Endothelial deficiency of L1 reduces tumor angiogenesis and promotes vessel normalization

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While tumor blood vessels share many characteristics with normal vasculature, they also exhibit morphological and functional aberrancies. For example, the neural adhesion molecule L1, which mediates neurite outgrowth, fasciculation, and pathfinding, is expressed on tumor vasculature. Here, using an orthotopic mouse model of pancreatic carcinoma, we evaluated L1 functionality in cancer vessels. Tumor-bearing mice specifically lacking L1 in endothelial cells or treated with anti-L1 antibodies exhibited decreased angiogenesis and improved vascular stabilization, leading to reduced tumor growth and metastasis. In line with these dramatic effects of L1 on tumor vasculature, the ectopic expression of L1 in cultured endothelial cells (ECs) promoted phenotypical and functional alterations, including proliferation, migration, tubulogenesis, enhanced vascular permeability, and endothelial-to-mesenchymal transition. L1 induced global changes in the EC transcriptome, altering several regulatory networks that underlie endothelial pathophysiology, including JAK/STAT-mediated pathways. In particular, L1 induced IL-6–mediated STAT3 phosphorylation, and inhibition of the IL-6/JAK/STAT signaling axis prevented L1-induced EC proliferation and migration. Evaluation of patient samples revealed that, compared with that in noncancerous tissue, L1 expression is specifically enhanced in blood vessels of human pancreatic carcinomas and in vessels of other tumor types. Together, these data indicate that endothelial L1 orchestrates multiple cancer vessel functions and represents a potential target for tumor vascular-specific therapies.

Introduction

Cancer growth strictly depends on the expansion of the host vasculature, which not only supplies oxygen and nutrients to the tumor tissue, but also provides cancer cells with the metastatic route to colonize distant organs. Therefore, angiogenesis represents a critical process during tumor initiation and malignant progression (1). Different strategies have been developed to reduce angiogenesis and thus control tumor progression, as exemplified by the inhibition of VEGF-dependent pathways. However, while some cancer types show a certain degree of therapeutic response, the benefits of such antiangiogenic agents are transient and the initial response is often followed by the establishment of resistance and escape mechanisms, leading to tumor relapse (2, 3). This highlights the need for a more comprehensive understanding of the biological processes that underlie tumor vascularization, which, in turn, would set the stage for additional angiogenesis-targeted therapies.

Compared with their normal counterparts, tumor vessels are aberrant in almost all aspects of their structure and function. They are heterogeneous and tortuous, branch chaotically, and have an uneven vessel lumen. In addition, they frequently lack pericyte coverage and show an abnormal basement membrane, resulting in vascular instability and altered permeability. These vessel abnormalities generate a promalignant microenvironment, characterized by hypoxia, low pH, and high fluid pressure, which can select for more malignant cancer cells and facilitate their dissemination through leaky vessels, thus causing poor response to therapy (4). These findings raise the question of whether tumor vessel normalization provides an alternative therapeutic opportunity in order to reduce metastatic spread and enhance tumor responses to chemotherapy and radiotherapy (4). However, the characteristics that make tumor-associated endothelial cells (ECs) different from normal ECs are not yet fully identified, and their properties are usually extrapolated from the behavior of ECs during vascular development. Moreover, while it is well known that different and morphologically distinct EC types, such as tip, stalk, and phalanx cells, coexist during normal vascularization, the heterogeneity of cancer-associated ECs is still poorly understood. Hence, a deeper understanding of the phenotypical heterogeneity and specific molecular signature of tumor vasculature is essential to elucidat-
Besides the nervous system, L1 is expressed in many human cancers, including ovarian and endometrial carcinoma, pancreatic ductal adenocarcinoma (PDAC), melanoma and glioblastoma. L1 expression confers motile and invasive properties to tumor cells, supporting cancer growth, metastasis, and chemoresistance and often acting as a marker of poor prognosis (8). L1 has also been detected in the hematopoietic system, in particular in immune cells of myelomonocytic and lymphoid origin (9), and we have previously reported L1-dependent transmigration of dendritic cells across the endothelium (10).

An intriguing aspect of L1 biology is its expression in the vascular system: while no or very little L1 is detectable in the vasculature of most normal tissues, its level is markedly increased in the vasculature of pathological angiogenesis and to identifying novel cancer vessel-specific markers.

L1 (also known as L1CAM or CD171) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily and is composed of an extracellular portion, containing 6 Ig-like domains and 5 fibronectin type III repeats, followed by a transmembrane region, and a highly conserved cytoplasmic tail (5). L1 was discovered and characterized as a cell-adhesion molecule in the nervous system (6), where it is involved in neurite outgrowth and fasciculation as well as cell adhesion and migration. In addition to homophilic binding, L1 can establish cis- or trans-interactions with different binding partners, such as integrins, CD24, neurocan, neuropilin-1, and other members of the neural cell adhesion family (7).

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Figure 1. Ablation of endothelial L1 in Tie2-Cre;L1floxed mice and related effects on tumor development and mouse survival. (A) Sections of normal pancreas and Panc02 tumors from L1floxed and Tie2-Cre;L1floxed mice were costained for L1 (green) and the vascular marker PECAM-1 (red), followed by confocal analysis. Arrows indicate vessels coexpressing PECAM-1 and L1; arrowheads indicate L1-positive nerves that served as internal control. Scale bars: 10 μm. (B and C) The volume (B) and weight (C) of pancreatic tumors from L1floxed and Tie2-Cre;L1floxed mice were recorded 14 days after Panc02 injection. Data represent mean ± SEM from 10 mice per group. (D) Images of explanted tumors. (E) Survival rates in Panc02 tumor-bearing L1floxed (n = 14) and Tie2-Cre;L1floxed mice (n = 13). **P < 0.01; ***P < 0.001.
L1 is associated with reduced tumor growth and increased apoptosis. This effect was accompanied by an increase in TUNEL-positive apoptotic cells in Tie2-Cre;L1floxed tumors (Supplemental Figure 4A), while cancer cell proliferation, as assessed by staining for either Ki-67 or phospho-histone H3, was not affected (Supplemental Figure 4, B and C). These data indicated that vascular L1 deficiency does not affect immune cell migration into cancer tissue and, therefore, any effect on tumor behavior upon Tie2 promoter–driven ablation of L1 in Tie2-Cre;L1floxed mice should be attributed to endothelial L1 rather than to L1 expressed in immune cells.

We then analyzed Panc02 tumors 14 days after implantation. Pancreatic tumor burden was markedly reduced in Tie2-Cre;L1floxed mice (Figure 1, B and C), implicating vascular L1 in cancer growth. This effect was accompanied by an increase in TUNEL–positive apoptotic cells in Tie2-Cre;L1floxed tumors (Supplemental Figure 4A), while cancer cell proliferation, as assessed by staining for either Ki-67 or phospho-histone H3, was not affected (Supplemental Figure 4, B and C). These data indicated that vascular L1 deficiency is associated with reduced tumor growth and increased apoptosis.

A macroscopical examination revealed that tumors of L1floxed mice exhibited intensely red areas, suggesting high tumor vascularization and/or hemorrhages. Notably, this feature was greatly reduced in tumors from Tie2-CreL1floxed mice (Figure 1D), possibly implicating endothelial L1 in tumor angiogenesis and/or vascular permeability.

Vascular L1 also appeared to be involved in the dissemination of Panc02 tumors, since only 29% of Tie2-Cre;L1floxed mice (2/7) showed metastatic lesions in different abdominal organs, while metastases were detected in 71% of L1floxed mice (5/7) (Table 1). Consistent with the reduced cancer growth and dissemination, tumor-bearing Tie2-Cre;L1floxed mice also showed longer survival times than their control littermates (Figure 1E). Overall, these results indicated that the ablation of endothelial L1 results in decreased tumor malignancy.

**Endothelial L1 regulates tumor angiogenesis and vascular normalization.** To determine whether endothelial L1 plays any role in cancer vascularization, we measured the microvessel density in tumor tissue. Panc02 tumors in Tie2-Cre;L1floxed mice exhibited a reduced number of vessels as compared with L1floxed mice, indicating that endothelial L1 is involved in tumor angiogenesis (Figure 2, A and B). These findings were further supported by in vivo
disruption of endothelial polarity, with no or very low level of collagen IV, the irregular distribution of podocalyxin within the vessel wall, and a lack of VE-cadherin–marked junctions (Figure 2E).

In contrast, the vasculature of Tie2-Cre;L1floxed tumors displayed abundant deposition of collagen IV in the basement membrane (Figure 2, E and F), localization of podocalyxin at the luminal surface, and a regular pattern of VE-cadherin localization, consistent with its accumulation at cell-cell contacts (Figure 2E). These findings implicated L1 in the maintenance of endothelial polarity.

Vascular abnormalities such as defective pericyte coverage, altered polarity, and disorganized endothelial junctions, lead to disruption of endothelial barrier function and, hence, to vascular leakage, thus implying that L1 might regulate vascular permeability. To test this hypothesis, we assessed the extravasation of intravenously injected 40-kDa dextran in Panc02 tumors. Indeed, tumor vascular permeability to dextran was remarkably higher in control, L1floxed mice as compared with Tie2-Cre;L1floxed mice (Figure 2, G and H), thus implicating L1 in tumor vessel leakiness.

Taken together, these observations point to endothelial L1 as a causal player in enhanced tumor angiogenesis as well as in the disruption of endothelial polarity, with no or very low level of collagen IV, the irregular distribution of podocalyxin within the vessel wall, and a lack of VE-cadherin–marked junctions (Figure 2E).
L1 orchestrates the angiogenic behavior of ECs. The reduced tumor microvessel density upon ablation of L1 in the endothelium prompted us to test whether L1 is involved in the critical EC processes that underlie angiogenesis, i.e., proliferation, migration, and tubulogenesis. Toward this goal, we employed immortalized mouse lung ECs (luECs) because, due to the expression of moderate levels of endogenous L1 (Supplemental Figure 7A), they were amenable to both gain- and loss-of-function studies. The luECs were stably transfected with the murine L1 cDNA (Supplemental Figure 7, A and B). As shown in Figure 4A, forced expression of L1 resulted in increased luEC proliferation. This result was confirmed in a classical in vivo assay for EC proliferation (18), where luECs were injected subcutaneously into immunodeficient mice and allowed to form hemangioma-like lesions. As shown in Figure 4B and C, hemangiomas formed by L1-transfected luECs exhibited a markedly higher growth rate than those formed by control luECs, consistent with our in vitro results on L1-induced proliferation of ECs. To further validate and extend these observations, we employed a loss-of-function approach. The knockdown of L1 with 2 different siRNAs (Supplemental Figure 8A) was accompanied by a decrease in luEC proliferation compared with control siRNA-transfected cells (Supplemental Figure 8B). In addition, L1 silencing also reduced the proliferative response of luECs to TNF-α stimulation (Supplemental Figure 8C). Thus, L1 plays a pivotal role in EC proliferation.

Next, a scratch-wound assay was used to evaluate the possible effect of L1 on EC migration. L1-transfected luECs showed a higher migration rate than control cells (Figure 4D and Supplemental Figure 7C). Conversely, L1 gene silencing resulted in decreased luEC migration (Supplemental Figure 8, D and E). These data demonstrate that L1 is causally involved in EC migration.

We also assessed the effect of L1 in in vitro angiogenesis assays, determining the ability of ECs to form tube-like structures in 3D reconstituted extracellular matrix. As shown in Figure 4E and Supplemental Figure 7D, forced expression of L1 significantly enhanced luEC tube formation as compared with control ECs.

Thus, L1 regulates EC proliferation, migration, and tubulogenesis, considered to be key cellular processes during the angiogenic cascade.
Our data on Tie2-Cre;L1 floxed mice indicated that L1 modulates the permeability of tumor vessels. Since vascular permeability is strictly related to the organization and function of cell-cell junctions in the endothelium, we determined whether L1 affected the expression and localization of junctional proteins in luECs. Staining for 2 prototypical adhesion molecules of the endothelium, PECAM-1 and VE-cadherin, showed that the architecture of cell-cell boundaries was disorganized in L1-transfected luECs (Figure 4F), despite the fact that the total level of either adhesion molecule was unaffected (Supplemental Figure 9A). This observation was consistent with the in vivo data showing that the altered localization of VE-cadherin in the vasculature of Panc02 tumors was reverted upon endothelial ablation of L1 in Tie2-Cre;L1 floxed mice (Figure 2E). We found no differences in the levels of other components of the interendothelial adhesion complexes, such as 4 cadherin family members (α, β, γ, and p120-catenin) and junctional adhesion molecule-A (Supplemental Figure 9B). We then tested whether L1 affected the expression of claudin-5, an essential component of tight junctions widely implicated in the barrier function of endothelium (19). The level of claudin-5 mRNA was significantly downregulated in L1-overexpressing luECs as compared with mock-transfected cells (Figure 4G), indicating that L1 negatively regulates its expression. The immunoblotting analysis also confirmed the decrease of claudin-5 in L1-transfected luECs (Supplemental Figure 10A). In line with these findings, the immunofluorescence (IF) staining of Panc02 tumors revealed that no or very little claudin-5 was present in L1 floxed tumor vessels, while the protein was readily detectable in the tumor vasculature of Tie2-Cre;L1 floxed mice (Supplemental Figure 10B), implicating L1 in the regulation of claudin-5 expression in ECs.

Interendothelial junctions play a key role in the integrity of vascular barrier (20). Therefore, given the junctional alterations induced by L1, we tested whether endothelial permeability was modified by L1 overexpression. Indeed, the permeability of confluent luEC monolayers to FITC-labeled dextran was dramatically increased upon forced expression of L1 (Figure 4H), in agreement with the reduced permeability observed in the vasculature of Tie2-Cre;L1 floxed mouse tumors (Figure 2, G and H).
destabilizes the vasculature not only by altering pericyte coverage and collagen IV deposition, but also via EC-autonomous effects on the localization and/or expression of certain junctional components, therefore orchestrating intercellular adhesion.

**L1 promotes EndMT.** The morphological and functional changes that L1 imparted on ECs, such as increased permeability, loosening of cell-cell junctions and a migratory phenotype, represent typical aspects of the EndMT, a process that has been causally linked to cancer progression (12). In the course of EndMT, ECs gain the expression of mesenchymal markers. Therefore, to verify whether L1 induced bona fide EndMT, we tested whether it promoted the acquisition of mesenchymal markers.

The overexpression of L1 in luECs, indeed, resulted in the upregulation of S100A4, N-cadherin, fibronectin, and Id1 (Figure 5A), all events associated with EndMT (21). Notably, we also observed markedly reduced levels of collagen IV in L1-overexpressing luECs (Figure 5A), which, besides being another hallmark of EndMT (21), was consistent with the increased collagen IV deposition in L1-deficient Panc02 tumor vessels (Figure 2, E and F). Furthermore, L1-overexpressing luECs exhibited enhanced expression of the stemness-associated factors KLF4 and CD44 (Figure 5A), in line with the notion that EndMT is accompanied by the acquisition of a stem-like phenotype (21). Most of the markers above were validated by quantitative real-time PCR (qRT-PCR) (Figure 5B), implying a regulation at the transcriptional level. In contrast, the mesenchymal marker vimentin was not affected by L1 overexpression (Figure 5B). Besides mesenchymal markers, L1 was able to enhance the expression of transcription factors (TFs) that are thought to drive EndMT, such as Zeb1 and Zeb2, as well as Tbx20 (Figure 5B), which has been implicated in EndMT-like processes during heart development (22).

These data point to L1 as a regulator of EndMT, possibly implicating such an activity in L1-dependent alterations of EC behavior.

**L1 regulates EC transcriptome.** In an attempt to elucidate the molecular mechanisms underlying the multiple roles of L1 in endothelium, we checked whether manipulating its expression affected the EC transcriptome. To this purpose, we compared the gene expression profiles of L1-overexpressing luECs with those of control luECs by Affymetrix microarray technology, and we used the Significance Analysis of Microarrays (SAM) (23) to identify genes whose expression was altered by L1. This analysis revealed a remarkable effect of L1 overexpression on luEC transcription (Figure 6A), with 361 genes that were upregulated and 580 that were downregulated (q value < 5%; 1.5-fold change difference; Supplemental Table 1). From the list of L1-regulated genes, we selected 16 candidates for qRT-PCR validation, based on their biological relevance. For all of them, including Cdk6, Adams9, Hoxb9, Stmn2, Ebf1, Dil4, Vegfa, Vegfc, Ccnb1, Ili13r2 (Figure 6B), CD44, N-cadherin, S100A4, Tbx20, Klf4 (Figure 5B), and Cldn5 (Figure 4G), the regulation in L1-overexpressing luECs was confirmed by qRT-PCR. Many of these genes, including Stmn2, Ebf1, Dil4, Ili13r2, and Vegfc, exhibited a concordant L1 dependence upon siRNA-mediated silencing of L1 (Figure 6C), indicating that L1 is required and sufficient for their modulation.

Next, we used ingenuity pathway analysis (IPA) to analyze the effect of L1 overexpression on genes involved in biologically relevant functions. The “bio-functions” analysis revealed that L1 affects the expression profiles of several genes involved in cell-cycle regulation, DNA replication, cellular assembly, and organization (top 5 biofunctions, P value < 10^-7, Benjamini-Hochberg correction; Figure 6D), which is consistent with the L1-induced proliferation of ECs (Figure 4A). Importantly, IPA also identified genes involved in cell migration and development (Figure 6D). In particular, the gene expression profile of L1-transfected luECs was consistent with the activation of pathways involved in EC movement (Supplemental Figure 11), thus supporting our observations on L1-dependent functional changes (Figure 4, D and E).

**L1 regulates EC function via the IL-6/JAK/STAT3 pathway.** To get further insights about possible effectors of L1 biological function, we performed an unsupervised ingenuity upstream regulator analysis, which predicts the activation of specific gene expression modulators (i.e., TFs, microRNA, etc.). This analysis predicted the L1-induced activation of the TFs STAT1, STAT2, STAT3, IRF7, and ATF4. In particular, we identified a network of 105 L1-regulated genes downstream of the above-mentioned 5 TFs (Figure 7A).
Next, we checked whether STAT signaling is involved in L1-dependent regulation of EC function. Since STAT activation occurs through JAK-mediated phosphorylation, we treated luECs with the JAK inhibitor I (JAKi) which, indeed, repressed L1-induced phosphorylation of STAT3 (Figure 7F). Importantly, JAK blockade repressed L1-dependent EC proliferation and migration (Figure 7G and H). Together, our transcriptomic, biochemical, and cell biological data support the notion that L1 regulates EC function via the JAK/STAT pathway.

Endothelial L1 is upregulated in human pancreatic carcinoma and in other tumor types. Based on our findings on the Panc02 orthotopic mouse model, we asked whether the expression of L1 in tumor vessels also occurs in human pancreatic carcinoma. Immunohistochemistry for L1 was performed on 18 tissue samples of PDAC and 11 samples of noncancerous pancreatic tissue, using
Figure 7. L1 regulates EC function via the IL-6/JAK/STAT3 pathway. (A) Gene network of L1-regulated genes. In bold, IPA-predicted upstream modulators. Lines connect modulators to direct targets, and colors indicate the consistency with the predicted activity with the expression change observed in L1-overexpressing luECs (i.e., target expression). Orange, consistent predicted activation of TFs; blue, consistent predicted inhibition of TFs; yellow, inconsistent predicted activation of TFs; grey, not defined activity. (B) qRT-PCR analysis of the indicated genes in mock- and L1-transfected luECs. Transcript levels were normalized as described in Methods and are shown as fold changes in L1-transfected cells relative to mock-transfected cells (n = 3). (C) The amount of IL-6 released in the culture medium by mock- and L1-transfected luECs was quantified by ELISA. (D) Immunoblotting analysis of mock- and L1-transfected luECs for IL-6Rα, phosphorylated STAT3, and total STAT3. (E) Immunoblotting analysis for phosphorylated and total STAT3 in mock- and L1-transfected luECs, treated either with anti-IL-6Rα antibody or with control IgG. (F) Immunoblotting analysis for phosphorylated and total STAT3 in mock- and L1-transfected luECs, treated either with vehicle (DMSO) or with 20 μM JAKi. Actin in D–F served as loading control. (G) Proliferation curves of mock- and L1-transfected luECs treated either with vehicle (DMSO) or with the indicated concentration of JAKi. (H) Mock- and L1-transfected luECs treated either with vehicle (DMSO) or with 20 μM JAKi were subjected to 24-hour migration assays. Data in G and H represent the mean ± SD from a representative experiment performed in triplicate. **P < 0.01; ***P < 0.001.
an antibody against PECAM-1 to identify the vessels on consecutive sections. L1 expression was markedly enhanced in PDAC vasculature as compared with noncancerous tissue (Figure 8, A and B), confirming and extending previous observations (11). The presence of L1 in vascular endothelium was further validated by confocal microscopy on PDAC tissue costained for L1 and for the endothelial marker VE-cadherin (Supplemental Figure 12). Taken together with our data on the Panc02 tumor model and on cultured ECs, the expression pattern of L1 in clinical samples supports the hypothesis that vascular L1 contributes to pancreatic malignancy.

To test whether the vascular expression of L1 also occurs in other cancer types, we performed immunohistochemical staining for L1 on tissue microarrays (TMAs) containing various tumors and their noncancerous tissue counterparts. As shown in Figure 9 and Supplemental Figure 13, in several tumor types, the percentage of L1-positive vessels was markedly higher as compared with their corresponding neoplastic tissues. Thus, the induction of L1 expression in the vasculature is common to a broad spectrum of human tumors.

Discussion
The expression of L1 in tumor vasculature has been reported in several cancer types, including breast, ovarian, colon, and pancreatic carcinoma (10,11), neural tumors (26), smooth muscle tumors (27), and melanoma (28). While vascular L1 has been implicated in the adhesion and transendothelial migration of L1-expressing cancer cells via homophilic binding (11, 29, 30), it remains elusive whether L1 induces cell-autonomous effects in tumor endothelium and whether this has an effect on cancer development.

Here, we report for what we believe is the first time that L1 orchestrates the EC behavior in tumor vasculature. In particular, the endothelial deficiency of L1 in a mouse model of cancer led to enhanced vessel stability and decreased tumor angiogenesis, resulting in reduced tumor growth and metastasis and prolonged mouse survival. Consistently, antibody-mediated targeting of L1 reduced tumor vascularization and enhanced vessel normalization, thus delaying tumor growth. These findings were supported by in vitro data that revealed that L1 induces EC proliferation, migration, and tubulogenesis, and confers a mesenchymal phenotype to ECs.

Previous studies have shown that a soluble form of the L1’s ectodomain enhances EC proliferation and migration (31–33), implying a model whereby exogenous (e.g., tumor cell derived) L1 stimulates ECs. While this remains a possibility, particularly in the case of L1-expressing tumors, our findings on the upregulation of L1 in cancer-associated vessels and on the proliferative and migratory response of L1-expressing ECs, point to a cell-autonomous effect of L1 in pathological vasculature.

It is noteworthy that L1 promotes the expression of various genes causally linked to tumor vascularization, such as VEGF-A, VEGF-C, Dll4, and HOXB9 (34–36). On the other hand, the endothelial expression of L1 itself is induced by classical angiogenic factors, including VEGF-A, ANGPTL4, TNF-α, and IFN-γ (10, 11). Taken together, these observations implicate L1 as a central hub in the transmission and amplification of angiogenic stimuli within the tumor microenvironment.

Our data also indicate that L1 promotes vascular permeability. Since VEGF-A, ANGPTL4, TNF-α and IFN-γ (i.e., the same cytokines that induce endothelial L1 expression) are among the most potent inducers of vascular permeability (37,38), it is conceivable that L1 acts as a general effector of cytokine-induced vascular permeability.

Notably, we discovered that L1 overexpression induces EndMT, a process that may underlie, or at least contribute to, many aspects of the EC response to L1, such as migration and tubulogenesis as well as increased tumor angiogenesis and vascular permeability (12). Various lines of evidence support the notion that EndMT recapitulates most of the cellular and molecular events occurring during epithelial-to-mesenchymal transition (EMT) (12). The role of L1 in EMT has been clearly established in various experimental models (39–41), including tumor cells in which the expression of L1 per se promotes EMT (42, 43). We now provide evidence that L1 expression is sufficient to confer a mesenchymal phenotype to ECs, which entails not only an increased migratory activity but also the upregulation of several mesenchymal markers, such as N-cadherin, CD44, S100A4/FSP1, and fibronectin, and even of TFs that are considered to be EMT/EndMT drivers, namely KLF4, Zeb1, Zeb2, and Tbx20 (22, 44, 45). These findings suggest that L1 acts as a key mediator of the multiple EndMT-inducing factors that can occur in tumor microenvironment and as a master orchestrator of cancer-associated EndMT (12).

One of the most intriguing and unexpected results of our study is the global effect of L1 on EC transcriptome, with the modulation of approximately 1,000 genes. While these include individual factors that per se might account for several aspects of the endothelial response evoked by L1, such as VEGF-A, VEGF-C, and Dll4 for tumor angiogenesis (see above), or occludin and claudin-5 for vascular permeability (19), our data rather implicate L1 in the control of whole gene networks, resulting in the modulation of signaling pathways that mediate the EC response.
In this context, it is noteworthy that L1 induces the expression of IL-6 and IL-6Rα. IL-6 is a potent angiogenic cytokine that promotes neovascularization in various solid tumors, and it has been proposed not only as a therapeutic target for antiangiogenic therapies but also as a biomarker to predict the response to such treatments (46). Thus, it is reasonable to speculate that the IL-6/IL6R axis acts as a key mediator of the angiogenic response elicited by vascular L1, an intriguing hypothesis that warrants further investigation. In addition, L1-mediated regulation of IL-6 expression might also occur in different biological contexts where IL-6 signalling has been implicated. For example, the IL-6/IL-6Rα system plays a major role in inflammation and in several cancer cell functions (47, 48), and our findings imply that the established function of L1 in both pathological conditions (8, 10) might be accounted for, at least to some extent, by the induction of IL-6 expression and activity.

Among the signalling cascades that are elicited by IL-6, the JAK/STAT pathway appears particularly important in the context of L1-dependent regulation of EC function. Indeed, L1 induces high phosphorylation of STAT3, a TF that plays a key role in EC activation and pathological angiogenesis (49, 50), consistent with the hypothesis that L1-dependent regulation of tumor vasculature is mediated by the IL-6/JAK/STAT pathway. Our data on the inhibition of L1-induced EC proliferation and migration upon blockade of JAK/STAT signaling not only support this hypothesis, but provide mechanistic insights into the role of L1 in pathological vessels and shed light on a signaling axis that links L1 to the JAK/STAT pathway. Given the broad spectrum of functions that have been ascribed to L1 in different cellular contexts, including cell-cell adhesion, axon guidance, tumor cell invasion, and stem cell self renewal (7, 8, 51), we propose that at least some of these activities are mediated by JAK/STAT signaling.

The expression and function of L1 in cancer vessels has relevant translational implications. First, it provides the rationale to test L1 as an imaging biomarker suitable for visualizing pathological angiogenesis. In this application, due to its absence or low levels in normal vasculature, L1 might show higher specificity than other molecular biomarkers that are currently in use or under clinical testing, such as integrins and VEGFR2 (52). The feasibility of this approach is supported both by the preclinical imaging of cancer vessels by targeting other immunoglobulin-like adhesion molecules such as NCAM (53) and by the suitability of L1 antibodies for tumor-imaging purposes as shown in mouse models (54). Second, based on the positive role of L1 in tumor neovascularization, in EC proliferation and migration, and in EndMT, it is reasonable to speculate that neutralizing L1 could represent a novel antiangiogenic strategy. Indeed, we showed that treating tumor-bearing mice with an L1-neutralizing polyclonal antibody delays tumor growth and reduces tumor vascularization, strengthening the rationale for developing L1-targeting agents as therapeutic tools. In this context, promising results have been obtained with anti-L1 monoclonal antibodies in preclinical models of solid tumors and of endometriosis (55, 56). Third, the observation that L1 destabilizes cancer-associated vessels implies that targeting endothelial L1 and interfering with its function might result in vessel normalization, a process that has been proposed to improve the delivery into the tumor of systemically administered chemotherapeutics (4). Thus, our results set the stage for exploring the clinical relevance of L1 expression and function in cancer vessels, possibly opening new avenues for targeted therapies of malignancies.

Methods

Mice
Tie2-CreL1floxed mice were generated in the C57BL/6 genetic background as previously described (10). Since the L1 gene is located on chromosome X (and hence, only 1 copy is present in the male genome), Cre-mediated ablation of L1 was expected to be more efficient in males. Therefore, only Tie2-Cre-positive males carrying the floxed L1 allele were used throughout the study, with L1floxed males serving as controls.
**In vivo models**

**Pancreatic carcinoma model.** The syngeneic mouse model of pancreatic cancer has been described previously (13). Briefly, 10- to 12-week-old C57BL/6 male mice were anesthetized by intraperitoneal injection of 500 mg/kg avertin (Sigma-Aldrich), the stomach was exteriorized via abdominal midline incision, and Panc02 tumor cells (1 × 106 cells in 30 μl PBS) were injected into the head of the pancreas using a 29-gauge needle. The intrapancreatic injection was considered successful with the appearance of a fluid bleb without intraperitoneal leakage. Peritoneum and abdominal wall were closed with individual surgical sutures. Where indicated, mice were treated every 48 hours with 7 mg/kg of affinity-purified anti-L1 polyclonal antibodies (obtained from rabbits immunized with mouse L1-Fc) or control, nonimmune rabbit IgG (Sigma-Aldrich), starting from the day after Panc02 injection. At day 14, primary tumors were removed, and tumor weight and volume were recorded. Tumor volume was calculated using the formula **V = \( \pi \times (d_1 \times d_2 \times d_3)/6 \)**, where **d1**, **d2**, and **d3** are the 3 tumor axes. To assess tumor invasion to adjacent organs and metastasis, mice were sacrificed at day 26 and subjected to whole-mount body fixation with 4% paraformaldehyde. Necropsy was performed by the Mouse and Animal Pathology Laboratory, Fondazione Filarete (Milan, Italy).

**Hemangioma model.** For EC transplantation, 1 × 104 mock- or L1-transfected mouse luECs in 200 μl of PBS was injected subcutaneously into the right flank of CD-1 nude (nu/nu) mice (9 weeks old, female; Charles River Laboratories) as previously described (18). Hemangioma volumes were recorded at the indicated time points and calculated using the formula **V = \( \pi \times (d^2 \times D)/6 \)**, where **d** and **D** are the minor and the major hemangioma axes, respectively.

**Chemicals and antibodies**

The JAK inhibitor 1 was provided by Calbiochem. The following monoclonal antibodies were used as indicated: anti-mouse L1 (clone S10.33 [ref. 10, 5 μg/ml in IF]; clone 555 [1 μg/ml in IF]; clone 324 [ref. 6] hybridoma supernatant [1:2 in IF]; and clone I4.2 [ref. 10] hybridoma supernatant [1:2 in Western blotting (WB)]); anti-human L1 (clone UJ127; 1:30 in IHC on human tissues; Thermo Scientific); anti-PECAM-1 (clone 2H8; 1:500 in IF on cells and mouse tissues); anti–claudin-5 (clone 4C3C2; 1:200 in IF, 1:250 in WB, Life Technologies); anti–β-catenin (clone 14/β-catenin; 1:1000 in WB; BD Biosciences); anti–γ-catenin (clone 15/γ-catenin; 1:1000 in WB; BD Biosciences); anti–CD44 (clone IM7; 1:100 in WB; BD Biosciences); anti–CD144 (clone DM1A; 1:2000 in WB; Sigma-Aldrich). The following polyclonal antibodies were used as indicated: anti-collagen IV (1:900 in IF and 1:400 in WB; Serotec); anti–fibrin/fibrinogen (1:1000 in IF; Dako); anti–fibronectin (1:4000 in WB; Abcam); anti-S100A4/FSP1 (1:1000 in WB; Millipore); anti–Id1 (C-20; 1:400 in WB; Santa Cruz Biotechnology Inc.); anti–IL-6Ra (1:1000 in WB; R&D Systems); anti–Ki-67 (1:100 in IF; Abcam); anti–KLF4 (1:1000 in WB; R&D Systems); anti–mouse L1 (obtained by rabbit immunization with mouse L1-Fc; 1 μg/ml in IF and WB); anti–PECAM-1 (M-20; 1:500 in WB; Santa Cruz Biotechnology Inc.); anti–NG-2 (1:200 in IF; Millipore); anti–phospho-histone H3 (1:250 in IF; Millipore); anti–podocalixin (1:400 in IF; R&D Systems); anti–VE-cadherin (C-19; 1:500 in WB and 1:200 in IF on human tissues; Santa Cruz Biotechnology Inc.); anti–β-tubulin III (1:500 in IF; Covance). Antibodies used for FACS analysis are listed in the corresponding section.

**Cells**

Mouse luECs were immortalized with polyoma middle T antigen as previously described (18) and cultured in MCDB131 medium (Gibco; Life Technologies) supplemented with 20% FBS (Invitrogen), 2 mM L-glutamine (Lonza), 1 mM Na-pyruvate (Gibco; Life Technologies), 100 μg/ml heparin (Sigma-Aldrich), and 50 μg/ml EC growth supplement (ECGS) obtained from calf brain. ECs were seeded on 0.1% gelatin (Sigma-Aldrich), except as otherwise indicated. To enhance EC adhesion, plates were coated with glataldehyde–crosslinked gelatin as follows. Plates were incubated overnight with 1% gelatin at 37°C followed by a crosslinking with 2% glataldehyde for 15 minutes at room temperature (RT). Glataldehyde was replaced with 70% ethanol for 1 hour at RT. After 5 washes with PBS, plates were incubated for 2 hours at 37°C with 2 mM glycine in PBS. Prior to cell seeding, plates were washed 3 times with PBS.

Murine Panc02 (pancreatic carcinoma) and MOVCAR7 (ovarian carcinoma) cell lines were provided by S. Sebens (Institute for Experimental Medicine, Kiel, Germany) and D. Connolly (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA), respectively, and cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS, 2 mM L-Glutamine, and 1 mM Na-pyruvate. All cells were cultured in a humidified incubator with 5% CO₂.

**Cell transfection and RNA interference**

Mouse cDNA encoding full-length L1 was cloned into the pcDNA3.1/Hygro(−) expression vector (Invitrogen) using standard DNA cloning procedures. The parental pcDNA3.1/Hygro vector and pcDNA3.1/Hygro-L1 were amplified in competent bacteria cells (TOP10) and purified using Maxi-Prep kits (QIAGEN) according to the manufacturer’s instructions. Transfection with either pcDNA3.1/Hygro or pcDNA3.1/Hygro-L1 was performed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were selected with 300 μg/ml of Hygromycin-B (Roche) and maintained under selection conditions as a bulk cell population.

Stealth RNAi Duplexes and the corresponding Medium GC Stealh RNAi Control Duplexes (Invitrogen) were used to knock down L1 in murine ECs. The following L1 target sequences were used: 5′-CCUG-GUAACCCGGACCAUCAUCA-3′ (siRNA A) and 5′-UGCACC-CUUUCUCUCAAUUGCGCUC-3′ (siRNA B). ECs were subjected to 2 rounds of transfection with 40 nM siRNA the 2 days prior to the experiment. Transfection was performed with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions.

**Cell proliferation assay**

ECs were seeded at a density of 2 × 10⁴ cells/well in 96-well plates coated with glataldehyde–crosslinked gelatin. After overnight incubation in medium containing 5% FBS, cells were stimulated with...
medium containing 20% FBS, 100 μg/ml heparin, and 50 μg/ml ECGS. Where indicated, cells were treated with JAK inhibitor 1 or vehicle (DMSO) or with 3 μg/ml of polyclonal anti-L1 antibodies or control, nonimmune rabbit IgG throughout the assay. Cells were fixed at 0, 24, 48, and 72 hours, followed by staining with 0.1% crystal violet in 20% methanol. Bound dye was solubilized with 10% acetic acid, and the absorbance at 590 nm was measured. Cell growth was normalized on absorbance measured at 0 hours. The experiments were performed in quintuplicate and repeated at least 3 times.

**Cell migration assay**

To assess cell migration, we employed the wound-healing assay. Briefly, confluent monolayers of luECs seeded on fibronectin-coated (1 μg/cm²) 24-well plates were starved for 24 hours in medium containing 0.5% FBS medium. Monolayers were wounded with a plastic pipette tip to induce EC migration into the wound, and images were acquired at 0 and 24 hours. Where indicated, cells were pretreated for 1 hour with 20 μM JAK inhibitor 1 or vehicle (DMSO), or with 3 μg/ml anti-L1 polyclonal antibodies or control, nonimmune rabbit IgG; such treatments were maintained throughout the assay. Optical images of the wounds were acquired at 0 and 24 hours. The width of the wounds was measured with ImageJ (http://imagej.nih.gov/ij/) software, and the distance covered luECs, expressed as μm in 24 hours, was calculated according to this formula: (wound width at 0 - wound width at 24 hours)/2. The experiments were repeated 3 times, each time with triplicate wells.

**Tube formation assay**

A Matrigel-based tubulogenesis assay was performed to assess the ability of ECs to form an organized capillary-like network. Confluent ECs were starved overnight with medium containing 1% FBS. Growth factor-reduced Matrigel (BD Biosciences) was thawed overnight at 4°C on ice, and, the day of the assay, plated on the bottom of a 96-well plate and left at 37°C for 1 hour for gelification. Thereafter, 1 × 10⁴ cells/well were seeded on Matrigel and incubated at 37°C. Where indicated, cells were treated with 3 μg/ml anti-L1 polyclonal antibodies or control, nonimmune rabbit IgG. Optical images of the wells were acquired after 8 hours at ×4 magnification. The tubes in each well were manually counted. The experiments were repeated 3 times, each time with triplicate wells.

**Vascular permeability assays**

**In vitro.** The luECs were cultured on Transwell inserts (clear, 24-well plate, 0.4-μm pores; 6.5 mm diameter; tissue culture–treated; Costar), coated with glutaraldehyde–crosslinked gelatin, and allowed to form confluent monolayers (typically after 4 days). The day of the experiment, the medium in the upper chamber was replaced with complete medium containing 1 mg/ml FITC-labeled 40-kDa dextran (Sigma-Aldrich). At each time point, 50 μl of medium in the bottom chamber were taken, and fluorescence was measured at 488 nm. The medium taken from the bottom chamber was replaced each time with fresh complete medium in order to maintain a constant volume in the bottom chamber. The experiments were performed in quintuplicate and repeated 3 times.

**In vivo.** Texas red-labeled 40-kDa dextran (Life Technologies) was administered intravenously by retroorbital injection into anesthetized Panc02 tumor-bearing mice (0.25 mg/mouse). Thirty minutes after injection, mice were sacrificed and their tumors were fixed in 2% paraformaldehyde and embedded in paraffin. Sections were then subjected to IF staining with rat anti-CD34 (BD; clone RAM34; 1:100) and rabbit anti–Texas red antibodies (1:100; Life Technologies). Vascular permeability was assessed as the ratio between the number of leaky vessels (i.e., vessels showing perivascular, extravasated Texas red staining) and the total number of CD34-positive vessels in 5 randomly selected fields (13).

**Gene expression profiling**

ECs were seeded on plates coated with glutaraldehyde–crosslinked gelatin and cultured in complete medium for 4 days to reach confluence. Total RNA was extracted with RNeasy Mini Kit (QIAGEN). Quality control of the RNA samples was performed using Agilent Bioanalyzer 2100 (Agilent Technologies). Three different RNA extractions were processed for each of the cell lines under analysis. Each sample was labeled and hybridized to a Mouse Gene 1.0 ST GeneChip array according to the manufacturer’s instructions (Affymetrix). Data were normalized using the Robust Multi-array Average (RMA) (ref. 58; raw and normalized data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE45859)).

All analyses were performed on log₂ data using parametric tests. BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used to run SAM analysis (http://www-stat.stanford.edu/~tibs/SAM/). A false discovery rate of less than 5% (i.e., the q value) was used to select statistically significant differentially expressed genes. Cluster 3.0 for Mac OS X (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and Java Treview (http://jtreeview.sourceforge.net) were used for hierarchical clustering analysis using the uncentered correlation metric and centroid clustering method.

Ingenuity Pathway Analysis and Upstream Regulator Analysis were performed using the online available web tool (http://www.ingenuity.com/). For gene network analysis, only direct relationships in mammals (i.e., human, mouse, and rat) were considered. P values for biofunction enrichment were corrected for multiple testing using the Benjamini-Hochberg correction.

**Immunoblotting**

ECs were cultured on petri dishes coated with glutaraldehyde–crosslinked gelatin for 4 days. Where indicated, cells were incubated for 2 or 24 hours with 2 μg/ml goat polyclonal anti–IL-6 R (R&D Systems) or control goat IgG. Total proteins were extracted by solubilizing cells in boiling Laemmli buffer (4% SDS, 16% glycerol, 40 mM Tris-HCl [pH 6.8]). Lysates were incubated for 15 minutes at 90°C to allow protein denaturation and then centrifuged for 5 minutes at 14,000 g to discard cell debris. The supernatants were collected, and the concentration of protein was determined using a BCA Protein Assay Kit (Pierce) according to the manufacturer’s instructions. Equal amounts of protein were separated on SDS polyacrylamide gel (SDS-PAGE), transferred to a Protran Nitrocellulose Transfer Membrane (Whatman), and blocked for 1 hour at RT in TBS, 0.1% Tween 20 containing 5% nonfat milk or BSA (blocking solution). The membranes were incubated overnight at 4°C or for 1 hour at RT with primary antibodies diluted in blocking solution. Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Bio-Rad). The signal was detected by the ECL system (GE Healthcare) using Hyperfilm (Amersham Biosciences). The molecular masses of proteins were estimated relative to the electrophoretic mobility of contransfected prestained protein marker Precision Plus Protein Standards (Bio-Rad).
qRT-PCR analysis
ECs were cultured on petri dishes coated with glutaraldehyde–cross-linked gelatin for 4 days. Total RNA was isolated by extraction with RNeasy Mini Kit (Qiagen), and 1 μg was reverse-transcribed with random hexamers (SuperScript Vilo cDNA Synthesis Kit; Invitrogen) according to the manufacturer’s instructions. cDNA (5 ng) was amplified in triplicate in a reaction volume of 15 μl with the TaqMan Gene Expression Assay (Applied Biosystems) and an ABI/Prism 7900 HT thermocycler (Applied Biosystems) using a pre-PCR step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 5°C and 60 seconds at 60°C. Preparations of RNA template without reverse transcriptase were used as negative controls. For each sample, the expression level was normalized against the geometric mean of the housekeeping genes encoding GAPDH and 18S. Normalized expression changes were determined with the comparative threshold cycle (Ct) method (59).

IL-6 detection
ECs were seeded on plates coated with 0.1% gelatin and cultured in complete medium for 4 days to reach confluence. At day 4, medium was replaced by fresh complete medium, and supernatants were collected after 24 hours. Mouse IL-6 was quantitated using the Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions.

Immunohistochemistry
The clinicopathological data of PDAC patients are described in Supplemental Table 2. Formalin-fixed, paraffin-embedded specimens were prepared from both cancerous (n = 18) and noncancerous pancreatic tissue samples (n = 11). Sections were rehydrated through xylene and graded alcohols. Antigen retrieval was accomplished using 1 mM EDTA and 0.05% Tween. Samples were incubated with 3% H2O2 for 5 minutes, followed by blocking in 2% goat serum in PBS for 1 hour. Primary antibodies were incubated for 2 hours at RT in 2% goat serum. Horseradish peroxidase–conjugated secondary antibodies were used. Samples were developed with DAB and counterstained with hematoxylin.

The vascular expression of L1 on tissue sections was measured as the ratio between L1-positive vessels and the total number of vessels identified by PECAM-1 staining on consecutive sections. The analysis was performed on 10 fields per sample at ×40 magnification.

FACS analysis
Tumors were collected 14 days after Panc02 injection, washed in PBS, and digested in RPMI medium containing 0.1% collagenase type 1 (Gibco; Life Technologies) and 10 μg/ml DNase (Roche) for 45 minutes at 37°C. Digested tissue was passed 10 times through a 20-gauge needle and filtered through a 40-micron–sized mesh. After red blood cell lysis, cells were resuspended in FACS buffer (PBS, 1% FBS, 0.05% NaN3) and subjected to FACS staining. After a blocking incubation in FACS buffer containing 1% normal mouse serum (Sigma-Aldrich) for 45 minutes on ice, cells were stained for 45 minutes on ice with the indicated antibodies against different immune cell markers. Finally, cells were washed 3 times and fixed with 1% PFA. Percentage of positive cells was measured by FACS Calibur flow cytometer and analyzed with CellQuest software.

The following monoclonal antibodies, all purchased by BD Biosciences except otherwise indicated, were used for FACS staining: PE anti-CD4 (clone RM4-5; 1:200), PE anti-CD8 (clone 53-6.7; 1:50), PE anti-CD11b (clone M1/70; 1:100), PE anti-CD11c (clone HL3; 1:50), FITC anti-CD14 (clone rmC5-3; 1:50), FITC anti-CD19 (clone 1D3; 1:200), FITC anti-CD45 (clone 104; 1:100), PerCP anti-CD45 (clone 30-F11; 1:100), FITC anti-F4/80 (Caltag Laboratories, clone CI:A3-1; 1:100); PE anti-Gr-1 (clone RB6-8C5; 1:50); and PE anti-1-A/I-E (clone M5/114.15.2; 1:200).

Statistics
Data are expressed as mean ± SEM, except as otherwise indicated. Student’s 2-tailed nonpaired t test or ANOVA and Bonferroni’s multiple comparison tests were used to determine statistical significance (GraphPad Prism 4). Tumor-free survival was drawn using the Kaplan-Meier method and compared by the log-rank test. Differences were considered significant at P < 0.05.
Study approval
Mouse housing and all experimental animal procedures were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health. Specimens of PDAC and noncancerous pancreatic tissue were obtained upon informed consent from patients operated at University Hospitals Leuven. All tissue samples used for the TMAs were collected via standardized operative procedures approved by the Institutional Ethical Board of the European Institute of Oncology, and informed consent was obtained for all samples linked with clinical data.

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