micro-osmotic pumps (Alzet 1004, Durec, US) (n = 6); c) SAR131675 (VEFGFR3 inhibitor), (100 mg/kg/day), by oral *gavage* (n = 5). At the end of a 3-week treatment, mice were sacrificed and FFPE liver tissue sections were evaluated for lymphatic microvessel density (LMVD) by immunohistochemistry for Lyve-1. See Supplementary Methods and Fig. S4B for further details. Procedures involving animals and

their care conformed to the institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, December 12, 1987), and were approved by the Ethical Committee of the University of Padua (OPBA) and the italian Ministry of Health (Authorization n. 293/2017-PR).

Quantification of lymphangiogenic growth factors

ELISA was performed from liver myofibroblasts and CAF culture media following incubation with recombinant human (rh) PDGF-D (100 ng/ml, 24 h, R&D Systems) to quantify secretion of the lymphangiogenic growth factors VEGF-A, VEGF-C, VEGF-D, Ang-1 or Ang-2. See Supplementary Material for details.

Western blotting

Western blotting was performed to assess the aforementioned immunophenotype of LECs, liver myofibroblasts and CAFs, and activation of p44/42, JNK and PI3K/AKT/mTOR pathway in LECs exposed to fibroblast conditioned medium after PDGF-D stimulation. Details of Western blotting experiments are reported in Supplementary Methods, and Table S2.

In vitro assessment of lymphangiogenesis

Effects of conditioned medium harvested from fibroblasts after PDGF-D stimulation and from EGI-1 cells on LEC transwell migration and 3D tube formation assay were studied as reported in Supplementary Materials. Details of conditioned medium preparation are given in Supplementary Methods. These experiments were performed with or without antagonism of PDGFRβ (on fibroblasts), VEGFR2 and VEGFR3 (on LECs). VEGF-A and VEGF-C served as positive controls. To see if PDGF-D could exert some direct effects on LECs, LEC transwell migration was also evaluated following exposure to PDGF-D at the same conditions (100 ng/ml, 24 h).

In vitro assessment of lymphatic intravasation by CCA cells

Effects of conditioned medium harvested from fibroblasts after PDGF-D stimulation were studied even on transendothelial electric resistance (TEER) and transendothelial migration (TrEM) of EGI-1 cells transduced with a lentiviral vector encoding EGFP reporter gene, as previously described.[14] In these conditions, we also tested the effects of binding VEGF-A and VEGF-C to prevent ligand interactions with its cognate receptor, by bevacizumab (250 μg/ml)[16] and anti-VEGF-C antibody (10 μg/ml)[17]. See Supplementary Methods.

MTS assay

MTS assay was performed to check toxicity on LECs of all inhibitors (SU5416, SAR131675, navitoclax, bevacizumab) and to see if conditioned medium of PDGF-D-stimulated fibroblasts, VEGF-A, and VEGF-C induced LEC proliferation. See Supplementary Methods.

Statistical analysis

Results are shown as the mean ± standard deviation. Statistical comparisons were performed using Student's *t* test (Origin8 software, OriginLab). A *p* value <0.05 was considered significant. The number of experiments are reported in Table S2.

Results

CCA specimens show a rich lymphatic vascularization

To evaluate the extent of tumor-associated lymphangiogenesis and angiogenesis, we quantified microvascular density of podoplanin$^+$ and Lyve-1$^+$ lymphatic vessels (LMVD) and of CD34$^+$ blood vessels (blood microvessel density [BMVD]) in human CCA samples. We compared results obtained in CCA with those obtained in HCC, a primary liver malignancy typically characterized by a rich blood vascularization. Clinical data of both cohorts of patients are shown (Table S3). Compared to HCC, where extensive and strong BMVD and negligible LMVD were observed, CCA showed a marked increase in LMVD, but a less remarkable BMVD (Fig. 1A-C). These findings confirm that expansion of the lymphatic vasculature is a defining feature of the typically desmoplastic CCA, in contrast to the scarcity of lymphatic vessels observed in HCC. Importantly, since podoplanin was reported to be displayed by CAFs, we observed high coincident expression of podoplanin and Lyve-1 by dual immunofluorescence, with scant extra-lymphatic expression of podoplanin in CCAs. Thus, we confirmed that podoplanin is highly specific to identify the lymphatic vasculature in CCA (Fig. S1).

In human CCA specimens, CAFs and LECs are in close vicinity and reciprocally express VEGF ligands and receptors

We then investigated the spatial relationships between the lymphatic vasculature and CAFs in CCAs. Double immunostaining for α-SMA (CAF marker) and podoplanin (LEC marker) revealed that CAFs and LECs are in close vicinity within the tumor reactive stroma of human CCAs (Fig. 2A). By dual immunofluorescence, we further observed that CAFs expressed VEGF-A and VEGF-C, whereas their cognate receptors VEGFR2 and VEGFR3 were displayed by LECs (Fig. 2B-E and Fig. S5). Some immunostaining for VEGF-A and VEGF-C could also be observed in inflammatory cells populating the tumor stroma. Conversely, in tumoral bile ducts, VEGF-A expression was patchy, and much weaker than in CAFs, whereas VEGF-C was constantly negative (Fig. 2F,G and Fig. S5). Overall, these data are consistent with the hypothesis that paracrine signals between CAFs and LECs are responsible for the generation of a rich lymphatic plexus within the tumor microenvironment.

Immunophenotype of liver fibroblasts, CAFs and LECs

We characterized the distinctive profiles of liver fibroblasts by Western blotting. Fibroblasts and CAFs expressed α-SMA, PDGFRβ, vimentin, and podoplanin, while fibroblasts and LECs expressed podoplanin, vimentin, VEGFR2 and VEGFR3 (Fig. S2 and Fig. S3A).

PDGF-D stimulates secretion of VEGF-A and VEGF-C, but not of VEGF-D, Ang-1 and Ang-2 by human fibroblasts

To assess whether PDGF-D could provide fibroblasts with pro-lymphangiogenic functions, we stimulated primary human fibroblasts with PDGF-D, and evaluated the secretion of VEGF-A, VEGF-C, VEGF-D, Ang-1 and Ang-2, by ELISA. We found that PDGF-D-treated fibroblasts had a significant and markedly increased secretion of both VEGF-A and VEGF-C; whereas, Ang-1 was insensitive to PDGF-D stimulation and remained at much lower levels (Table 1). Of note, VEGF-D and Ang-2 secretion was never detectable. Notably, upon PDGF-D-stimulation, CAFs from both CCA and renal carcinoma increased VEGF-A

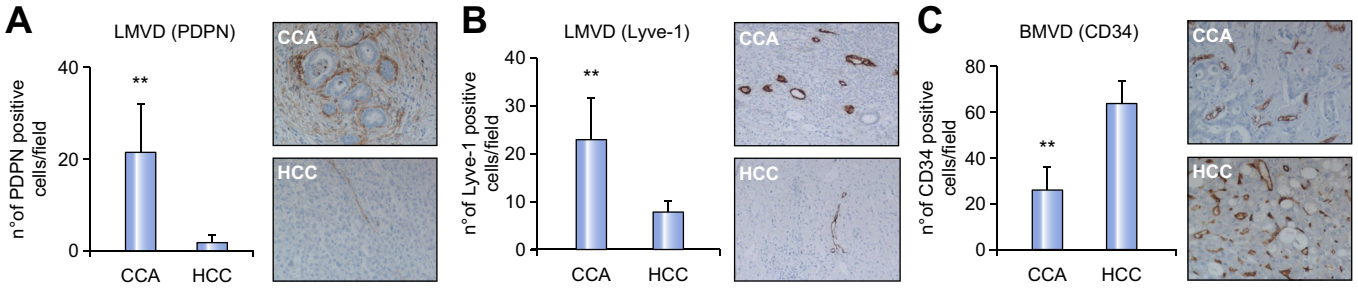

Fig. 1. In cholangiocarcinoma lymphatic microvascular density is much more preponderant than in hepatocellular carcinoma, at variance with blood microvascular density. (A-B) In human archival paraffin sections, LMVD was more extensively represented in CCA compared to HCC, as shown by IHC for podoplanin and Lyve-1 (lymphatic endothelial cell marker). (C) On the contrary, BMVD, evaluated as number of CD34$^+$ (blood endothelial cell marker) cells, was increased in HCC samples. Right-side of the plots, representative pictures of podoplanin$^+$ (A), Lyve-1$^+$ (B), and CD34$^+$ (C). Structures are shown for CCA and HCC; some faint expression of podoplanin is also expressed by CAFs. n = 6; $^*p$ <0.01, using 2-tailed *t* test. Original magnification: 200×. BMVD, blood microvascular density; CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; LMVD, lymphatic microvascular density; PDPN, podoplanin.

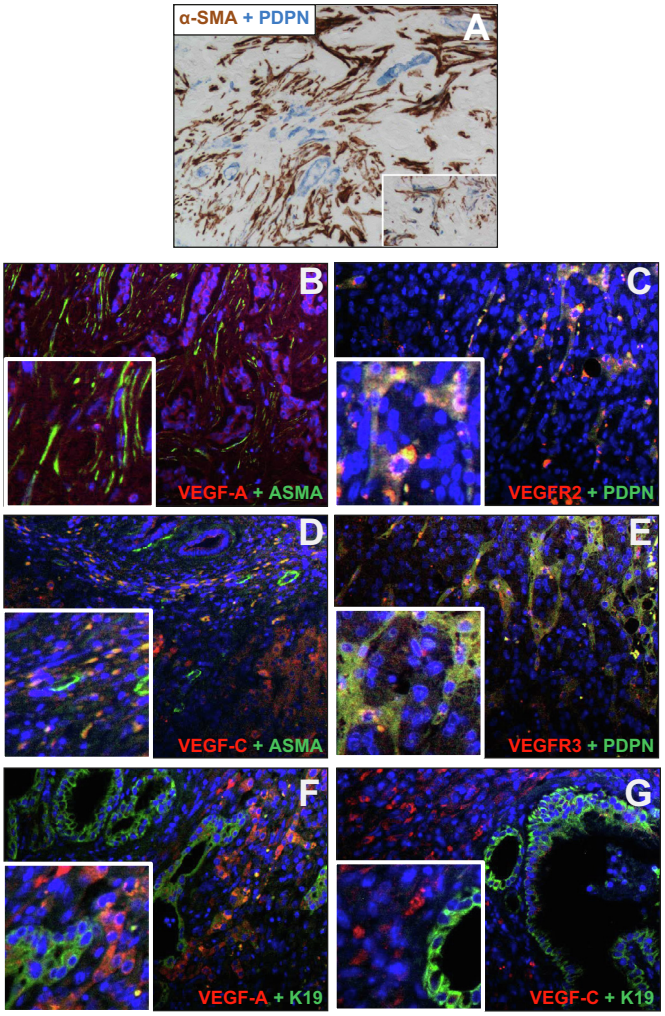

Fig. 2. In human cholangiocarcinoma specimens, lymphatic endothelial cells closely align with cancer-associated fibroblasts. (A) Within the stroma of CCA, LECs were localized in close proximity to CAFs, as shown by dual IHC for podoplanin (blue) and α-SMA (brown). (B, D) α-SMA$^+$ CAFs (green) expressed VEGF-A and VEGF-C (red), while (C, E) podoplanin$^+$ LECs (green) reciprocally expressed VEGFR2 and VEGFR3 (red). (F) In malignant cholangiocytes (K19, green), expression of VEGF-A was weak and uneven (red), whilst (G) VEGF-C (red) was constantly not expressed. Original magnification: A-G, 200×; Insets: 400×. CAFs, cancer-associated fibroblasts; IHC, immunohistochemistry; LECs, lymphatic endothelial cells; PDPN, podoplanin.

Table 1. Assessment of lymphangiogenic growth factors secreted in the supernatant by cultured human fibroblasts exposed to PDGF-D.

	Ctrl	PDGF-D
Ang-1	190.57 ± 84.68	162.27 ± 84.10
Ang-2	ND	ND
VEGF-A	188.51 ± 87.56	1152.48 ± 297.11**
VEGF-C	696.47 ± 119.20	1715.30 ± 579.83**
VEGF-D	ND	ND

ND, not detectable.
**p <0.01 *vs.* Ctrl, using 2-tailed t test.

and VEGF-C secretion levels to an extent comparable with fibroblasts (Fig. S3B). Thus, we used fibroblasts in the following experiments because they are easy to handle.

ERK and JNK signaling mediates VEGF-A and VEGF-C secretion by fibroblasts stimulated with PDGF-D

We then examined the signaling pathways that mediate VEGF-A and VEGF-C secretion by fibroblasts, upon PDGFRβ activation by PDGF-D. VEGF-A and VEGF-C were measured in supernatants from fibroblasts challenged with PDGF-D, with or without inhibitors of PDGFRβ (imatinib mesylate), or of its downstream effectors ERK (U0126) and JNK (SP600125). PDGFRβ antagonism and ERK or JNK inhibition significantly counteracted the increase in VEGF-A and VEGF-C secretion induced by PDGF-D, with comparable efficacy (Fig. 3A,B). Overall, these data indicate that 2 distinct pathways downstream of PDGFRβ, one dependent on ERK and another dependent on JNK, are activated in fibroblasts by PDGF-D, and cooperate to modulate both VEGF-A and VEGF-C secretion.

Conditioned medium from PDGF-D-activated fibroblasts is a strong stimulator of LEC migration

LEC recruitment is a prerequisite for tumor lymphangiogenesis.[7] To understand whether PDGF-D empowered fibroblasts with the ability to attract LECs, we studied LEC migration in Boyden chambers. We found that conditioned medium from fibroblasts treated with PDGF-D significantly increased the migration of LECs compared to controls, an effect completely abolished by treating fibroblasts with imatinib. The pro-migratory effect of the conditioned medium from PDGF-D-stimulated fibroblasts on LECs was comparable to that exerted by VEGF-A or VEGF-C, whilst no direct effect by PDGF-D on LEC migration was observed (Fig. 4A,B). Of note, Western

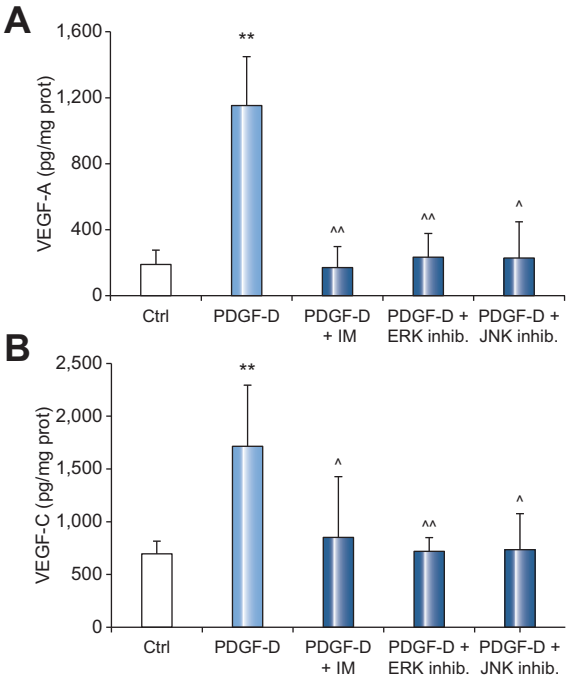

Fig. 3. PDGF-D-stimulated secretion of VEGF-A and VEGF-C by human fibroblasts is dependent on ERK and JNK activation. (A, B) While evaluating the intracellular signaling mediating VEGF-A and VEGF-C secretion by fibroblasts, we found that ERK and JNK inhibition abrogated the stimulatory effects of PDGF-D on both VEGF-A (A) and VEGF-C (B) secretory levels, similarly to the PDGFRβ inhibitor, imatinib mesylate (IM). n = 5–7 experiments in duplicate; $^{**}p$ <0.01 *vs.* Ctrl; $^{\wedge}p$ <0.05 *vs.* PDGF-D; $^{\wedge\wedge}p$ <0.01 *vs.* PDGF-D, using 2-tailed *t* test.

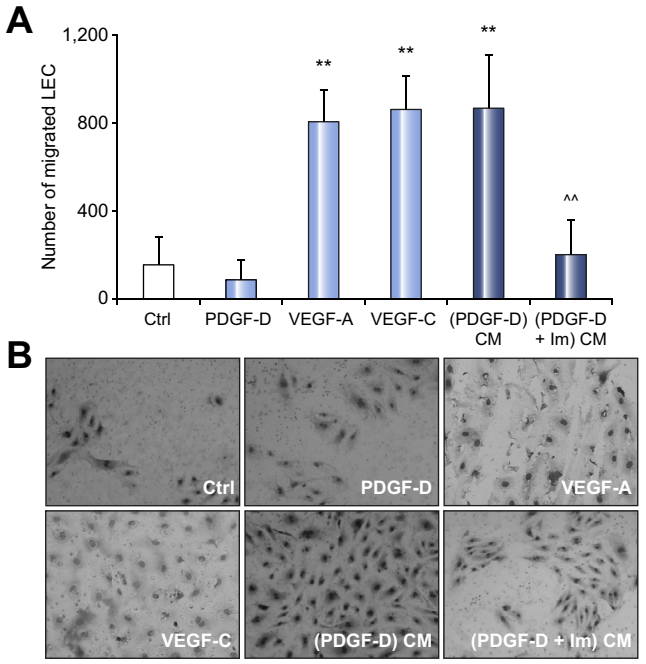

Fig. 4. Upon PDGF-D stimulation, cultured human fibroblasts promote lymphatic endothelial cell recruitment, *in vitro*. (A) CM from fibroblasts exposed to PDGF-D ((PDGF-D) CM), potently stimulated LEC migration, an effect prevented by PDGFRβ antagonism with IM ((PDGF-D + IM) CM). This effect was reproduced by VEGF-A and VEGF-C, but not by the PDGF-D itself. (B) Representative pictures of Boyden Chamber inserts (M = 200×). n = 4–11 experiments; $^{**}p$ <0.01 *vs.* Ctrl; $^{\wedge\wedge}p$ <0.01 *vs.* (PDGF-D) CM, using 2-tailed *t* test. CM, conditioned medium; IM, imatinib mesylate; LEC, lymphatic endothelial cells.

blotting analysis confirmed that LECs express VEGFR2 and VEGFR3, but not PDGFRβ, consistent with their complete lack of response to PDGF-D (Fig. S3A). Moreover, LECs migratory effects were independent of proliferation, since conditioned medium from PDGF-D-stimulated fibroblasts, as well as VEGF-A and VEGF-C exerted only a slight proliferative effect on LECs (Fig. S6A-C). LECs exposed to fibroblast conditioned medium upon PDGF-D stimulation did not show any activation of the p44/42 MAPK, JNK, and PI3K/AKT/mTOR pathways (Fig. S7A-E).

Conditioned medium from PDGF-D-activated fibroblasts, but not from EGI-1 cells, stimulates tube formation in 3D cultures of LECs

After showing the recruiting effect of conditioned medium on LECs, we sought to evaluate whether PDGF-D-stimulated fibroblasts could also drive the generation of tubular vascular structures. To this end, we generated 3D cultures of LECs, and exposed them to condition medium from either PDGF-D-stimulated fibroblasts or EGI-1 cells. Then, we performed an AngioTool software-based tubulization assay, whereby we evaluated the length of vascular branches, the number of junctions between branches and the percentage area covered by vessels.[18] Compared to controls, PDGF-D-stimulated fibroblast conditioned medium significantly increased all 3 readouts of vasculogenesis, similarly to VEGF-A and VEGF-C, while EGI-1 conditioned medium was ineffective, except for a minimal increase in the vessel area. Importantly, the effects of PDGF-D-stimulated fibroblast conditioned medium were abrogated by antagonizing either PDGFRβ on fibroblasts, or VEGFR2 (by SU5416) and VEGFR3 (by SAR131675) on LECs (Fig. 5A-D). Of note, SU5416 and SAR131675 were not cytotoxic on LECs at the chosen experimental doses, as shown by a dose-response MTS assay (Fig. S8A,B). It is important to remember that VEGF-C can act by binding to either VEGFR2 or VEGFR3, whereas VEGF-A only binds to VEGFR2.[19] Altogether, these data argue for the concept that following PDGF-D activation, fibroblasts potently stimulate lymphangiogenesis, which is not affected by EGI-1 cells.

Conditioned medium from PDGF-D-activated fibroblasts enhances the permeability of LEC monolayers

The high permeability of the lymphatic vasculature caused by defective tight junctions is conducive to tumor cell invasion.[20] Thus, to evaluate the effect of PDGF-D-stimulated fibroblasts on the permeability of lymphatic vessels, we allowed LECs to become confluent, and then measured TEER across the endothelial monolayer. We found that TEER was significantly impaired by conditioned medium from PDGF-D-stimulated fibroblasts (Fig. 6A), and this effect could be blocked by treatment of fibroblasts with imatinib. Interestingly, VEGF-C also increased the permeability of the LEC monolayer, whereas VEGF-A did not. Consistently, blocking of VEGFR3 but not VEGFR2 on LECs exposed to conditioned medium from PDGF-D-stimulated fibroblasts restored the TEER to the basal levels, similarly to the reversion induced by anti-VEGF-C and anti-VEGF-A antibodies (Fig. 6A). Overall, these results suggest that fibroblasts activated by PDGF-D can perturb the integrity of the lymphatic endothelial barrier by secreting VEGF-C, which is indeed capable of triggering the formation of intercellular gaps between adjacent LECs.[7]

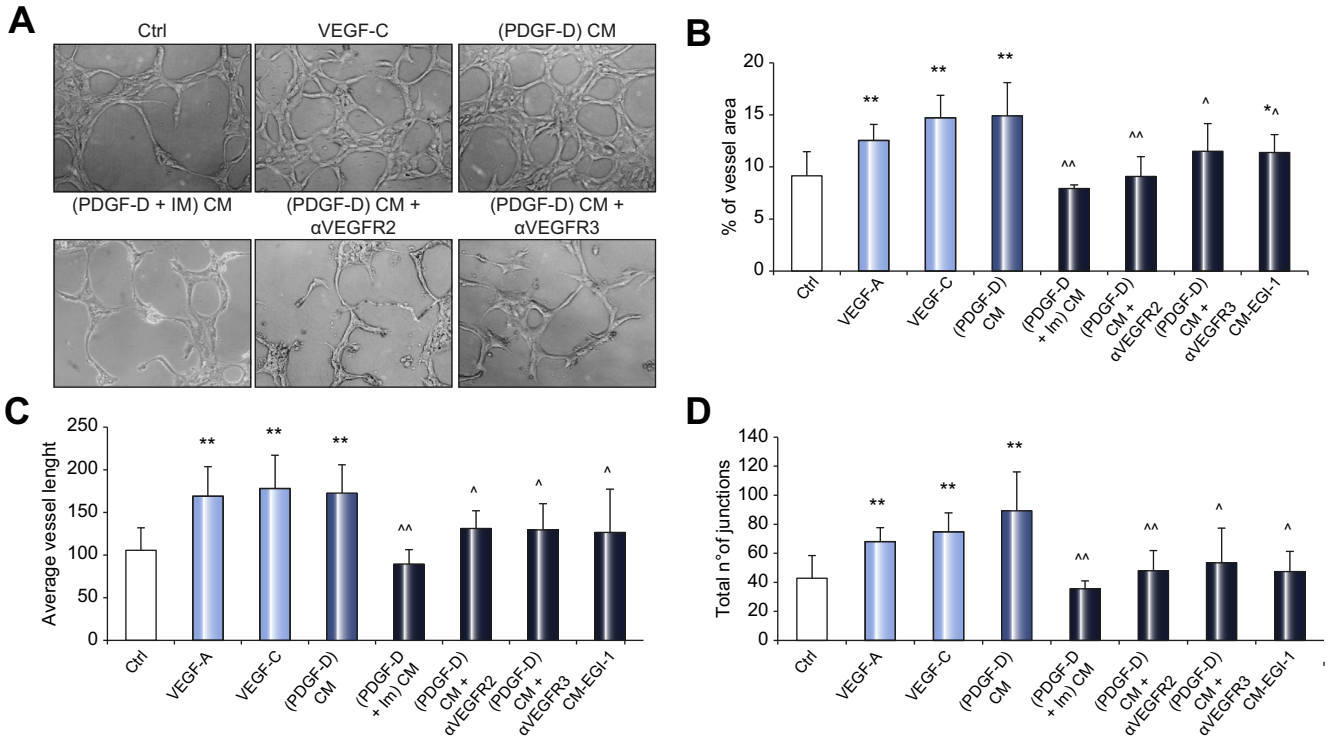

Fig. 5. Upon PDGF-D stimulation, liver fibroblasts, unlike EGI-1 cells, exert multiple lymphangiogenetic functions, inducing lumen formation, tubular branching and tubular lengthening of cultured lymphatic endothelial cells *in vitro*. (A) Representative micrographs of LEC tubulization and branching in a fibronectin/matrigel sandwich for the main treatment conditions. (B-D) CM from PDGF-D-treated fibroblasts ((PDGF-D) CM) induced 3D cultured LECs to significantly increase the vessel area (B), the vessel length (C) and the number of junctions (D), with respect to controls. These effects were significantly attenuated by PDGFRβ antagonism in PDGF-D-treated fibroblasts ((PDGF-D + IM) CM), as well as by pre-treatment of LECs with inhibitors of VEGFR2 ((PDGF-D) CM + αVEGFR2) or VEGFR3 ((PDGF-D) CM + αVEGFR3). Similar effects to CM from PDGF-D-treated fibroblasts were obtained with VEGF-A and VEGF-C, but not with CM from EGI-1 cells, which induced only a slight increase in the vessel area (B). n = 6–14 experiments; **p* <0.05 *vs.* Ctrl; ***p* <0.01 *vs.* Ctrl; ^*p* <0.05 *vs.* (PDGF-D) CM; ^^*p* <0.01 *vs.* (PDGF-D) CM, using 2-tailed *t* test. Original magnification: 200×. CM, conditioned medium; IM, imatinib mesylate; LECs, lymphatic endothelial cells.

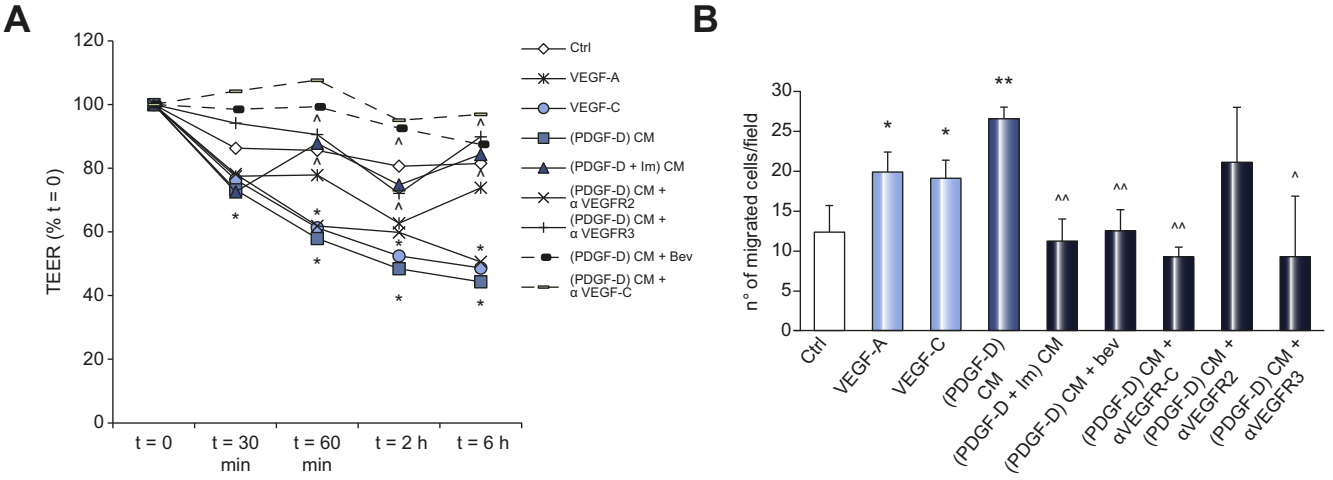

Fig. 6. Upon PDGF-D stimulation, liver fibroblasts reduce transendothelial resistance of lymphatic endothelial cell monolayers and stimulate transendothelial migration of cholangiocarcinoma cells (EGI-1-EGFP). (A) CM from PDGF-D-treated fibroblasts ((PDGF-D) CM) dramatically impaired the integrity of the lymphatic endothelial barrier, more effectively than VEGF-C and VEGF-A. This effect was only partially counteracted by the concomitant treatment with αVEGFR2 ((PDGF-D) CM + αVEGFR2), but completely abrogated by the supplementation with αVEGFR3 ((PDGF-D) CM + αVEGFR3) or with bevacizumab ((PDGF-D) CM + Bev) or anti-VEGF-C ((PDGF-D) CM + αVEGF-C), as well as by PDGFRβ antagonism in PDGF-D-treated fibroblasts ((PDGF-D + IM) CM). (B) In TrEM experiments, CM from PDGF-D-treated fibroblasts ((PDGF-D) CM) enabled the CCA cell line EGI-1-EGFP to cross the LEC monolayer (similar to VEGF-A and VEGF-C), an effect blunted by the treatment of PDGF-D-stimulated fibroblasts with PDGFRβ antagonist ((PDGF-D + IM) CM), or with bevacizumab ((PDGF-D) CM + Bev) or anti-VEGF-C ((PDGF-D) CM + αVEGF-C), or by the treatment of LEC with αVEGFR3 ((PDGF-D) CM + αVEGFR3), but not with αVEGFR2 ((PDGF-D) CM + αVEGFR2). For TEER, n = 4–15 experiments in duplicate. For TrEM, n = 3–4; **p* <0.05 *vs.* Ctrl, ***p* <0.01 *vs.* Ctrl, ^*p* <0.05 *vs.* (PDGF-D) CM, ^^*p* <0.01 *vs.* (PDGF-D) CM, using 2-tailed *t* test. CCA, cholangiocarcinoma; CM, conditioned medium; IM, imatinib mesylate; LECs, lymphatic endothelial cells; TEER, transendothelial resistance; TrEM, transendothelial migration.

Conditioned medium from PDGF-D-activated fibroblasts induces transendothelial migration of CCA cells across LEC monolayer

After showing that the permeability of LEC monolayers increased upon exposure to conditioned medium from PDGF-D-treated fibroblasts, we sought to determine whether the migration of CCA cells through the endothelial barrier was promoted as well. We seeded EGFP-expressing EGI-1 cells on top of a LEC monolayer and added conditioned medium from PDGF-D-activated fibroblasts. The number of trans-LEC migrated CCA cells was considerably increased in the conditioned medium-treated group, compared with controls. This effect was almost completely inhibited by antagonizing either PDGFRβ on fibroblasts, or VEGFR3 (but not VEGFR2) on LECs, and by neutralizing VEGF-A and VEGF-C (Fig. 6B, and Fig. S9). Toxicity of the different compounds on LECs was excluded by MTS (Fig. S8A-C).

Depletion of CAFs by navitoclax markedly decreases the lymphatic vascularization and reduces lymph node metastases in a syngeneic rat model of CCA

To confirm the pivotal role played by CAFs in directing tumor lymphangiogenesis *in vivo*, we used a well-established syngeneic rat model of CCA.[15,21,22] This model shows an intense expression of PDGF-D by CCA cells. Selective depletion of CAFs through apoptosis induced by navitoclax was accompanied by a significant decrease in the LMVD but not in the BMVD compared to control rats (Fig. 7A), without affecting the expression of PDGF-D on CCA cells (Fig. S10). This effect was accompanied by a decrease in lymph node metastases that was significant at the peritoneal region, and with a tendency towards significance at the para-aortic region (Fig. 7B). Notably, possible toxicity-related effects on hypovascularization by navitoclax were clearly ruled out by MTS assay (Fig. S8D). These data support the concept that CAFs may represent a valuable target to inhibit lymphangiogenesis and lymphatic dissemination in CCA.

VEGFR2 and VEGFR3 antagonism markedly decreases the lymphatic vascularization in a xenograft mouse model of CCA

To see if interference with the PDGF-D-driven sequence involving CAFs and LECs could significantly hamper tumor-associated lymphangiogenesis *in vivo*, we used a xenograft model of CCA in SCID mice generated by intraportal injection of human EGI-1 cells secreting PDGF-D.[15,21,22] Antagonism of both VEGFR2 (SU5416) and VEGFR3 (SAR131675) led to a significant decrease in the LMVD compared to controls (Fig. S11). Again, a possible toxicity of both compounds on LECs was excluded by MTS assay (Fig. S8A-B). These data provide *in vivo* evidence that acting on the cross-talk mechanisms governed by PDGF-D represents a useful strategy to halt CCA-associated lymphangiogenesis.

Discussion

The generation of an extensive lymphatic vascularization within the tumor reactive stroma is a key event in CCA progression. Indeed, early metastatic dissemination through the lymphatic vessels occurs in 60–70% of patients with CCA, often impairing the efficacy of curative treatments.[23,24] However, the molecular mechanisms modulating lymphangiogenesis in CCA are still poorly understood, and more generally, tumor-associated lymphangiogenesis itself is still a major knowledge gap in cancer research.

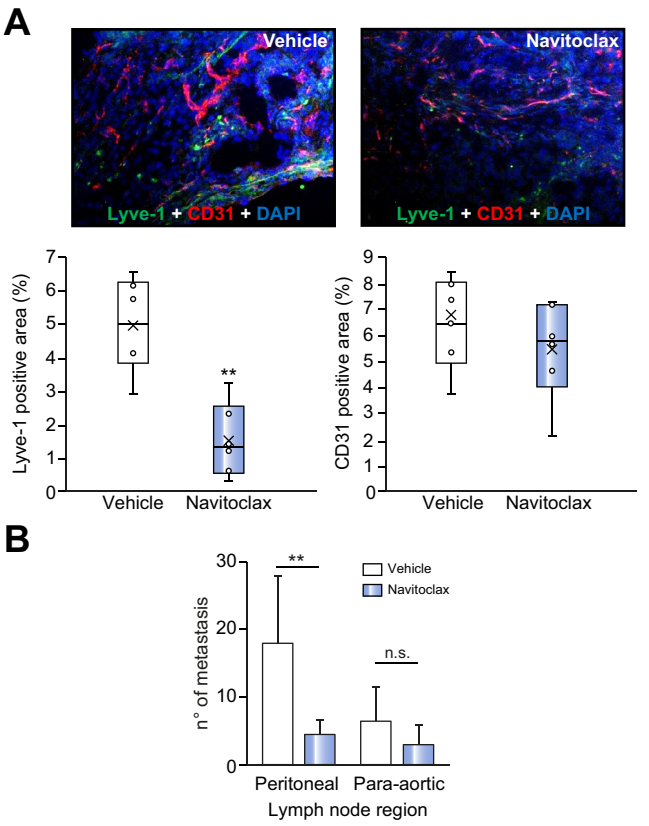

Fig. 7. In a syngeneic rat model of cholangiocarcinoma, targeting cancer-associated fibroblasts by navitoclax is associated with decreased lymphatic vascularization and lymph node metastasis. (A) In Fischer 344 male rats transplanted with BDE-neu rat CCA cells, selective depletion of CAFs by navitoclax was accompanied by a significant decrease in Lyve-1$^+$ LEC without affecting CD31$^+$ blood endothelial cells compared to untreated rats. Up-sided, representative images of CCA sections, with dual immunofluorescence for CD31 (red) and Lyve-1 (green), show the stark differences in lymphatic and blood vessels between navitoclax and vehicle groups. (B) Concomitantly, navitoclax led to a reduction in the number of lymph node metastases that was significant at the peritoneal region (p <0.05), and close to significance at the para-aortic region (p = 0.068). (n = 6 for each group). Original magnification: 100×. $^{**}p$ <0.01 *vs.* Vehicle, using 2-tailed t test. CAFs, cancer-associated fibroblasts; CCA, cholangiocarcinoma; LECs, lymphatic endothelial cells.

Epithelial-mesenchymal interactions are potent enhancers of CCA aggressiveness. PDGF-D is an important candidate in this cross-talk, due to its key role in the activation of stromal cells and generation of the tumor reactive stroma, as well as its potential druggability.[11,14] PDGF-D plays a number of roles related to tumor-promotion, by fostering cancer cell proliferation and invasiveness, fibroblast recruitment and activation, and aberrant extracellular matrix deposition.[15,25–27] As we have previously shown, PDGF-D expression is upregulated in malignant cholangiocytes under hypoxic conditions.[14] In this study, we provide evidence that in CCA, cancer cell-derived PDGF-D triggers a multi-step paracrine sequence centered around CAFs. This paracrine signaling leads to recruitment of LECs into the tumor microenvironment, as well as to their assembly into highly branched and leaky tubular structures. These newly formed lymphatic vessels can be easily invaded by cancer cells resulting in early metastatic dissemination. However, it must be underlined that malignant cholangiocytes can also secrete PDGF-B, that like PDGF-D binds PDGFRβ[28], possibly contributing to the pro-lymphangiogenic activities of PDGF-D.

First, we observed that CCA samples displayed a striking expansion of the lymphatic vascular bed compared to HCC samples. These findings support the notion that the lymphatic vasculature represents a preferential route for CCA cells to escape from the primary site of growth. Moreover, we found that the lymphatic structures within the CCA stroma were closely surrounded by CAFs. This physical proximity is most likely related to the reciprocal expression of VEGF-A and VEGF-C (which were negligibly expressed in malignant cholangiocytes) by CAFs and of their cognate receptors VEGFR2 and VEGFR3 by LECs, suggesting an intimate cross-talk between the 2 cell types. It is interesting to note that a similar pattern of expression was found in other desmoplastic epithelial cancers, such as colorectal[29] and ovarian[30] carcinomas. However, other studies performed in South-East Asia reported VEGF-C expression by malignant cholangiocytes in the intrahepatic variant of CCA[31], although the unique environmental risk factors of CCA in this geographic area might account for the discrepancy with our findings.

In an effort to reproduce the biological interactions occurring within the tumor microenvironment, we next stimulated human fibroblasts with recombinant PDGF-D. The pro-secretory effects of PDGF family members (especially, PDGF-B) on VEGF ligands have been documented in various cell types, including hepatic stellate cells[32] and pulmonary fibroblasts.[33] However, no study has focused on the pro-lymphangiogenic properties of PDGF-D, the PDGF isoform specifically expressed by CCA cells. In this study, we demonstrated that PDGF-D potently elicits the secretion of VEGF-C and VEGF-A by myofibroblasts, without affecting the release of other lymphangiogenic growth factors, such as VEGF-D, Ang-1, and Ang-2. The PDGF-D-dependent expression of both VEGF isoforms by fibroblasts was mediated by 2 pathways, namely ERK and JNK. Thus, these data add another piece to the puzzle of pleiotropic functions played by CAFs in response to PDGF-D: CAFs are not only activated, but can also induce tumor lymphangiogenesis.

To address this concept further, we assessed the influence of conditioned medium from PDGF-D-stimulated fibroblasts on LEC motility, vascular assembly and permeability. In order to rule out confounding effects possibly related to the presence of residual exogenous PDGF-D, we preliminarily evaluated the expression of PDGFRβ on LECs. We found that LECs did not express PDGFRβ, while strongly expressing both VEGFR2 and VEGFR3. PDGFRβ expression on LECs of murine and rat origin and only at the mRNA level was reported by Cao *et al.*[34] Other studies in mice reported PDGFRβ expression on LECs from large lymphatic vessels, whilst it was negative on LECs lining the small vessels.[35]

To evaluate the lymphangiogenic properties of conditioned medium from PDGF-D-treated fibroblasts, we employed the classic Boyden chamber system and the AngioTool software, which allows a reproducible quantification of different morphological and spatial parameters of newly formed vessels, including vessel length, the percentage of area covered by vessels, and the number of branch points.[36] Cultured LECs exposed to conditioned medium from PDGF-D-stimulated fibroblasts were recruited to assemble branched vascular networks, with a negligible effect on proliferation. The larger surface area covered by lymphatic structures increases the likelihood of contact with tumor cells, thereby favoring their access to the lymphatic system, an important factor for lymph node metastasis.[7,37] Notably, unlike conditioned medium from liver myofibroblasts, conditioned medium from EGI-1 cells did not play significant pro-lymphangiogenic functions.

In our model, enhanced lymphangiogenesis was dependent on the sequential activation of PDGFRβ on fibroblasts and of VEGFR2 or VEGFR3 on LECs. In this regard, it is worth noting that the blockade of PDGFRβ resulted in a stronger inhibition of vasculogenesis compared to the specific antagonism of VEGFR2 or VEGFR3. These observations hinted at the possibility that additional soluble factors released from PDGF-D-stimulated fibroblasts might act in concert with VEGF-A and VEGF-C in order to promote tumor lymphangiogenesis.

Although the correlation between the expression of lymphangiogenic growth factors and the propensity of the tumor to develop lymph node metastasis has been described in different cancer types,[38–40] the molecular mechanisms driving the invasion of tumor cells into lymphatic vessels remain elusive. Thus, to evaluate whether the pro-lymphangiogenic functions of CAFs could also promote the entry of invasive cancer cells into the lymphatic system, we assessed the TEER across LEC monolayers, and migration of CCA cells across the LEC monolayers. Indeed, LEC monolayers challenged with conditioned medium from PDGF-D-stimulated fibroblasts showed an increased permeability compared to controls, consistent with the recent observation that in colorectal cancer, VEGF-C makes the lymphatic endothelial barrier looser by weakening tight and adherent junctions.[41] Furthermore, CCA cells were able to cross LEC monolayers more easily upon endothelial cell exposure to conditioned medium from PDGF-D-stimulated fibroblasts. Notably, both effects were reversed by blocking either PDGFRβ on fibroblasts or VEGFR3 on LECs, or by neutralizing VEGF-A and VEGF-C, but not blocking VEGFR2. This suggests that even though VEGF-C and VEGF-A possess partially overlapping functions in the initial establishment of the lymphatic vascular network, the increase in endothelial permeability is primarily an effect of VEGF-C. Taken together, these data demonstrate that PDGF-D is indeed a key factor secreted by CCA cells to recruit myofibroblasts and then turn them into potent pro-lymphangiogenic players, essential for permitting the lymphatic invasion and dissemination of tumor cells.

To confirm that CAFs are central actors in tumor lymphangiogenesis in CCA, we used a syngeneic rat model of CCA.[21,22] This animal model is characterized by a highly desmoplastic tumor mass expressing PDGF-D and early metastasis. Selective depletion of CAFs was achieved by treating rats with the anti-apoptotic protein inhibitor navitoclax.[15] The ability of navitoclax to selectively target CAFs is dependent on the distinctive expression profile of Bcl-2 family members (increased expression of Bax proteins and absence of Mcl-1 expression) compared with quiescent fibroblasts and CCA cells.[15] Importantly, CAF depletion was paralleled by a stark decrease in the lymphatic vascular system embedded in the tumor mass, coupled with a significant reduction in lymph node metastases at the peritoneal region, without affecting the blood vascularization. This suggests that lymphatics strictly depend on cues originating from CAFs, whereas tumoral neoangiogenesis can be sustained by signals derived from other cell types. Turning to a xenograft model of CCA induced by intraportal injection of EGI-1 cells, we found that therapeutic inhibition of the PDGF-D-driven multistep mechanism at the level of VEGFR2 (SU5416) and VEGFR3 (SAR131675) expressed by LECs significantly dampened the tumor-associated lymphangiogenesis.

In conclusion, our results unveil the presence of a paracrine loop within the tumor reactive stroma of CCA, unleashed by PDGF-D, involving CCA cells, CAFs and LECs, and able to orchestrate tumor-associated lymphangiogenesis and tumor cell intravasation (see graphical abstract). This process is a prerequisite for the dissemination of cancer cells to the regional lymph nodes, which is an early, frequent and feared event in CCA, precluding potential curative therapies. This working model provides a series of putative molecular targets for therapeutic interventions aimed at halting the metastatic dissemination of CCA. Several molecules are available to interfere with the different arms of the described paracrine mechanism, encompassing VEGFR inhibitors, PDGFR inhibitors, and inhibitors of CAF-specific anti-apoptotic proteins.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

MC was involved in study concept and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. SB was involved in acquisition of data, analysis and interpretation of data, and drafting of the manuscript. JM provided the experimental *in vivo* rodent model and critically revised the manuscript for important intellectual content. MV, AM, CF, CM, MCM, EM and VM were involved in acquisition of data, and analysis and interpretation of data. TS and MM provided technical and material support with human samples. GN, LDO and SI provided technical and material support with xenograft experiments. CS, RF and SI critically revised the manuscript for important intellectual content. MS and FL were involved in study concept and design, analysis and interpretation of data, revision of the manuscript, fund raising, and study supervision.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.12.004.

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Author names in bold designate shared co-first authorship

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