Nodal-Induced L1CAM/CXCR4 Subpopulation Sustains Tumor Growth and Metastasis in Colorectal Cancer Derived Organoids.

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Abstract

Background

Colorectal cancer (CRC) is currently the third leading cause for cancer-related mortality. Cancer stem cells have been implicated in colorectal tumor growth, but their specific role in tumor biology, including metastasis, is still uncertain.

Methods

Increased expression of L1CAM, CXCR4 and NODAL was identified in tumor section of patients with CRC and in Patients-derived-organoids (PDOs). The expression of L1CAM, CXCR4 and NODAL was evaluated using quantitative real-time PCR, western blotting, immunofluorescence, immunohistochemistry and flow cytometry. The effects of the L1CAM, CXCR4 and NODAL on tumor growth, proliferation, migration, invasion, colony-formation ability, metastasis and chemoresistance were investigated both *in vitro* and *in vivo*.

Results

We found that human colorectal cancer tissue contains cancer stem cells defined by L1CAM^{high}/CXCR4^{high} expression that is activated by Nodal in hypoxic microenvironment. This L1CAM^{high}/CXCR4^{high} population is tumorigenic, highly resistant to standard chemotherapy, and determines the metastatic phenotype of the individual tumor. Depletion of the L1CAM^{high}/CXCR4^{high} population drastically reduces the tumorigenic potential and the metastatic phenotype of colorectal tumors.

Conclusion

In conclusion, we demonstrated that a subpopulation of migrating L1CAM^{high}/CXCR4^{high} is essential for tumor progression. Together, these findings suggest that strategies aimed at modulating the Nodal signaling could have important clinical applications to inhibit colorectal cancer-derived metastasis.

Background

Colorectal cancer (CRC) is currently the third leading cause for cancer-related mortality¹. Despite growing comprehension in tumor biology, the treatment efficiency in CRCs has not improved significantly over the past decade. Several studies on CRC have been focused on the role of cancer stem cells (CSCs). Indeed, cells bearing stem cell properties are involved in self-renewal, tumor progression, apoptosis resistance and cancer relapse following treatment²³.

Nodal belong to transforming growth factor (TGF)- β super family of growth and differentiation factors expressed in various tissues. Through binding with its receptors, Nodal exerts its biological effects by activating the intracellular signaling pathway. Nodal receptors are trans-membrane serine/threonine

kinases, including type I (ActRI, named ALK4 and ALK7) and type II (ActRII) receptor. ActRI combines with ActRIIs to form a complex to transduce Nodal signals. After Nodal binding, ActRI is capable to phosphorylate Smad2 and Smad3. Phosphorylated Smad2/3, together with Smad4, translocate to the nucleus where they regulate the transcription of NODAL target genes⁴⁵. Interestingly, Nodal pathway may determine the tumor cell progression and metastatic ability by regulating cancer stem cells self-renewal in CRC⁶. Besides, Nodal is activated in hypoxic tumor microenvironment⁷. Hypoxia enables a number of events in the tumor microenvironment that lead to the expansion of aggressive clones from heterogeneous tumor cells thus promoting a lethal phenotype⁸.

Overexpression of L1 cell adhesion molecule (L1CAM; CD171) has been reported in numerous human cancers, including breast, kidney and lung⁹. In colorectal cancer cells, L1CAM promotes cell growth and survival and L1CAM secreted from tumor cells makes these cells more invasive and mobile and thus, more aggressive¹⁰. Conversely, L1CAM has an anti-oncogenic function in pancreatic cancer¹¹.

In CRC has been demonstrated that abnormal expression of Chemokine (C-X-C motif) receptor 4 (CXCR4) plays a crucial role in the invasion and liver metastasis¹². CXCR4 belongs to G protein-coupled receptor superfamily, which selectively binds to stromal cell-derived factor 1 (SDF-1, also called CXCL12) to promote cancer metastasis¹³. Corroborating this data, high expression of CXCR4 correlates with poor prognosis in CRC patients¹⁴¹⁵.

Here, we identified a subpopulation of L1CAM^{high}/CXCR4^{high} CSCs activated by Nodal signaling in hypoxic microenvironment that is not only endowed with tumor-initiating properties, but is also capable of forming liver metastasis.

Methods

Bioinformatics analysis

Survival was analyzed using the <http://genomics.jefferson.edu/proggene/filter.php>. A Median Group cut-off (50% high vs 50% Low) was used. GSE40967 contains gene expression profile data of 566 colon cancer patients. *NODAL* transcript expression in colon tumor (T, n=272) and normal epithelium (N, n=41) was analyzed using the <http://gepia2.cancer-pku.cn/#survival>.

Culture of patient-derived tumor organoids

Human biological samples used to expand tumoroids were obtained from individuals treated at Hospital del Mar and Hospital de la Santa Creu i Sant Pau, under informed consent and approval of the Tumor Bank Committees according to Spanish ethical regulations. The study followed the guidelines of the Declaration of Helsinki and patients identity of pathological specimens remained anonymous in the context of this study. MTA 27/04/2017 n.324 CRC cells with high EphB2 levels were FACS sorted from dissociated tumors and cultured embedded in Matrigel (Basement Membrane Matrix Low Concentration,

BD) with Advanced DMEM/F12, 10 mM HEPES, 1× Glutamax; 1× B-27 without retinoic acid, 1× N-2, 20 ng/ml bFGF (basic fibroblast growth factor); 50 ng/ml EGF (epidermal growth factor), 1 μM LY2157299 and 10 μM Y-27632. Under these conditions, cells with high EphB2 levels expanded as tumor organoids that we could propagate indefinitely, whereas cells with medium or low EphB2 levels did not. All cells were tested weekly for mycoplasma contamination with negative results.

Immunohistochemistry

Immunostainings were carried out using 4 μm tissue sections according to standard procedures. Briefly, after antigen retrieval, samples were blocked with Peroxidase-Blocking Solution (Dako, S202386) for 10 min at RT, and then primary antibodies were incubated overnight. Slides were washed with EnVision™ FLEX Wash Buffer (Dako, K800721) and the corresponding secondary antibody was incubated with the sample for 45 min at RT. Samples were developed using 3,3'-diaminobenzidine, counterstained with hematoxylin and mounted. See Antibodies section. Images were acquired using a digital image scanning (Nanozoomer 2.0HT, Hamamatsu) and cropped using NDP.view2.

Immunofluorescence

L1CAM-PE, Ki67-FITC, CXCR4-FITC, E-CADHERIN-FITC antibody and Phalloidin-TRITC (see Antibodies section) were incubated for 30 minutes at room temperature in the dark. Unconjugated pSMAD2 and NODAL were incubated over night at 4°C and the day after counterstained with fluorescent secondary antibody. Cell nuclei were stained with DAPI and slides were mounted in Glycerol/PBS/Phenylenediamine for observation using an SP5 or SPE confocal microscope (Leica).

Antibodies. The following primary antibodies were used:

Antibody	Manufacturer and Reference	Technique and dilution used
Phospho-Smad2	Cell Signaling, Ref. 3108	Immunohistochemistry and Immunofluorescence (1/100), Western Blot (1/1000)
E-Cadherin-FITC	BD Bioscience Ref. 612130	Immunofluorescence (1/50)
Phalloidin-TRITC	Sigma Ref. P1951	Immunofluorescence (1/2500)
L1CAM-PE	eBioscience, Ref. 12-1719-42	Immunofluorescence and Flowcytometry (1/200)
Ki67-FITC	eBioscience, Ref. 11-5699-42	Immunofluorescence (1/200)
CXCR4-FITC	R&D systems, Ref. FAB170F-100	Immunofluorescence and Flowcytometry (1/100)
NODAL	Abcam, Ref: ab55676	Immunohistochemistry and Immunofluorescence (1/200)

Quantitative RT-PCR (qRT-PCR)

RNA was extracted using Trizol Reagent (Invitrogen) and qRT-PCR was performed using TaqMan assays (Applied Biosystems) following manufacturer's instructions.

Flow cytometry and cell sorting

To identify CSCs anti-human membranous L1CAM-PE, Anti-human CXCR4-FITC and were used. 7AAD (BD) was used for exclusion of dead cells. Samples (n>6) were run on the FACS Canto II (BD) and data were analysed using FlowJo 9.2 (Ashland, OR).

Protein isolation and western blot analysis

Cells were lysed with RIPA buffer (50mM Tris-HCl at pH 7.6, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM EDTA plus proteases and phosphatases inhibitors) for 1hr at 4°C. Total protein quantification was performed with Bio-Rad Protein Assay Dye Reagent concentrate. A total of 50µg of protein was separated on 15% SDS–PAGE gels at 100 V and transferred to PVDF membranes for

2 hours at 200mA. PVDF membranes were hybridized with mouse antibodies against L1CAM (HPA005830, Sigma-Aldrich), HIF1a (2015-1, Epitomics), GAPDH (ab9483, Abcam), NODAL (ab55676, Abcam), CXCR4 (ab124824, Abcam), pSmad2 (3108; Cell Signaling) and Smad2 (#5339; Cell Signaling), treated with peroxidase-conjugated goat anti-mouse or anti-rabbit Ig secondary antibody (DPVR-HRP, Immunologic), and then visualized by enhanced chemiluminescence (ECL Nova 2.0 XLS071, 2050 Cyanagen). n>6.

Lentiviral shRNA delivery

As lentiviral shuttle backbone we used a pLKO shRNA plasmid (Mission SIGMA). As control we used pLKO shRNA scramble expression vectors. Cells were then transduced with lentiviral particles in the presence of polybrene (8ug/ml, Sigma). The cells were seeded at a density of 30,000 cells per well in a 24-well plates and allowed to adhere overnight. The next day, the cells were infected with the lentiviral particles for 6 hours. Stably transduced cells were obtained using puromycin resistance.

Organoids growth and treatment

2000 colon CSCs were plated in a 25 µl drop of matrigel in 48-well flat bottom plates in presence of rNODAL recombinant protein (50 ng mL⁻¹) or SB431542 (10 µM). Media were changed every 2 days including fresh rNODA or SB431542. At the indicated time points the organoids were counted in order to follow the growth of seeded cells. Counting was performed using ImageJ software. Triplicate wells were assayed for each condition and standard deviation (s.d.) was determined.

Plasmid construct and transfection

NODAL expressing plasmids and empty vectors (pcDNA3.1) were obtained from Genscript (New Jersey, USA). Transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) following the manufacturers' instructions.

Apoptosis assay

Attached and floating cells were collected, resuspended and stained with Annexin V (550474; BD Bioscience) after incubation with Annexin V binding buffer (556454, BD PharMingen). Cells were then incubated with PI. Samples were analysed by flow cytometry using a FACS Canto II (BD), and data were analysed using DIVA Software.

Cell cycle assay

To synchronize the cell cultures, the cells were seeded in 6-well plate in growth medium with 10% FBS overnight. Then the cultures were rinsed by PBS and changed to serum free medium. After serum starvation for 24 hours, the cells were passaged and released into cell cycle by addition of serum. For FACS analysis, cell samples were harvested at indicated time points. Cells were trypsinised, washed in PBS, centrifuged, and pellets were fixed in 200µl of 70% ethanol and stored at -20°C until use. Cells were

centrifuged and pellets resuspended in 200µl of PBS with 10µg/mL of RNase A. Cells were incubated for 1 hour at 37°C prior to resuspension in PI. Cell-cycle analysis was carried out by flow cytometry (CANTO II). Data were analysed by DIVA software.

Xenotransplantation experiments

All experiments with mouse models were approved by the animal care and use committee of the Barcelona Science Park (CEEAPCB), the Catalan Government (P18-R5-09) and by the local ministry (IACUC protocol #992/2017-PR).

Cells were injected subcutaneously, intra-caecum or intra-splenically in 5 to 6 weeks old NSG mice (Jackson Labs), which were followed until sacrifice. Tumor appearance was assessed by palpation.

In vivo treatment of established colorectal cancer

Single-cell suspensions were subcutaneously implanted and when the tumors reached 20mm² the mice were randomized to the respective treatment groups. Size and weight of the colorectal tumors were monitored. 5-FU was administered twice a week for 21 days (30 mg/kg i.p.). SB431542 was used at 25 mg/kg, by oral gavages twice daily for 3 weeks. Control mice were treated with vehicle.

Statistical Analyses

Results for continuous variables are presented as means ± standard deviation (SD) unless stated otherwise of at least three independent experiments. Treatment groups were compared to the independent samples t test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. The disease-free interval of patients was calculated using the Kaplan–Meier method, and differences among subgroups were assessed by the log-rank test. Experiments were performed a minimum of three independent times and always performed in independent triplicate samples. qPCR were repeated a minimum of five independent times in triplicate. $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPAD Prism7. Correlation analysis were performed applying the Pearson's correlation coefficient.

Results

Clinical impact of L1CAM, CXCR4 and Nodal signaling in CRC

In a first step, we aimed to identify colorectal biomarkers with CSC features in tissue samples derived from patients with colorectal cancer. We observed that *L1CAM* and *CXCR4* overexpression was associated with reduced Overall Survival (OS) by Kaplan–Meier survival analysis ($p < 0.05$) (**Figure 1A**). One of the key players in the (TGF)-β super family is NODAL, which has been detected at higher levels in CRC tissue compared to adjacent non-cancerous tissue⁴. We analyzed the level of *NODAL* transcripts in a series of CRC (T, n=272) and adjacent normal colonic epithelium (N, n=41) tissue samples from human patients. Interestingly, we found an increase of *NODAL* expression in tumor samples as compared to

healthy tissue samples (**Figure 1B**). Histological analyses revealed that L1CAM, CXCR4 and ALK4 (the Nodal-receptor) have a similar expression pattern (**Figure 1C, insets**) and were anatomically localized in the bulk tumor and in the invasive front of colorectal cancer samples (**Figure 1C**). All samples from patients with colorectal cancer reproducibly demonstrated the presence of L1CAM, CXCR4 and ALK4 - positive cells in the invasive front with histological evidence for cell dissemination (**Figure 1C**). The high number of L1CAM^{high} and CXCR4^{high} cells in cancer tissue, compared to the normal epithelium (**Figure 1C**), most likely results from their oncogenic transformation.

To further investigate this phenotype we analysed the expression of L1CAM in Patient-Derived-xenograft (PDx) generated from primary tumors injected in the caecum (IC) of immunocompromised mice, and their matched Patient-Derived-Organoids-xenograft injected either in the caecum (PDOx_IC), which were also able to give rise Liver Metastasis (LiMets) (**Figure 1E**), or subcutaneously (PDOx_SC) (**Supplementary Figure 1A**). The histological profile of L1CAM was conserved in all the sections analysed (e.g., PDx_IC, PDOx_IC and PDOx_SC), with PDOx typically showing a more cuboidal appearance, but otherwise a similar cellular morphology to the primary specimen (**Figure 1E**). Quantification of KI67 positive cells showed that L1CAM^{high} cells have a similar proliferation profile in the human CRC and in the PDOx_SC (**Supplementary Figure 1B and 1C**). Interestingly, the L1CAM^{high} cells have a nuclear activation of pSMAD2 and are NODAL-positive in both human CRC and PDOx_SC (**Supplementary Figure 1B**). Finally, also the CXCR4 expression profile is similar in both samples (**Supplementary Figure 1B**). To more broadly assess the expression profiles of *L1CAM*, *CXCR4* and *NODAL* genes in CRC, we performed a quantitative PCR (qPCR) analysis on PDOx_SC, PDOx_IC, PDOx_LiMets (**Figure 1F**) and PDOs (**Supplementary Figure 1E**). These results were consistent with the immunostaining and revealed similar expression levels among the different injections. Of note, by flow cytometry we that L1CAM^{high}/CXCR4^{high} population significantly increased in the PDOx_LiMets derived cells compared with the PDOx_SC derived cells (**Figure 1F**), suggesting that this double population plays a role in the secondary tumor.

Nodal signaling and L1CAM^{high}/CXCR4^{high} populations are both up-regulated in a hypoxic environment

Hypoxic condition regulates the cancer stemness in colorectal cancer¹⁶. Western blot analysis showed that L1CAM levels were up-regulated, in a time-dependent manner, under hypoxic condition (i.e., 1% O₂), with a peak of expression after 18 hours (**Figure 2A**). NODAL similarly increased in the culture media after 18 hours in hypoxic condition (**Figure 2B, upper panel**); while CXCR4 expression peaked later at 20 hours (**Figure 2B, lower panel**). The expression profile of *NODAL*, *L1CAM* and *CXCR4* was confirmed by qPCR (**Figure 2C**) and immunofluorescence analysis (**Figure 2D**). Under low oxygen level we also detected, by immunofluorescence (IF) on PDO#2, a nuclear translocation of pSMAD2 (**Figure 2D**). Flow cytometry analysis confirmed that L1CAM^{high}/CXCR4^{high} double population is significantly augmented in low oxygen compared to the normoxic condition (**Figure 2E and 2F**). Notably, the increase of L1CAM population in low oxygen condition strictly relies on NODAL signaling. Indeed, *NODAL* knock down (**Figure 2G**) prevented increased expression of L1CAM in hypoxic condition as shown by FACS analysis (**Figure**

2H and **2I**). Conversely, CXCR4 expression was not affected upon NODAL downregulation (data not shown).

Nodal induces an L1CAM^{high}/CXCR4^{high} double population with CSC properties

High Nodal activity characterizes the colon cancer stem cell (CSC) population ⁴ and its expression is augmented in hypoxic condition as demonstrated above. To better understand the effect of Nodal on tumor cell behavior, we forced the system by treating the PDOs with NODAL recombinant protein (rNODAL). We performed either a short (7 days) or a long (12 days treatment (for 7 days) with rNODAL in presence or absence of the inhibitor of the Nodal/Activin receptor ALK4 (SB431542); and a long treatment with rNODAL for 12 days (**Figure 3A**). We treated three different PDOs, PDO#1 (smad4 mutant, no responsive to the treatment) and two smad4 proficient PDOs (i.e., PDO#2 and PDO#5). While short NODAL treatment increased the tumor initiation frequency (TIC) in PDO#2 and PDO#5 of 2 and 2.3 times, respectively, it does not alter TIC in the PDO#1, as expected (**Figure 3B**). Moreover, the short NODAL treatment significantly increased the number of organoids (**Figure 3C** and **3D**) and their size (**Figure 3E**), compared to the untreated control. Both L1CAM and CXCR4 expression was strongly induced by NODAL, in the PDO#2 and PDO#5, while it was repressed by pharmacological inhibition of Nodal signaling (**Figure 3F** and **3G**). Western blot analysis of pSMAD2 confirmed that Nodal signaling was properly activated in PDO#2 and PDO#5 (**Figure 3F**), but not in the PDO#1 cells (Supplementary Figure 2A). These findings were further confirmed by IF (**Figure 3H**) and qPCR (**Figure 3I** and **Supplementary Figure 2B**). Moreover, both NODAL overexpressing PDO#2 and PDO#5 showed marked increase in *L1CAM* mRNA compared to the mock control PDOs (**Supplementary Figure 2C**).

Of note, the short NODAL treatment reduced the cells death, while co-treatment with SB431542 inhibitor reverted this effect and decreased the percentage of live cells (**Supplementary Figure 3A** and **3B**). In line with these findings, while expression of the cell cycle inhibitors *CDKN1A*, *CDKN1B* and *CDKN1C* decreased, *CYCLIN-D1* and *Ki67* increased upon short Nodal treatment (**Figure 3I**). Finally, FACS-based EDU incorporation assay showed increased S-phase (**Supplementary Figure 3C** and **3D**). It has been reported that Nodal promotes an aggressive phenotype in several types of cancers by inducing EMT ¹⁷¹⁸. However, Nodal treatment did not alter the expression of key EMT genes, including *CDH1*/E-cadherin, *SNAIL* and *VIMENTIN* (**Figure 3I**).

We then assessed the effect of long NODAL treatment (i.e., 12 days). Of note, we found that it induced morphological changes in the PDOs, which became flat and showed L1CAM^{high}/CXCR4^{high} spreading cells (**Figure 3H** and **Supplementary Figure 4A**). While long NODAL treatment induced both *L1CAM* and *CXCR4* overexpression as observed with short NODAL (**Figure 3I**), it did not alter the expression of cell cycle (**Figure 3I**). Conversely, long NODAL treatment determined a significant decrease of *CDH1* and increase of *SNAIL1* and *VIMENTIN* (**Figure 3I** and **Supplementary Figure 4B**), suggesting an induction of a mesenchymal phenotype.

In order to evaluate the impact of the L1CAM expression on tumor growth, we knockdown (KD) its expression in PDO#2 by ShRNAs. *L1CAM* KD PDO#2 cells showed a reduced proliferative capacity compared to the Sh scramble control (**Supplementary Figure 4C**), and were unable to generate tumors when subcutaneously injected in nude mice (**Supplementary Figure 4D**). In agreement with these data, *L1CAM* knockdown significantly downregulated the expression levels of stem cell-related genes, including *EPHB2* and *OLFM4* (**Supplementary Figure 4E**).

Taken together these data supported the idea that Nodal signaling tightly control the cells behavior in a time-dependent manner.

Identification of migrating $L1CAM^{high}/CXCR4^{high}$ subpopulation in CRC organoids

To better understand the biology of $L1CAM^{high}/CXCR4^{high}$ double population we FACS-sorted the cells from PDO#2 and PDO#5 and analyzed their capacity to form organoids (**Figure 4A** and **Supplementary Figure 5A**). The $L1CAM^{high}/CXCR4^{high}$ cells exhibited the highest capacity to form organoids (**Figure 4B** and **4C**) that were also bigger in size compared to the other populations (i.e., $L1CAM^{low}/CXCR4^{low}$, $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{low}/CXCR4^{high}$) (**Supplementary Figure 5B**). These results were further confirmed in the second generation (P1) (**Figure 4B** and **4C**). Accordingly, we found that cells $L1CAM^{high}/CXCR4^{high}$ cells showed higher expression of colon CSC markers (i.e., *LGR5*, *OLFM4* and *EpHB2*) and lower expression for the epithelial differentiation-associated gene (i.e., *KRT20* and *MUC2*) compared to the $L1CAM^{low}/CXCR4^{low}$ population (**Supplementary Figure 5C**). Furthermore, $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{high}/CXCR4^{high}$ populations also showed increased expression of the chemoresistant gene *ABCG1* and *CDH1*, while expression of the mesenchymal genes (i.e., *SNAIL 1* and *VIMENTIN*) was downregulated (**Figure 4D** and **Supplementary Figure 5D**), suggesting that these populations were more resistant to chemotherapeutic treatment and showed a more mesenchymal traits. Corroborating these data, time lapse indicated that $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{high}/CXCR4^{high}$ populations showed visible capacity to spread and move through the Matrigel (**Figure 4E** and **4F**, **Supplementary Figure 5E**). Moreover, the $L1CAM^{high}/CXCR4^{high}$ population started to spread earlier (i.e., 2 hours after plating) than the $L1CAM^{high}/CXCR4^{low}$ (i.e., 3 hours after plating). We never observed spreading of the $L1CAM^{low}/CXCR4^{low}$ and $L1CAM^{low}/CXCR4^{high}$ population even after 24 hours after plating (**Figure 4F**). The higher aggressiveness of $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{high}/CXCR4^{high}$ was also confirmed by Boyden chamber invasion assay (**Figure 4G** and **4H**). Then, we evaluated the metastatic potential of isolated colorectal $L1CAM^{low}/CXCR4^{low}$, $L1CAM^{low}/CXCR4^{high}$, $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{high}/CXCR4^{high}$ populations. More specifically, we intrasplenically injected the four populations and we did not observe any metastasis in the mice injected with the $L1CAM^{low}/CXCR4^{low}$ population. On the contrary, we observed a sequential reduction in the disease free survival (DFS) and increase number of metastasis in the mice intrasplenically injected with: $L1CAM^{low}/CXCR4^{high}$, $L1CAM^{high}/CXCR4^{low}$ and $L1CAM^{high}/CXCR4^{high}$ populations (**Figure 4I**).

Nodal inhibition chemosensitizes $L1CAM^{high}/CXCR4^{high}$ to chemotherapy

Colorectal cancers are fundamentally resilient to treatment because the malignant cells survive to the chemotherapy. Because CSC may play a crucial role in treatment resistance, we performed vitality analyses (i.e., CCK8 assay) of both L1CAM^{low}/CXCR4^{low} and L1CAM^{high}/CXCR4^{high} cells after exposure to the standard chemotherapeutic agent 5-fluorouracile (5-FU). We found that L1CAM^{high}/CXCR4^{high} cells showed dramatic drug resistance to 5-FU compared to autologous L1CAM^{low}/CXCR4^{low} cells (**Figures 5A**). To demonstrate the *in vivo* relevance of these findings, we analyzed tumor samples from mice bearing colorectal cancer after injection of 10⁶ PDO#5 cells and receiving either vehicle or 5-FU treatment (biweekly with 25 mg/kg by i.p. administration for 21 days) when tumors reached 20mm³. Based on our idea that Nodal signaling regulates the aggressiveness of the tumor cells through L1CAM and CXCR4, we investigated whether inhibition of Nodal signaling by SB431542 translates into increased progression-free survival in pre-established colorectal cancers. Over time, both control and SB431542 treated animals bore large, life-limiting tumors and succumbed within 87 days after tumor implantation. 5-FU alone significantly prolonged survival due to inhibition of tumor growth, but all animals showed progressive disease. Interestingly, the combination of SB431542 and 5-FU significantly increased the long-term survival compared to 5-FU alone, with 100% survival at day 87 (**Figure 5B**).

Harvesting the tumors after the last round of SB431542 treatment administration revealed efficient *in vivo* targeting of the Nodal pathway (**Figure 5C**) with subsequent downregulation of L1CAM and CXCR4 (**Figure 5D**). Although the tumors in the 5-FU treated mice were significantly smaller compared to vehicle-treated mice (**Figure 5B**), a significant enrichment of L1CAM^{high} and CXCR4^{high} had occurred (**Figure 5D**). Excitingly, the double combination (i.e., SB431542 + 5-FU) almost completely eliminated the cells L1CAM^{high} and CXCR4^{high} cells (**Figure 5D**).

Taken together, these data demonstrate that double therapy is capable of eliminating tumor-promoting colorectal CSCs *in vivo*, leading to long-term progression-free survival.

Discussion

L1CAM^{high} population has been recently defined as the regenerative origin of metastasis-initiating cells in colorectal cancer¹⁹. For further clinical insights, we reported that high expression of L1CAM, together with CXCR4, significantly correlated with poor overall survival in CRC patients. In addition, we also observed a higher expression of NODAL in tumor tissue compared to normal colonic epithelium. Remarkably, we found that Nodal receptor ALK4, L1CAM and CXCR4 were co-expressed in the same cells in patients biopsies. Surprisingly, we found that those with the highest levels of NODAL and active NODAL signaling (pSMAD2 positive) had also very high L1CAM expression levels. Furthermore, tumor derived from PDOx_SC exhibited co-expression of L1CAM and NODAL or pSMAD2, suggesting again that L1CAM expression may be functionally linked to NODAL signaling. It is still unclear on how intracellular or secreted L1CAM plays a critical role in stemness maintenance and tumor progression/metastasis of CSCs. One possible mechanism involves direct regulation of CSC stemness-related genes via activation of L1CAM by external NODAL signals. pSMAD2/3-activated-L1CAM may trigger stemness-related genes

expression in downstream signaling pathways¹¹. Alternatively, the metastatic properties of L1CAM secreted may be responsible for its effects on cancer progression. Ganesh K. *et al.* recently reported that L1CAM interacts with metastatic genes involving the EMT process¹⁰. It has been extensively reported that EMT is involved in the maintenance of colorectal CSC-like cells^{20,21}, suggesting that NODAL can similarly regulate L1CAM through disruption of E-CADHERIN and withdrawal of REST from L1CAM promoter. This mechanism might mediate stemness maintenance function of L1CAM. However, there is no evidence that NODAL signals activate the REST axis to regulate L1CAM/CSC function. Another mechanism, by which L1CAM may affect CSCs, involves its secretion into the extracellular matrix. It may also be possible that secreted L1CAM triggers non-canonical TGF- β signaling via upregulation of ERK, and eventually promotes migration and invasion of CRC cells in monolayer culture. In our study, we first demonstrated that the canonical NODAL signaling is directly linked to L1CAM expression and that L1CAM enhances the cell proliferation and differentiation in spheroidal culture, as well as regulates the stemness of colon CSCs. L1CAM may be a potent colon CSC as well as a cellular contextual oncogene.

Here we report, for the first time to our knowledge, a correlation between L1CAM^{high}/CXCR4^{high} and the NODAL signaling pathway in colon CSCs and CRC patients was reported for the first time. Indeed, we found that under low oxygen condition NODAL is up-regulated and positively regulates the L1CAM and CXCR4 expression, thereby maintaining stemness. In CRC patients, hypoxic condition and elevated mRNA levels of NODAL are associated with poor outcome²² and stemness²³, respectively. In our study, we focused on high NODAL-expressing cells, L1CAM^{high}/CXCR4^{high} population in colon CSCs, and the role of L1CAM^{high}/CXCR4^{high} in the regulation of stemness in CRC cells.

Low oxygen levels induce the expression of the embryonic morphogen Nodal²⁴ and L1CAM has been showed to be regulated by SMAD2/SMAD4 complex in different systems²⁵. Low levels of oxygen induced secretion of NODAL in PDOs and regulate the expression of L1CAM and CXCR4 in a time dependent manner, suggesting a *consecutio temporum* of NODAL expression followed by L1CAM and ending with CXCR4. This temporal expression indicates that the appearance of the metastatic population implies as a first step the NODAL secretion. Notably, NODAL KD under hypoxic condition prevents the increase of L1CAM levels, clearly showing that NODAL is required for L1CAM activation. On the contrary, CXCR4 levels are not affected in the absence of NODAL and still increase in low oxygen condition, indicating the absence of a linear correlation between NODAL and CXCR4 expression. To deep inside the mechanism, we observed that the exogenous treatment with recombinant NODAL increased L1CAM and CXCR4 level in CRC organoids in a time dependent manner, with different readout. The acute/short treatment (7 days) induces a proliferate phenotype, increases the number and the size of organoids and boosts the cell vitality. Moreover, spheroidal culture cells overexpressing NODAL exhibits enhanced L1CAM (but not CXCR4) expression and its secretion, suggesting that L1CAM expression may be functionally linked to NODAL signaling. On the other hand, the long treatment with rNODAL leads to the appearance of L1CAM^{high}/CXCR4^{high} population with a less proliferative phenotype but significantly more invasive capacity.

Finally, the *in vivo* co-treatment of implanted tumors with 5-FU and the SB431542 (ALK4 inhibitor) significantly reduces the tumor formation and stabilizes the tumor size over time. This findings are also observed in pancreatic cancer ²⁶ indicating that inhibiting NODAL signaling is a powerful strategy to eradicate or at least stabilize the disease to a chronic level. We speculate that a triple combination of 5-FU+SB43152 and a CXCR4 inhibitor (e.g., AMD070) or L1CAM inhibitor would eradicate totally the tumor. In fact, the intrasplenic injection of the 4 sorted populations (i.e, L1^{low}/CX^{low}, L1^{low}/CX^{high}, L1^{high}/CX^{high}) clearly indicate that the double negative population was unable to migrate and give rise metastasis.

Conclusion

In conclusion, a novel role of L1CAM in the regulation of colon CSCs, and subsequent regulation of the stemness and aggressive metastatic properties of colon CSCs was identified for the first time. Our study identified an important therapeutic target and raised the possibility that L1CAM^{high}/CXCR4^{high} population-targeting drugs could be used to suppress CSC-related metastasis following conventional therapy.

Abbreviations

CRC: Colorectal cancer

CSC: Cancer Stem Cells

PDO: Patients-Derived-Organoids

ALK4: Activin receptor type-1B

CXCR4: Chemokine (C-X-C motif) receptor 4

OS: Overall Survival

IC: intra-caecal

SC: subcutaneous

LiMtes: Liver Metastasis

5-FU: 5-Fluorouracile

Declarations

Availability of data and materials

The data supporting the conclusions of this article are presented within the article and its additional files.

Ethics approval and consent to participate

The research was approved by the Tumor Bank Committees according to Spanish ethical regulations.

Consent for publication

All subject have written informed consent.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' Contributions

D.D.C. designed, planned and performed experiments, and analyzed the results. X.H. helped design and performed in vivo experiments, and helped analyze the results. M.S. performed the immunohistochemistry experiments. G.M. revised the manuscript. E.L. conceptualized and supervised the project, performed in vivo experiments, analyzed results and wrote the manuscript.

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Figures

Figure 1

Figure 1

Figure 1

Figure 2

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 4

Figure 5

Figure 5

Figure 5

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