Microenvironment-Modulated Metastatic CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>−</sup> Lung Cancer–Initiating Cells Sustain Tumor Dissemination and Correlate with Poor Prognosis

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Abstract

Metastasis is the main reason for lung cancer–related mortality, but little is known about specific determinants of successful dissemination from primary tumors and metastasis initiation. Here, we show that CD133<sup>+</sup>/CXCR4<sup>+</sup> cancer-initiating cells (CIC) directly isolated from patient-derived xenografts (PDX) of non–small cell lung cancer are endowed with superior ability to seed and initiate metastasis at distant organs. We additionally report that CXCR4 inhibition successfully prevents the increase of cisplatin-resistant CD133<sup>+</sup>/CXCR4<sup>+</sup> cells in residual tumors and their metastatization. Immuno-phenotypic analysis of lung tumor cells intravenously injected or spontaneously disseminated to murine lungs demonstrated the survival advantage and increased colonization ability of a specific subset of CD133<sup>+</sup>/CXCR4<sup>+</sup> with reduced expression of epithelial cell adhesion molecule (EpCAM<sup>+</sup>), which also shows the greatest in vitro invasive potential. We next prove that recovered disseminated cells from lungs of PDX-bearing mice enriched for CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>−</sup> CICs are highly tumorigenic and metastatic. Importantly, microenvironment stimuli eliciting epithelial-to-mesenchymal transition, including signals from cancer-associated fibroblasts, are able to increase the dissemination potential of lung cancer cells through the generation of the CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>−</sup> subset. These findings also have correlates in patient samples where disseminating CICs are enriched in metastatic lymph nodes (20-fold, P = 0.006) and their detection in primary tumors is correlated with poor clinical outcome (disease-free survival: P = 0.03; overall survival: P = 0.05). Overall, these results highlight the importance of specific cellular subsets in the metastatic process, the need for in-depth characterization of disseminating tumor cells, and the potential of therapeutic strategies targeting both primary tumor and tumor–microenvironment interactions. Cancer Res; 75(17): 3636–49. ©2015 AACR.

Introduction

Despite novel therapeutic strategies, survival rates for lung cancer have not greatly improved in recent years, mostly due to late diagnosis and limited efficacy of available pharmacologic treatments (1). Cancer-initiating cells (CIC), functionally defined as the subset of cancer cells responsible for tumor generation and maintenance (2, 3), could also account for tumor recurrence and metastatic seeding due to their inherent stem-like and chemoresistant properties (4).

In this context, specific subsets of tumor cells endowed with both stemness and motility properties have been defined as migrating CICs (5, 6), and activation of the epithelial-to-mesenchymal transition (EMT) process, which is pivotal in metastatic progression, has been shown to modulate stemness properties of cancer cells, indicating a possible connection between external cues and the generation of potential seeds of metastasis (7–9). The subset of CICs deputed to tumor dissemination could therefore be specifically modulated by tumor microenvironment stimuli, as recently observed for colon metastatic CICs (10). Studies in transgenic mouse models also indicate that dissemination is an early event and is
supported by tumor cells that have undergone EMT and possess CIC features (11, 12).

Increasing evidence also shows that chemotherapy might select for intrinsically chemoresistant CICs that could be responsible for primary tumor recurrence (13, 14). Accordingly, we previously demonstrated that a distinct subset of CD133⁺ lung CIC coexpressing the chemokine receptor CXCR4 is spared by cisplatin treatment (15). The CXCL12/CXCR4 axis plays a crucial role in mediating tumor cell survival and the metastatic process (16); more recently, it has been proven to sustain CICs, through activation of self-renewal pathways (17), and to guide their metastatic dissemination in a human pancreatic tumor model (6).

In the present study, through the analysis of a variety of lung cancer patient–derived xenografts (PDX; ref. 18) and clinical samples, we phenotypically characterize and functionally prove the existence of a novel subset of CICs, regulated by the microenvironment, driving lung tumor dissemination and metastasis and we identify CXCR4 signaling as a potential target for novel therapeutic strategies.

Materials and Methods

List of antibodies

Antibodies used for FACS analysis were anti-human PE CD133/1, FITC CD326 (EpCam) (Miltenyi Biotech); APC CD187 (CXCR4; BD Pharmingen); anti-mouse PerCP-eFluor 710 MHC class I, anti-human PerCP-eFluor 710 CD31 (eBioscience); and anti-human PC5 CD34, PC5 CD45 (Reckman Coulter). Anti-human FITC cytokeratin (CK3-6H5, Miltenyi Biotech) was used for immunofluorescent staining. For IHC analyses, following antibodies were used: anti-human CD133/1 (AC133, Miltenyi Biotech), CXCR4 (CXCR-4000-RM, Biotrend), keratin, Pan Ab-1 (Clone AE1/AE3, Dako), CD34 (clone QBdN/10, Neomarkers), and low-molecular-weight cytokeratins (clone AE1/AE3/PCK26, Ventana Medical Systems). Detailed protocols for flow cytometry, immunofluorescence, and IHC staining are in Supplementary Materials and Methods.

Tissue dissociation and culture

Clinical specimens were obtained from a consecutive series of 97 consenting patients (17 also had matching lymph nodes available). The protocol was approved by the Internal Review and the Ethics Boards of the Fondazione IRCCS Istituto Nazionale Tumori of Milan.

The protocol for primary tumors, lymph nodes, PDXs, and murine lungs tissue dissociation was already described (15). Culture method to obtain cancer tissue–originated spheroids (CTOS) and murine lung tissue spheroids was adapted from Kondo and colleagues (19). Protocols are detailed in Supplementary Materials and Methods.

Cell lines and treatments

All tumor cell lines were cultured in RPMI with 10% FBS at 37°C under 5% CO₂. The LT73 primary cell line was derived from a surgical specimen of a male patient with lung adenocarcinoma. The H460 cell line (lung large cell carcinoma) was purchased from ATCC and authenticated by DNA short tandem repeat (STR) profiling.

To induce EMT, cell cultures were treated with TGFβ (5 ng/mL; Peprotech) for 5 up to 15 days.

CTCE-9908 inhibitor and corresponding scramble control peptide were kindly provided by British Canadian BioSciences Corp, and used for in vitro cell culture treatment at 10 μg/mL for 30 minutes at 37°C in the appropriate growing medium (RPMI-1640 plus 1% FBS or in stem cell medium), before FACS analysis or in in vitro invasion assays, detailed in Supplementary Materials and Methods.

Animal studies

In vivo experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori, according to institutional guidelines.

PDX were established from primary lung cancers from patients undergoing surgical resection, who gave their informed consent and expanded as previously described (18).

For in vitro combination therapy, cisplatin (Teva Pharma) was administered i.v. at 5 mg/kg every 7 days for 3 weeks, starting when tumors were palpable (≥50 mm³). CTCE-9908 was administered i.p. (50 mg/kg) once every 5 days after cisplatin administration for 3 weeks.

A human-in-mice model of bone metastasis was established, as previously reported (20). A bone metastasis assay was performed by injecting s.c. close to human bone implant 1 × 10⁶ cells sorted from PDX models for CD133 and CXCR4 or by intracardiac (i.c.) route, injecting in the left ventricle 1 × 10⁶ sorted cells in 100 μL physiologic solution. See also Supplementary Materials and Methods.

Integrated strategies to detect disseminating tumor cells in murine lung

To set up the most robust strategy to detect disseminating tumor cells (DTC) in murine lungs, intravenous injection of scalar doses of H460 cells was performed in SCID mice (n = 2 mice for each dose) and lungs were analyzed 2 hours after injection by FACS, real-time PCR, and IHC.

For FACS analysis, injected tumor cells were identified using a negative gating strategy to exclude 7-aminoactinomycin D (7-AAD⁺) dead cells and mouse H2K⁺ cells that was able to specifically detect as few as 10⁴ single tumor cells in murine lungs (Supplementary Fig. S1A and Supplementary Materials and Methods).

For detection of tumor cells in murine lungs by real-time PCR, analysis of human-specific β2 microglobulin (B2M) housekeeping gene expression was performed (Supplementary Fig. S1B and Supplementary Materials and Methods).

IHC analysis on formalin-fixed, paraffin-embedded (FFPE) lung tissue for pan-cytokeratins (CK) expression was able to robustly identify single cells when injected at 10⁴. To detect lower number of single cells, at least 5 serial sections of FFPE lung tissue, cut at 1.5-μm thickness, had to be analyzed (Supplementary Fig. S1C).

Results

The subset of CD133⁺ CXCR4⁺ lung CICs is endowed with increased seeding and metastatic potential

To verify the presence of a metastatic subset in lung CICs, we analyzed by FACS 78 primary human non–small cell lung cancer (NSCLC) for CD133 and CXCR4 expression: double-positive cells accounted for 0.3% of total tumor cells (median value range, 0%–8%) and represented 20% of CD133⁺ cells (median value...
CD133\(^+\) CXCR4\(^+\) cells have enhanced metastatic potential. A, percentages of CD133\(^+\) CXCR4\(^+\) cells in paired primary tumors and lymph node metastases (n = 17). P = 0.01. B, percentage of human tumor cells detected by flow cytometry in murine lungs 3 months and 6 months after i.v. injection of CD133\(^+\) CXCR4\(^+\), CD133\(^+\) CXCR4\(^-\), CD133\(^-\) CXCR4\(^+\) cells. n = 13 mice per group at 3 months and n = 7 mice per group at 6 months. *** P ≤ 0.001. C, representative images of hematoxylin and eosin histologic sections of murine lungs scanned with Aperio ePathology System, 6 months after injection of CD133\(^+\) CXCR4\(^+\), CD133\(^+\) CXCR4\(^-\), and CD133\(^-\) CXCR4\(^+\) cells sorted from LT111-PDX, LT73, and H460. Areas of tumor invasion for each model are shown on the right at higher magnification (×40); yellow arrows, representative infiltrating metastatic cells; green arrows, polymorphonuclear leukocytes associated with tumor foci. D, invasion of murine lungs was calculated by scanning four different layers of each histologic section in C and quantifying the percentage of invaded areas for each single lung with ImageScope Software. Bars are the mean percentage of invaded area ± SEM. * P ≤ 0.05; ** P ≤ 0.01. E, representative FACS analysis of LT111 CD133\(^+\) CXCR4\(^+\) cell-generated metastasis, 6 months after injection; tumor cells are identified as mouse H2K\(^-\) (left dot plot) and analyzed for CD133 and CXCR4 expression (right dot plot). IHC staining confirming CD133 and CXCR4 positivity is shown on the right.

**Figure 1.**
Identification of Lung Cancer Metastasis-Initiating Cells

To dissect the metastatic potential of different subpopulations of lung CICs, CD133+CXCR4+ and CD133−CXCR4− cells sorted from different PDXs (n = 5), from a primary cell line freshly established from a lung adenocarcinoma sample (Lung Tumor 73, LT73) and from H460 cells were injected in the tail vein of SCID mice. CD133+CXCR4− non–CICs were used as control. To detect and monitor during time tumor cells in murine lungs, we set up and validated a method on the basis of flow cytometric analysis, not relying on specific human antigens expression and supported by qRT-PCR and IHC analysis (Supplementary Fig. S1A–S1C). Three months after injection, rare tumor cells were detectable in murine lungs and were consistently higher in the groups injected with CD133+CXCR4+ (2-fold increase +/+ vs. +/−: 5-fold increase +/+ vs. −/−, P = 0.001; Fig. 1B). After an extended period of time (6 months), CD133+CXCR4+ cells, sorted from one PDX model (LT111) and LT73 and H460 cell lines, showed their full metastatic potential and greatest ability to colonize murine lungs compared with other injected groups as revealed by both FACS analysis (4.5-fold increase, $P = 0.0003$ +/+ vs. +/−: 6.4 fold increase, $P = 0.0006$ +/+ vs. −/−; Fig. 1B) and histologic analysis (Fig. 1C): software-assisted quantification of macroscopic areas of tumor invasion clearly demonstrated the higher degree of infiltration of lung parenchyma of double-positive cells compared with moderate and slight degree of infiltration of single-positive and double-negative cells ($P = 0.0003$ +/+ vs. +/−: $P = 0.0004$ +/+ vs. −/−; Fig. 1D). A relevant inflammatory infiltrate, mainly consisting of polymorphonuclear leukocytes, was also observed associated with tumor foci (Fig. 1C) possibly explaining the long latency periods before metastatic cells proliferation. The phenotype of injected double-positive cells remained unchanged during early colonization steps, implying that their survival advantage enables them to support metastasis initiation (Fig. 1E).

To confirm the high metastatic properties of CD133+CXCR4+ cells and their selective advantage in seeding different organs, we exploited an innovative model of humanized mice implanted with human bone (20, 21), the second more frequent metastatic site of NSCLC. The implanted human bone, detected in the mouse flank by X-rays examination, was viable as indicated by the presence of bone marrow cells, mineralized areas, stromal cells, and neovascularization (Fig. 2A). Unsorted tumor cells and CD133−CXCR4−, CD133+CXCR4−, and CD133−CXCR4− subsets were isolated from two PDXs (LT59 and LT111) and injected both s.c. and intracardially in mice bearing bone, to model different aspects of bone seeding. CD133+CXCR4+ cells were able to colonize human bone generating metastatic lesion after both s.c. and i.c. injection with high efficiency (65%, 9 of 14 mice), conversely CD133+CXCR4− and CD133−CXCR4− cells did not (Fig. 2B and Supplementary Table S1). Enhanced bone

![Figure 2](image_url)

**Figure 2.** CD133+CXCR4+ cells efficiently initiate bone metastasis. A, left, X-ray imaging of mouse implanted with human bone fragment in the flank. Right, hematoxylin and eosin on human implanted bone showing viable osteocytes, stromal cells, human vessels (top). IHC staining for human CD34 (bottom) confirmed the presence of human vessels (magnification, ×40). B, IHC staining for low molecular cytokeratins of bone metastasis derived from CD133+CXCR4+ cells (magnification, ×10) and inset at higher magnification (×60). C, trichrome staining of human implanted bone in mice injected with CD133+CXCR4+ (top) and CD133−CXCR4− (bottom) showing the presence of new collagen fibers (stained in blue). D, mean values ± SD of the osteoid thickness of human bone in CD133+CXCR4+ cell-injected mice compared with all other groups (n = 4), $P < 0.05$. E, hematoxylin and eosin staining of lung of mouse bearing human bone after s.c. injection of CD133+CXCR4+ cells showing massive lung metastatization (magnification, ×10; left) and inset of tumor cells at ×60 magnification (right).
Figure 3.
CXCR4 inhibition prevents metastatic dissemination of chemoresistant CD133^+ CXCR4^+ cells. A, invasion assay performed on PDX cells treated with CTCE-9908 or scramble peptide and chemoattracted with SDF-1 (left) or FBS (right). After 72 hours, invaded cells were counted in four random fields of the inserts; data, mean value ± SD. *, *P < 0.05. B, relative frequency of CD133^+ CXCR4^+ cells in invading cells fraction, recovered after treatment with CTCE-9908 and scramble peptide, compared with PDX total population (n = 4 models in duplicate experiments). *, *P < 0.05. C, fold change in CD133^+ and CXCR4^+ cells in treated H460 xenografts compared with control, evaluated by FACS. Data are mean % ± SD. n = 4 per group. D, qRT-PCR for stemness genes expression in cisplatin or cisplatin + CTCE-9908–treated H460 xenografts. Untreated control tumors were used as calibrator. E, representative IHC staining for human cytokeratins of H460 lung metastases in control, cisplatin, and cisplatin + CTCE-9908–treated groups. Magnification, ×10. Percentage of CD133^+ and CXCR4^+ cells in H460 lung metastases (F) and in LT111 DTCs (G) in control and treated groups, as evaluated by flow cytometry. Data, mean value ± SD. n = 4 mice per group. *, *P < 0.05; **, *P ≤ 0.01; ***, *P ≤ 0.001.
remodeling, revealed by large areas of new bone apposition and an increased osteoid thickness due to metastasis formation, was evident in mice injected with CD133⁺CXCR4⁺ cells compared with all other groups (P < 0.05; Fig. 2C and D). Notably, CD133⁺CXCR4⁺ cells injected both s.c. and i.c. in mice carrying human bone implants also showed significantly higher ability to disseminate and initiate lung metastasis, with extensive replacement of normal lung parenchyma with cancer cells (Fig. 2E), compared with CD133⁺CXCR4⁻ and CD133⁻CXCR4⁺ cells or unsorted cells (CD133⁺CXCR4⁺ vs. others groups, P = 0.007; Supplementary Table S1).

Altogether, these results prove the high dissemination and colonization potential of CD133⁺CXCR4⁺ cells and their ability to support metastasis development at distant organs.

Inhibition of the CXCR4 pathway prevents metastatic spread of chemoresistant CD133⁺CXCR4⁺ cells

To block the migration of the CD133⁺CXCR4⁺ metastatic subset, we tested the CXCR4 inhibitor CTCE-9908, a small peptide analogue of SDF-1, previously shown to be effective in preventing metastatic dissemination in different cancer models (22–25).

In vitro CTCE-9908 treatment could block invasion of PDX-derived tumor cells (n = 4 PDX models) induced by both SDF-1 and FBS (Fig. 3A). Interestingly, we also verified that the invading fraction of PDXs cells attracted with a nonspecific signal (FBS) was 10-fold enriched in CD133⁺CXCR4⁺ cells compared with total population and that CTCE-9908 inhibitor was able to specifically block their invasion (4-fold change; Fig. 3B), thus pointing at CXCR4 axis as a pivotal mediator of lung CIC invasion.

To test the efficacy of CXCR4 inhibition in a clinically relevant therapeutic setting, we treated in vivo metastatic H460 xenografts with CTCE-9908 in combination with standard chemotherapy. We previously reported that cisplatin treatment, although effective in reducing tumor size, induces enrichment of chemoresistant CICs in the residual tumor (15). Combination treatment with CTCE-9908 had a similar efficacy in inhibiting subcutaneous tumor growth as cisplatin (Supplementary Fig. S2A), but it was able to prevent the relative increase of CD133⁺CXCR4⁺ cells and concomitant upregulation of stemness genes induced by chemotherapy alone (Fig. 3C and D).

FACS analysis of H460 lung metastases in control mice demonstrated a 10-fold enrichment for CD133⁺CXCR4⁺ cells compared with matched s.c. xenograft (P < 0.05), confirming their pivotal role also in the spontaneous metastasis development (Supplementary Fig. S2B).

Cisplatin treatment primed the dissemination of the CD133⁺CXCR4⁺ subset (2-fold change) inducing a boost in metastasis formation (1.8-fold change) compared with control group (Fig. 3E and F). Combination therapy with CXCR4 inhibitor was able to counteract cisplatin effect, drastically reducing metastatic formation (2-fold decrease) and concomitantly decreasing the subsets of CD133⁺ and CD133⁺CXCR4⁺

Figure 4. CD133⁺CXCR4⁺ and EpCAM⁻ cells are crucial in early steps of colonization. A, time course analysis of i.v. injected LT111, LT73, and H460 tumor cells. Arrow on the left indicates increasing metastatic behavior of tested tumor cells. Percentages of tumor cells (left, y-axis) and relative fold increase in CD133⁺CXCR4⁺ and EpCAM⁻ subsets are evaluated by FACS analysis of murine lungs. Data represent mean ± SEM. n = 2 mice at given time point. B, qRT-PCR analysis for stemness genes expression of H460 cells, 2 hours, 1 and 3 weeks after i.v. injection in murine lung. H460 parental cell line was used as calibrator.
Figure 5. DTCs are enriched in CD133⁺CXCR4⁺EpCAM⁺CICs and generate tumors with distinctive features. A, percentages of the CD133⁺CXCR4⁺EpCAM⁻ subset within CD133⁺ cells in total PDX cells and in the corresponding invading cells fraction recovered after the invasion assay, evaluated by FACS as shown on the right. n = 5 PDXs. P = 0.01. B, representative image of lung spheroids culture and immunofluorescence staining for human CKs on lung spheroids cytospins, identifying single human DTC (top). Representative qRT-PCR amplification plot (bottom) showing increased expression of human B2M gene in lung spheroids (red circle) compared with lung tissue from PDX-bearing mice (green circle). (Continued on the following page.)
cells in lung metastasis compared with cisplatin-treated group (2.6- and 2.3-fold change, respectively; Fig. 3E and F and Supplementary Fig. S2C).

Next, we also proved in a highly disseminating PDX model (LT11) that in vivo combination treatment with CTCE-9908 had a modest effect on tumor growth inhibition (Supplementary Fig. S2D) but was able to prevent tumor spread associated with cisplatin treatment (1.5-fold change) and to efficiently reduce the fraction of disseminated CD133+/CXCR4+ CICs to murine lungs (4.7-fold change; Fig. 3G and Supplementary Fig. S2E).

We additionally verified in cell lines that CXCR4 is also frequently expressed (mean, 70%; range, 50%-90%) in another subset of CICs previously identified as chemoresistant (CD133+/ABCG2+; ref. 15) and that CXCR4 inhibition could also prevent enrichment of CD133+/CXCR4+/ABCG2- induced by chemotherapy (data not shown).

Our results suggest that combination treatment with CXCR4 inhibitor could specifically impair dissemination of chemoresistant and metastatic CD133+/CXCR4+ cells.

CD133+/CXCR4+ and EpCAM+ CICs have survival advantage at ectopic sites in early steps of colonization

To examine the role of CD133+/CXCR4+ cells in different steps of the metastatic cascade, we injected i.v. 1 × 10^6 unsorted lung tumor cells endowed with different metastatic behavior (LT11-PDX cells, weakly metastatic; LT73 cell line, moderately metastatic; H460 cell line, highly metastatic), and we monitored in time course experiment which tumor cell subsets were preferentially selected to drive specific steps of metastasis development. 

Flow cytometric analysis of murine lung 2 hours after injection confirmed the presence of tumor cells that maintained the same phenotype of original tumor cells (Supplementary Fig. S3A). After 1 week, the number of tumor cells drastically decreased (20-fold) with a concomitant enrichment for the CD133+/CXCR4+ subset in the surviving tumor fraction (Fig. 4A). We also detected in LT73 and LT111 surviving cells a significant increase for a fraction of CD133+/CICs negative for the expression of epithelial antigen EpCAM compared with parental injected tumor cells (18-fold increase), whereas the parental H460 cell line does not express at all EpCAM marker (Fig. 4A). Surviving LT111 cells remained in murine lung, without proliferating, for up to 3 months and stably showed enrichment for CD133+/CXCR4+ and CD133+/EpCAM+ subsets (Fig. 4A). Conversely, during metastatic growth of LT73 and H460 cells, both subsets of CD133+/CXCR4+ and CD133+/EpCAM+ cells, diminished and reverted to the original percentage of the parental cell line when metastases reached macroscopic size (Fig. 4A and Supplementary Fig. S3B).

Expression of stemness genes followed the same trend of CD133+/CXCR4+ cells in different phases of metastatic development (Fig. 4B).

In summary, these findings confirm the primary role of CD133+/CXCR4+ CICs in metastasis process, due to a superior survival advantage and colonization ability, and reveal the existence of the CD133+EpCAM- subset strongly enriched in early steps of colonization.

The subset of CD133+/CXCR4+ EpCAM- CICs sustains lung cancer dissemination and is endowed with high tumorigenic and metastatic potential

To further investigate the involvement of the CD133+/CXCR4+ and EpCAM- subsets in a preclinical model of spontaneous tumor dissemination, we analyzed lungs of PDX-bearing SCID mice.

While generally no histologic metastases were evident, FACS analysis was able to detect a subset of DTCs in 9 of 11 PDX models (n = 6 adenocarcinoma; 1 large cell carcinoma, LCC; 2 squamous cell carcinoma models, SCC), in a percentage (range, 0.001%-0.01%) consistent with that estimated from human B2M expression by qRT-PCR (data not shown).

Lung DTCs were already detectable at early stages of subcutaneous tumor development and remained stable during tumor growth (Supplementary Fig. S4A). Notably, immunophenotyping of lung DTCs revealed a constant increase for the fraction of CD133+/CXCR4+ CICs (P = 0.004) and in particular a predominant enrichment for the subset of CD133+/EpCAM- cells (P = 0.007) compared with parental subcutaneous tumors (Supplementary Fig. S4A and S4B), confirming evidence obtained in i.v. time course experiments.

Moreover, an in vitro invasion assay on dissociated PDX cells (n = 5) proved that CD133+/CXCR4+ EpCAM- cells represented the CIC subset more significantly enriched in the invading fraction compared with total population (90-fold, P = 0.01; Fig. 5A).

To functionally characterize the subset of lung DTCs enriched in disseminating CICs, we adapted an in vitro culture method useful to enrich for tumor cells (19), from 6 different PDXs (4 adenocarcinoma, 1 SCC, 1 LCC), we generated cultures of CTOS and corresponding lung tissue spheroids, highly enriched in DTCs compared with freshly dissociated lung tissue (range, 70- to 145-fold increase) as also confirmed by qRT-PCR analysis of human cytokeratins (Fig. 5B and Supplementary Fig. S4C).

Real-time analysis of lung spheroids cultures showed a strong upregulation for stemness genes and for a panel of EMT-related genes in DTCs compared with matching PDX-CTOS, subtending an enrichment for the subset of CD133+ stem-like cells endowed with mesenchymal features (i.e., EpCAM-; Fig. 5C).

To functionally test tumorigenic properties of DTCs, we s.c. injected in NOD-SCID mice 1 × 10^5 lung spheroids dissociated cells, containing approximately 200 DTCs and 1 × 10^5 PDX-CTOS dissociated cells generated from LT59 PDX. DTCs were able to originate tumors that closely resembled histology and phenotype of original PDX (Fig. 5D) but contained a larger amount of...
Figure 6. Microenvironment stimuli generate disseminating CICs. A, percentage of CD133+ cells evaluated by FACS in the parental LT73 cell line and in LT73-EMT cells. n = 10 independent experiments. P = 0.0001. B, relative mRNA expression of EMT-associated genes and stemness genes in LT73-EMT cells, MSC, and CAFs. CAF cells were used as calibrator for analysis. (Continued on the following page.)
CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> cells compared with parental xenograft (Fig. 5E). Moreover, cells from DTC tumors maintained a slightly increased expression of EMT-related genes compared with CTOS tumors and exclusively possessed the ability to proliferate in vitro as spheres in anchorage-independent condition (Supplementary Fig. S4D and S4E).

Remarkably, in vivo s.c. injection of cells from DTC tumors demonstrated an enhanced ability to generate secondary tumors (Fig. 5F) and higher lung dissemination ability compared with CTOS tumor (disseminated tumor cells: 0.6% ± SD 0.1 vs. 0.09% ± SD 0.03; Fig. 5G).

Finally, intravenous injection of LT59 lung sphereoids dissociated cells (~200 DTCs) in NOD-SCID mice generated lung metastases, which again showed an increased fraction CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> cells, whereas up to 1 × 10<sup>8</sup> LT59 cells and LT59-CTOS cells failed to initiate metastasis (Fig. 5H).

Our evidence proves the existence of an invasive and highly tumorigenic CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> subset that specifically drives lung tumor dissemination.

Microenvironment stimuli modulate the subset of CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> lung disseminating CICs

The mesenchymal properties displayed by CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> disseminating CICs, lead us to investigate the possible involvement of EMT activation in their generation/modulation (26).

Induction of EMT through TGFβ treatment of the LT73 tumor cell line (LT73-EMT) evaluated as morphologic changes, modulation of EMT-related genes, and increased invasive potential (Supplementary Fig. S5A–S5C), caused a 10-fold increase in the fraction of CD133<sup>+</sup> CICs (P = 0.0001) with a concomitant upregulation of stemness gene expression (Fig. 6A and Supplementary Fig. S5D). We also verified that LT73-EMT cells showed an expression pattern for EMT and stemness-related genes more similar to mesenchymal stem cells (MSC) and then to differentiated adult mesenchymal cells (i.e., fibroblasts derived from primary lung tumors; Fig. 6B).

Increase of CD133<sup>+</sup> CICs in LT73-EMT cells was functionally proved by enhanced in vivo tumorigenic potential and ensuing tumors stably maintained an augmented content of CD133<sup>+</sup> cells compared with LT73 parental tumors (Supplementary Fig. S5E and S5F).

Examination of CD133<sup>+</sup> CIC subsets in LT73-EMT cells demonstrated a 3.8-fold increase (P = 0.04) for metastatic CD133<sup>+</sup> CXCRI<sup>+</sup> cells that was mainly driven by the expansion of CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> subpopulation (13.5-fold increase, P = 0.02; Fig. 6C).

In PDXs and primary CTOS cultures (n = 5), EMT induced by TGFβ was able to enrich for CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> disseminating CICs (15-fold change, P = 0.05; Fig. 6D and Supplementary Fig. S5G), associated with an increased in vitro invasiveness and upregulation of EMT and stemness genes (Supplementary Fig. S5H and S5I).

To evaluate whether the CIC enrichment was due to an expansion of the pre-existing CD133<sup>+</sup> pool or de novo generation, we depleted CD133<sup>+</sup> cells by FACS from the LT73 cell line, creating the LT73-CD133<sup>−</sup> line that stably maintained a CD133<sup>+</sup> phenotype during in vitro culturing (Fig. 6E). TGFβ treatment of LT73-CD133<sup>−</sup> cells induced the generation of a fraction of CD133<sup>+</sup> cells (mean ± SD, 0.14% ± 0.05%), similar to that observed in LT73 parental cells (mean ± SD, 0.09% ± 0.02%), that however exhibited a lower expression of EpCAM compared with endogenous CD133<sup>+</sup> cells (Fig. 6E). Upon TGFβ withdrawal, LT73-CD133<sup>−</sup> cells slowly reverted to an epithelial phenotype, whereas the fraction of de novo generated mesenchymal CD133<sup>+</sup> cells persisted, indicating a stable reprogramming (Fig. 6E and Supplementary Fig. S5J and S5K). Consistently, tumors derived from s.c. injection of LT73-CD133<sup>−</sup> EMT appeared with a shorter latency period than LT73-CD133<sup>−</sup> cells and maintained a similar fraction of de novo generated CD133<sup>+</sup> cells (Supplementary Fig. S5N and S5O).

To verify whether physiologically relevant tumor microenvironment stimuli could show similar proficiency in generating lung CICs, LT73-CD133<sup>−</sup> cells were cocultured with conditioned medium (CM) from cancer-associated fibroblasts (CAF) isolated from primary human lung tumors. A variable but constantly detectable fraction of de novo CD133<sup>+</sup> cells was observed after exposure to different CMs, mirrored by a parallel modulation of EMT genes (Fig. 6F and G), indicating that also stimuli from microenvironment are able to expand tumor cells with mesenchymal traits associated with CIC generation.

Because stromal SDF-1 has been proven to induce EMT activation in different tumor types (27, 28), we explored the role of SDF-1 as a possible mediator of CAF effects on tumor cells. In line with reported evidence, we confirmed that in vitro treatment of the LT73 cell line with SDF-1 resulted in upregulation of EMT genes (Supplementary Fig. S5P). Treatment of LT73-CD133<sup>−</sup> cells with CMs and CXCR4 inhibitor CTCE-9908 demonstrated a partial prevention of EMT genes modulation compared with CMs alone, thus confirming that SDF-1 represents one of the CAF-secreted factors that mediate EMT activation in tumor cells (Fig. 6G).

CM-generated CD133<sup>+</sup> CICs were functionally associated with an enhanced in vitro tumorigenicity compared with untreated CD133<sup>+</sup> cells (Fig. 6H), and interestingly, the ensuing xenografts displayed a CD133 fraction reflecting the proficiency of CAF-CMs to create CICs in vitro (Fig. 6I). Notably, the relative percentages of de novo generated CD133<sup>+</sup> CXCRI<sup>+</sup> EpCAM<sup>-</sup> cells detected in CM-treated LT73-CD133<sup>−</sup> tumors correlated with their disseminating potential to murine lungs (Fig. 6I and J).

(Continued)
Our data demonstrate a dynamic phenotype of lung CICs and show that stimuli from tumor microenvironment primarily generate the subset of CD133\(^+\)CXCR4\(^+\)EpCAM\(^{-}\)CICs functionally associated with tumor dissemination.

Disseminating CICs can be detected in lymph nodes of lung cancer patients and correlate with poor clinical outcome

Comparison of lymph node metastases and matched synchronous primary NSCLC tumors (\(n=17\)) revealed a 20-fold enrichment in the fraction of CD133\(^+\)CXCR4\(^+\)EpCAM\(^{-}\) cells in metastases (\(P=0.006\); Fig. 7A), confirming in a clinical setting our preclinical data for the existence of CD133\(^+\)CXCR4\(^+\)EpCAM\(^{-}\) CICs related to tumor dissemination and metastasis initiation.

To evaluate the potential clinical relevance of disseminating CICs, we analyzed, by FACS, 78 surgically resected primary NSCLC for expression of CD133, CXCR4, and EpCAM. As we previously reported, the overall frequency of CD133\(^+\) cells was generally low with a median value of 1.3% (interquartile range, 0.7%–2%). A subset of CD133\(^+\)CXCR4\(^+\)EpCAM\(^{-}\) cells was detected in 50% of cases, independently from the total amount of EpCAM\(^{-}\) tumor cells. The CD133\(^+\)CXCR4\(^+\)EpCAM\(^{-}\) fraction...
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accounted for 12.5% of total CD133<sup>+</sup> cells (range, 0.4%–100%). There was no correlation among the total amount of CD133<sup>+</sup> cells and clinical variables, including age, sex, histologic subtype, grade, or stage (Supplementary Table S2). The frequency of migrating CICs (CD133<sup>+</sup>/CXCR4<sup>+</sup> EpCAM<sup>-</sup>) within the CD133 pool was more frequent in SCCs (P = 0.017). Interestingly, both CD133<sup>+</sup> and CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> content was associated with the presence of chronic obstructive pulmonary disease (P = 0.025 and P = 0.011, respectively), indicating a possible role of the lung microenvironment in modulating stemness and disseminating properties. More importantly, while the total amount of CD133<sup>+</sup> cells did not correlate with clinical outcome, the presence of the CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> subset was negatively correlated with both disease-free survival (DFS, P = 0.033) and overall survival (OS, P = 0.055; Fig. 7B). The association of disseminating CICs with DFS remained significant also in subclass analyses when only nonsquamous tumors were analyzed (P = 0.038) and in a multivariate model even after adjustment for stage [P = 0.03; HR, 2.25; 95% confidence interval (CI), 1.05–4.82]. Particularly, in tumors with low content of CD133<sup>+</sup> cells (<2%), the presence of CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> metastatic CICs was associated in multivariate analysis with both shorter DFS (P = 0.03; HR, 3.34; 95% CI, 1.09–10.18) and OS (P = 0.05; HR, 3.10, 95% CI, 1.00–9.59), thus representing a risk factor for tumor relapse and poor prognosis in patients with NSCLC.

**Discussion**

Although metastasis has generally been considered a multistep process requiring acquisition of specific genetic alterations providing tumor cells with crucial advantages (29), plasticity is gaining attention as a fundamental trait for metastasis-prone tumors, and the identification of definite phenotypes linked to this process appears crucial for its understanding (30).

Here, we demonstrate that the subset of CD133<sup>+</sup>/CXCR4<sup>+</sup> lung CICs drives tumor cells seeding and metastasis initiation in lung cancer. The high metastatic potential of CD133<sup>+</sup>/CXCR4<sup>+</sup> cells and their enhanced ability to seed and colonize different organs (i.e., lung and bone) is substantiated in experimental lung metastasis as well as in an humanized murine model of bone metastasis: interestingly, in such an experimental model, the presence of a "human pre-metastatic niche" represented by implanted bone is conductive for generation of overt metastases in a considerable shorter time than in an experimental metastasis assay, stressing the importance of proficient niche to support and dictate tumor development and the challenge of recapitulating the human microenvironment in mouse (31–33).

Next, we prove for the first time that CXCR4 targeting is able to counteract the chemotherapy-induced metastatic spread of chemoresistant fractions of CD133<sup>+</sup>/CXCR4<sup>+</sup> and possibly also of CD133<sup>+</sup>/ABC2<sup>+</sup> CICs (15) that mostly express CXCR4 receptors, pointing at combination therapy with CXCR4 inhibitor as an attractive novel strategy to improve neoadjuvant and adjuvant therapy for NSCLC.

Time course analysis of phenotypic changes in i.v. injected tumor cells at different steps of metastatic cascade revealed in the first phase of colonization an enrichment for a subset of lung CD133<sup>+</sup>/CXCR4<sup>+</sup> cells negative for EpCAM; similar enrichment was observed in spontaneous early disseminated tumor cells detected in PDX preclinical models. These data are in line with evidence in different tumor types for an early tumor metastatic spread guided by cells endowed with mesenchymal and stem-like features (11, 12, 34). Besides the phenotypic characterization, we functionally prove that lung DTCs, surviving in ectopic murine soil without metastatic outgrowth, are indeed highly tumorigenic and metastatic when injected s.c. with Matrigel or i.v. in NOD/SCID mice, proving the metastasis initiation potential of the specific subset of CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> cells, as well as highlighting once more the need of a favorable metastatic niche to dictate tumor growth (32, 33, 35).

Remarkably, tumors generated from DTCs, as well as patient’s lymph nodes metastases, are enriched in the CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> CIC subset, demonstrating in a clinical setting, the relevance of this subpopulation in metastasis formation.

The mesenchymal features of disseminating tumor cells as well as the well-known link between EMT activation and acquisition of invasiveness and stem-like features prompted us to investigate the possible involvement of EMT in their generation (7, 36, 37). Our data indeed confirmed that EMT triggering generates CD133<sup>+</sup> cells and, more interestingly, we demonstrated for the first time that stimuli from tumor microenvironment, specifically those from CAF, could dictate de novo creation of the CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> CIC subset directly linked to EMT induction and acquisition of in vitro and in vivo increased dissemination. Similar data proving the capability of microenvironmental stimuli to convert progenitor cells into cancer stem cells with metastatic properties are reported in colon cancer (10).

Moreover our finding showing that CAF-secreted SDF-1 promotes EMT activation and likely the acquisition of stemness features in lung tumor cells, in line with evidence reported in colorectal cancer (27), strengthens the potential therapeutic use of CXCR4 inhibitor to prevent both dissemination of metastatic CICs and their generation through the impairment of tumor-stroma cross-talk.

The clinical relevance of our findings was demonstrated by the fact that the frequency of CD133<sup>+</sup>/CXCR4<sup>+</sup>/EpCAM<sup>-</sup> within the CIC pool in primary tumors represents an independent indicator of poor prognosis in surgically resected NSCLC patients, suggesting that dissection of the biologic complexity of primary tumors will be crucial to improve clinical management of lung cancer.

The prospective to identify the fraction of metastatic CD133<sup>+</sup>/CXCR4<sup>+</sup> CICs negative for EpCAM expression in circulating tumor cells from patients with lung cancer holds therefore great interest and implications from both a prognostic and therapeutic point of view, as recently highlighted in patients with breast cancer where isolated EpCAM<sup>-</sup> circulating tumor cells showed high invasiveness and ability to generate brain and lung metastases (38–40).

In conclusion, we provide evidence for the existence of novel human lung disseminating CICs regulated by microenvironmental signals able to complete all the steps of the metastatic cascade and related to clinical recurrence. We also propose CXCR4 targeting as a novel therapeutic option in neoadjuvant and adjuvant setting to counteract the dissemination of chemoresistant and metastatic lung CICs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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