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Laminin 521 enhances self-renewal via Stat3 activation and promotes tumor progression in Colorectal Cancer

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Abstract

Remodeling of basement membrane proteins contributes to tumor progression towards the metastatic stage. One of these proteins, laminin 521 (LN521), sustains embryonic and induced pluripotent stem cell self-renewal, but its putative role in cancer is poorly described. In the present study we found that LN521 promotes colorectal cancer (CRC) cell self-renewal and invasion. siRNA-mediated knockdown of endogenously-produced laminin alpha 5, as well as treatment with neutralizing antibodies against integrin α3β1 and α6β1, were able to reverse the effect of LN521 on self-renewal. Exposure of CRC cells to LN521 enhanced STAT3 phosphorylation, and incubation with STAT3 inhibitors Napabucasin and Stattic was sufficient to block the LN521-driven self-renewal increase. Robust expression of laminin alpha 5 was detected in 7/10 liver metastases tissue sections collected from CRC patients as well as in mouse liver metastasis xenografts, in most cases within areas expressing metastasis cancer stem cell markers such as c-KIT and CD44v6. Finally, retrospective analysis of multiple CRC datasets highlighted the significant association between high LN521 mRNA expression and poor clinical outcome in colorectal cancer patients. Collectively our results indicate that high Laminin 521 expression is a frequent feature of metastatic dissemination in CRC and that it promotes cell invasion and self-renewal, the latter through engagement of integrin isoforms and activation of STAT3 signaling.

Keywords: colon and rectal carcinoma, metastasis, extracellular matrix, cancer-initiating cells.

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1. Introduction

Although clinical outcomes have steadily improved over the last few decades for patients with colorectal cancer, survival remains poor for those who have developed metastatic disease to distant sites such as the liver, lung or peritoneum [1]. Characterizing cellular and molecular processes that drive metastasis progression therefore represents an essential step to better prevent or target metastatic disease. Previous studies have demonstrated that the development of metastases requires tumor cells to display invasive properties, enabling their dissemination from primary sites and transit through the lymphatic and/or blood stream, as well as survival and self-renewing properties that will drive their ability to initiate metastasis growth and colonize the distant site [2, 3]. Invasion and self-renewal are both sensitive to microenvironmental cues provided by cellular and non-cellular actors of the microenvironment. Among the latter, extracellular matrix molecules have been described as instrumental modulators of cell motility, invasion, and stemness [4]. In particular, remodeling of basement membrane proteins such as laminins during cancer development is thought to alter interaction of these proteins with receptors located at the surface of cancer cells, thereby leading to phenotypic modifications [5].

Laminins are large multimeric basement membrane proteins, with functional units constituted through the assembly of one alpha, one beta and one gamma isoform [6]. In the present work we analyzed the contribution of laminin alpha 5 (LAMA5), and more specifically of the laminin-521 isoform (LN521, constituted by one alpha 5, one beta-2 and one gamma-1 chain), in the regulation of self-renewal and invasion of colorectal cancer cells. Laminin-521 (LN521) was previously shown to be important in the maintenance of embryonic and induced-pluripotent stem cells [7, 8], and we hypothesized that exposure to LN521 could also promote self-renewal in colorectal cancer cells. Since long-term self-renewal is a hallmark of cancer stem cells (CSCs) and contributes to their driving role in cancer progression and poor clinical outcomes [9, 10], we also investigated whether LN521 may play a role in regulating phenotypic and clinical features that are classically associated with metastatic development, such as cell migration, invasion and patient survival.

2- Materials and Methods

Immunohistochemical staining, Extreme Limiting Dilution Analysis (ELDA), Protein and RNA quantitation, in vivo liver metastasis induction in immunocompromised mice, as well as analysis of associations between laminin expression and CRC patient survival, were performed as previously described [11, 12]. Details for these procedures are provided under Supplemental Materials and Methods.

2.1- Laminin coating

Tissue culture plates (Corning) were coated with either LN521 (BioLamina, LN521-01), LN-332 (BioLamina, LN-332-0202) or LN-111 (Cultrex, 3400-010-02) as described [8]. Briefly, laminins were diluted to 10ug/ml in DPBS containing Ca2+ and Mg2+ (Gibco), added to tissue culture plates, and incubated overnight at 4 °C. Coating volume was adjusted to the plate/well size and final laminin density was 1.7µg/cm^2, lower than previously reported for the maintenance of embryonic stem cells (5µg/cm^2)[8]. Cells were seeded at approximately 20% confluency on laminin-coated wells for 48hrs to reach 70%-80% confluency at the time of harvest.

2.2- Inhibition of Integrin / Laminin binding.

Integrin neutralizing antibodies were included in cell suspension at the time seeding onto tissue culture plates coated or not with LN521 to inhibit the binding between laminin and integrin. A complete list of integrin neutralizing antibodies and their respective isotype controls is provided in Supplemental Table SI.

2.3- Invasion assays

For invasion assays, cells were preincubated or not with laminin as described above for 24h, then seeded onto 24-well culture plate inserts (Millipore, 8µm pore size) that had been coated for 2hrs at 37°C with growth factor-reduced Matrigel (Becton Dickinson) diluted to 200ug/ml with DMEM/HAM F-12 media and mixed or not with 10ug/ml LN521. Cells were seeded within the top compartment in DMEM/HAM F-12 medium and DMEM/HAM F-12 + 10% FBS was used as chemoattractant in the bottom compartment. Cells were allowed to travel through the insert for 6 hrs. Thereafter, cells that failed to invade were removed using sterile cotton buds. After fixing with 4% paraformaldehyde, the insert membrane was stained with 1ug/ml DAPI, washed three times with 1X DPBS and mounted under a coverslip using a slide

using mounting solution. Pictures of the bottom side of the insert were taken from 5 randomly selected fields for each condition. The average number of cells was quantified using Image J. For invasion experiments followed by self-renewal analysis, cells were left to invade as above. After removal of cells that failed to invade using a cotton bud, invading cells were resuspended for 3min using Accumax (Sigma-Aldrich), washed pelleted and seeded in Extreme Limiting Dilution Analyses. In alternate well, invading cells were removed using a cotton but and Accumax was applied to the upper Transwell chamber to resuspend the non-invading cells and seed them in suspension to perform an ELDA.

3- Results

3.1- Laminin-521 promotes self-renewal and invasion of CRC cells

To determine whether exposure to LN521 impacted on the self-renewal of colorectal cancer cells, we first exposed human CRC cells to various laminin isoforms and subsequently resuspended and seeded them into an extreme limiting dilution analysis (ELDA) in suspension. The ability of these cells to generate at least one colonosphere per well under all limiting dilution conditions was quantified and the frequency of self-renewing cells was calculated as described previously [11, 13] using the http://bioinf.wehi.edu.au/software/elda/ webtool [14]. We compared the ability of LN521 to regulate self-renewal with that of LN332, which was previously shown to promote cancer stem cell survival in primary hepatocellular carcinoma (HCC) samples [15] and to collaborate with collagen XVII in enabling survival of tumor-initiating cells [16], as well as with that of LN111, which is often used to support ES/iPS cell cultures [17] and constitutes the main component of Matrigel.

The DLD-1 colorectal cancer cell line and CPP19 patient-derived colorectal cancer liver metastasis cells [18] were grown on tissue culture plates pre-coated or not with LN111, LN332 and LN521 (Supplemental Figure S1) before seeding in a limiting dilution assay in ultra-low adherence plates. The frequency of cells displaying self-renewing activity was significantly enhanced after exposure to laminin 521 compared to cells preincubated on non-coated wells (20.22% +/- 4.97 vs 9.69% +/- 3.01, respectively, for DLD-1 cells; 11.93% +/- 6.12 vs 3.71% +/- 0.27 for CPP19 cells) (Figure 1A-B and Supplemental Figure S1). In contrast, self-renewal was not significantly enhanced by preincubation on LN111-coated plates, while LN-332 preincubation only increased self-renewal of CPP19 cells but not of DLD-1 (Figure 1A-B).

This suggests that LN521-induced signaling may promote self-renewal in colorectal cancer cells.

To further investigate the potential of LN521 to further promote tumor progression, we quantified the invasive capacity of DLD-1 and CPP19 cells through a Matrigel layer after and/or during exposure to LN521 (Figure 1C) and assessed the self-renewal ability of invading cells (Figure 1D). Invasion of CRC cells through Matrigel was significantly promoted by LN521 preincubation and/or by addition of LN521 to the Matrigel (Figure 1C). To ascertain whether LN521 can jointly promote invasion and self-renewal, two essential characteristics for metastasis formation, we collected cells having invaded or not through a Matrigel layer and quantified their self-renewal capacity. Cells preincubated on LN521 displayed significantly enhanced self-renewal compared to those preincubated on uncoated wells, with the exception of non-invading CPP19 cells (Figure 1D). Additionally, we found that the promoting impact of LN521 on self-renewal frequency was stronger in invading compared to non-invading cells (Figure 1D). Collectively our results suggest that that LN521 is able to concomitantly promote invasion and self-renewal in colorectal cancer cells.

3.2- Laminin alpha 5 contributes to the LN521-induced promotion of self-renewal.

In view of the essential role played by laminin alpha chains in the recognition of cellular laminin receptors [19, 20] we hypothesized that laminin alpha 5 (LAMA5) might be instrumental for the self-renewal increase induced by LN521 on CRC cells. First, we sought to determine whether CRC cells were able to produce the three laminin chains that make up LN521. Indeed, expression of *LAMA5, LAMB2* and *LAMC1* RNAs was readily detected using RT-qPCR in DLD-1 and CPP19 cells, along with those encoding several other laminin chains such as LAMA3, LAMB1, LAMB3, and LAMC2 (Supplemental Figure S2).

We also quantified LAMA5 and LAMA3 RNA expression and performed ELDA self-renewal assays *in vitro* on 9 additional early-passage primary cell lines (6 from primary tumors, 3 from liver metastases) that we recently generated from CRC patients [11, 13]. Variable expression of both laminin alpha chain RNAs was detected across all CPP lines. Expression of LAMA5 was positively correlated to the self-renewal potential in these cells ($R2 = 0.7548$, $p = 0.0011$) (Figure 2A), whereas no correlation was found between expression of LAMA3 and self-renewal potential ($R2 = 0.0207$, $p = 0.6914$) (Figure 2B).

To establish whether endogenous LAMA5 is indeed able to contribute to the promotion of self-renewal, selective siRNAs were then used to down-regulate *LAMA5* RNA expression (Supplemental Figure S2), and siRNA-transfected cells were seeded in an ELDA assay to quantify their sphere-forming efficiency, in comparison with cells transfected with a control siRNA targeting β-galactosidase. While transfection with β-Gal siRNA had no detectable effect on self-renewal, down-regulation of LAMA5 RNA expression resulted in a two to three-fold decrease in the sphere-forming ability of CPP19 and DLD-1 cells, (47.29 +/- 8.9% and 29.56 +/- 3.87 %, respectively, compared to untransfected cells) (Figure 2C).

Collectively these results suggest that endogenous production of laminin alpha 5 contributes to the maintenance of self-renewal in human colorectal cancer cells.

3.3- Role of integrins in the promotion of self-renewal by LN521.

Laminins are able to regulate cell signaling and alter cell phenotypes by interacting with specific receptors located at the cell surface, such as integrins. LN521 has been shown to primarily interact with two integrin isoforms, α6β1 and α3β1 [6]. mRNAs encoding integrin α3, α6 and β1 chains were readily detected in CRC cells, along with α7 and β4 chains (Figure 3A). To investigate if interaction with either or both of α6β1 and α3β1 integrins contributed to the identified role of LN521 in enhancing self-renewal capacity, we made use of neutralizing antibodies targeting the α6, α3, and β1 chain of integrins and thereby blocking their binding to laminins. DLD-1 cells were grown on LN521-coated or control plates for 24hrs, then treated with α6, α3, or β1 integrin neutralizing antibodies, either individually or in combination (α6β1 or α3β1), and grown under these conditions for another 24hrs before being resuspended and analyzed for self-renewal potential using ELDA.

Apoptotic cell death in DLD1 and CPP19 cells was monitored during these experiments via detection of cleaved caspase 3 expression (Supplemental Figure S3A). Apoptosis levels were low in all samples (1.92 to 5.56% in CPP19, 0.54 to 2.05% in DLD1). For both cell lines, we detected a non-significant trend towards lower apoptosis in cells pre-coated with LN521 and a slight but significant increase in apoptosis for cells treated with anti-α6β1 antibodies (Supplemental Figure S3A). The ability of these antibodies to inhibit the LN521-induced internalization of integrin isoforms was confirmed using immunofluorescent staining in DLD-1 and CPP19 cells, with strongest effects measured for α3 and β1 integrins (Figure 3B). In both cell lines, the robust increase in self-renewal frequency induced by exposure to LN521 was strongly and significantly inhibited by all three integrin neutralizing antibodies in comparison

with cells treated with isotype control IgG (Figure 3C). Control DLD-1 cells treated with all three neutralizing antibodies also exhibited a small reduction of sphere-forming frequency in comparison with their matching isotype antibody-treated controls, while a similar effect was only detected after α3 integrin antibody treatment in CPP19 cells. Altogether these results demonstrate that integrins α3, α6 and β1 all contribute to the promoting effect of LN521 on self-renewal in DLD-1 and CPP19 colorectal cancer cells. Overall, the similar level of neutralizing efficiency shown by α3, α6 and β1 antibodies in these experiments suggests that both α and β integrin chains are required for this process, consistent with previous findings that both integrin chains contribute to interaction with extracellular ligands such as laminins and for initiation of signaling [21, 22].

3.4- Activation of Stat3 signaling during the promotion of self-renewal by LN521

We then attempted to identify tumor cell signaling pathways involved in the regulation of self-renewal by LN521, acting via its interaction with α3β1 and α6β1. We elected to prioritize the analysis of AKT and STAT3 pathways based on their previous identification as LN521 and integrin targets and on their known role in cancer progression and/or stemness [8, 23]. Effector proteins of these pathways were quantified using Western blotting after exposing colorectal cancer cells to laminins for 48h.

Preincubation with LN521 was found to significantly enhance the expression and phosphorylation of STAT3 in both DLD-1 and CPP19 cells, while LN111 had a similar effect on DLD1 cells only and LN332 did not significantly alter STAT3 expression or phosphorylation (Figure 4A). In contrast, preincubation with LN332 and LN521 had similar effects on AKT levels in both cell lines (Supplemental Figure S3B), suggesting that the LN521-specific self-renewal activation in these cells was unlikely to be mediated by this pathway.

To further determine if the detected increase of STAT3 expression and activity contributed to the enhancement of self-renewal induced by LN521, we first quantified the colonosphere-forming frequency of DLD-1 cells pre-incubated or not with LN521 and treated respectively with DMSO or with the STAT3 inhibitors Napabucasin and Stattic (Figure 4B-D). Both inhibitors were able to reduce not only the baseline but also the LN521-enhanced self-renewal down to similar levels, suggesting that STAT3 activation may be required for the stimulatory effect of LN521 on self-renewal. To verify this finding, we used RNA silencing to determine the impact of STAT3 expression knockdowns on the ability of endogenous LAMA5 to

stimulate self-renewal in CRC cells. Both pharmacological STAT3 inhibitors as well as siRNA-mediated LAMA5 down-regulation inhibited self-renewal to similar extent, and no significant additive effect was detected when both types of treatment were performed simultaneously (Figure 4C).

These results strongly suggest that STAT3 signaling is an important contributor to the effect of LN521 on colorectal cancer cell self-renewal.

Finally, because of their well-characterized role as activators of STAT3 signaling and their suspected contribution to cancer stemness [24, 25], we then sought to establish whether interleukins such as IL-6 and IL-11 could act as mediators of the LN521-driven self-renewing frequency increase. Our results indicated that preincubation of colorectal cancer cells on LN521-coated plates did not modify the expression of IL-6, IL-11 and their respective receptors, suggesting that it is unlikely to be the case (Supplemental Figure S3C).

3.5- LAMA5 expression in human and mouse colorectal cancer liver metastases.

The promoting effect of LN521 and LAMA5 on self-renewal and/or invasion suggests that it may contribute to the metastatic process in colorectal cancer *in vivo*. This hypothesis implies that LN521, or at least LAMA5, may be expressed within colorectal metastases to support their initiation and long-term maintenance. To verify this assertion, we used immunohistochemical staining to establish whether LAMA5 is expressed in liver metastasis tissue sections from CRC patients, as well as in an experimental mouse model of liver metastasis.

Immunohistochemical staining for LAMA5 was performed on chemo-naïve liver metastasis samples collected during surgical resection from patients with stage IV disease (n = 10). Metastatic cells were sensitively detected via staining for cytokeratin 19 (CK19), a marker for the detection of carcinoma metastasis to the liver in several cancers including colorectal [26]. LAMA5 expression was detected in 7/10 samples, and its expression was prominent in areas that expressed the c-Kit receptor, recently identified as an essential contributor to the undifferentiated stem-like tumor cell phenotype in colorectal cancer [27], as well as CD44v6, a marker for metastatic CRC stem cells [28] (Figure 5A and Supplemental Figure S4A). One of 10 samples analyzed exhibited a distinct pattern of expression, with detectable c-Kit and CD44v6 staining despite little to no detection of LAMA5 (Supplemental Figure S4B). In

comparison, specific LAMA3 staining was not detected in our liver metastasis samples, while it was readily detectable in primary colorectal tumor sections (Supplemental Figure S4C).

To generate liver metastases in mice, we xenografted CPP1 patient-derived CRC cells in the caecum of BALBc/nude immunocompromised mice and liver metastases were collected 6-9 weeks after tumor cell injection and paraffin-embedded. Corroborating our results in patient samples, tissue sections from these liver metastases were also found to express LAMA5 in similar areas as cKit (Figure 5B). The latter was also found to be expressed by neighboring mouse hepatocytes, in contrast to our finding in human liver. Most cells in CPP1-derived metastases were found to express CD44v6 (Figure 5B), suggesting that CPP1-derived tumors may be enriched for cancer stem cells or that expression of this marker may not be restricted to cancer stem cells in this model.

Our results suggest that LAMA5 expression is frequent in liver metastases from colorectal cancer and that tumor cells expressing metastatic cancer stem cell markers are often detected in LAMA5-rich areas within these samples.

3.6- Laminin-521 expression is associated with poor disease-free survival in patients with colorectal cancer

Collectively, results obtained above suggest that expression of LAMA5, combined or not with expression of the LAMB2 and LAMC1 chains, may contribute to tumor progression and to the development of metastases in colorectal cancer. Because self-renewal and metastatic progression are linked to poor clinical outcome in colorectal and other solid cancers, we retrospectively analyzed previously published CRC datasets to test whether the expression of mRNA that encode LN521 chains was associated with disease outcome.

To do so, we first used the cBioPortal webtool (http://www.cbioportal.org) to extract and analyze the colorectal adenocarcinoma dataset (n = 592 samples) from the TCGA PanCancer Atlas [29]. Despite the highly heterogeneous nature of this patient cohort (multiple tumor stages, various tumor subtype and treatment regimen…), we found that High LN521 RNA expression was significantly associated with both disease-free survival (Logrank test, $p = 0.0353$) and overall survival ($p = 0.0127$) (Supplemental Figure S5). In contrast, no association was found in the case of LN332 (Logrank test, $p = 0.115$ for disease-free survival, $p = 0.215$ for overall survival) or LN111 (Logrank test, $p = 0.908$ for disease-free survival, $p = 0.447$ for

overall survival). We also used several previously published GSE14333 [30], GSE24551−GPL11028 [31] and GSE41258 [32] datasets, as they contained large cohorts (>150) of colon cancer patients. We analyzed these datasets using the SurvExpress bioresource [33], allocating patients to either high or low laminin chain expressing subgroups using median expression a cutoff for subgroup allocation. High expression levels of LN521-encoding mRNAs were significantly associated with poorer survival in all three datasets, with respective Hazard Ratios of 2.58 (n = 226 patients, p = 0.002134), 1.70 (n = 160 patients, p =0.04118) and 2.01 (n = 243 patients, p = 0.00072) (Figure 6 and Supplemental Figure S6). The association of LN521 mRNA expression with survival was consistently stronger (higher Hazard Ratio) and more statistically significant than for LN332 or LN111-encoding mRNAs (Figure 6 and Supplemental Figure S6), as well as for mRNAs encoding LN511 (Supplemental Figure S7), another LAMA5-containing laminin isoform shown to regulate metastasis development in breast and other cancer [34].

Collectively these results suggest that LN521 expression is strongly associated with disease-free and overall survival in multiple colorectal cancer patient cohorts.

4- Discussion

In the present study we characterized the promoting role of laminin-521 on colorectal cancer cell self-renewal and invasion and found that endogenous production of the laminin alpha 5 chain contributed to this effect. We demonstrated that integrins α3β1 and α6β1 are likely mediators of the self-renewal promotion induced by laminin alpha 5 and that downstream activation of STAT3 signaling is required for the promotion of self-renewal by LN521. Finally, we also found that colorectal cancer liver metastases frequently contain laminin alpha 5-rich areas and that high laminin-521 expression correlates with poorer disease-free and overall survival in several colorectal cancer patient datasets.

Laminin alpha 5 was recently shown to promote growth and angiogenesis of liver metastases from colorectal cancer, and to inhibit the Notch signaling pathway in metastasis endothelial cells [35]. It may do so by interacting with the Basal Cell Adhesion Molecule (BCAM) to mediate interactions between endothelial and metastatic tumor cells, a mechanism possibly restricted to K-RAS mutated colorectal tumors [36]. Here, our main findings were that laminin alpha 5, either endogenously secreted by tumor cells or exogenously provided to colorectal cancer cells, significantly enhanced the invasive ability as well as promoting self-renewal of

colorectal tumor cells. We also detected frequent expression of laminin alpha-5 within human colorectal cancer liver metastases, often in regions that also express metastatic cancer stem cell markers such as cKIT and CD44v6. Together these results suggest that laminin alpha 5 and LN521 may contribute to a local environmental niche that sustains self-renewing cells during progression of colorectal cancer to the metastatic stage and/or upon arrival at the metastatic site.

The potential role of specific laminin chains in the maintenance of cancer stem cells has been reported in prior studies (summarized in [5]). Thus, laminin gamma-2 was shown to contribute to a putative cancer stem cell niche in hepatic cancer [15], while laminin alpha 2 was shown to have similar attributes in glioblastoma multiforme [37]. In addition, exposure to multiple laminin isoforms (411, 421, 511 and 521) was reported to enhance the clonogenicity of glioma stem cells [38]. However, our study is the first to demonstrate that laminin-521 is able to concomitantly enhance the invasive capability and the self-renewal of some colorectal cancer cells, thus providing them with a competitive advantage for metastasis initiation. In addition, the present work is also the first to demonstrate that the effect of LN521 on self-renewal can be detected in patient-derived cancer cells, where endogenous LAMA5 expression also correlates with enhanced self-renewal abilities.

LN521 was also found to significantly promote CRC cell invasion. The invasive ability of CRC cells was highest when they were exposed to LN521 during preincubation as well as during invasion through Matrigel as a Matrigel-LN521 mix. This result, which highlights the role of LN521 in promoting the invasion of colorectal cancer cells, is in accordance with finding in other cancers such as melanoma, where cancer cells produce several laminins including 521 and use them as a substrate for migration [39].

In addition, our findings indicate that LN521 acts to promote self-renewal vie engaging with integrin α3β1 and α6β1 at the surface of tumor cells. This result is not unexpected as these integrin isoforms were reported previously as primary interactors for LN521 in other model systems [6]. While other integrins chains including α5 [40], α2 [41] or β3 [42] contribute to the adhesion of colorectal cancer cells to their microenvironment in colorectal liver metastases, our results using neutralizing antibodies strongly suggest that the α3, α6 and β1 integrin chains are instrumental to mediate the self-renewal promoting effect of LN521.

Exposure to LN521 was shown to increase STAT3 expression and phosphorylation in both DLD-1 and CPP19 CRC cells. In addition, the clinically-relevant pharmacological STAT3

inhibitor Napabucasin, as well as the STAT3 activation and dimerization inhibitor Stattic, were both able to reverse the LN521-driven increase in self-renewal. These results indicate that STAT3 signaling mediates the effect of LN521 on CRC cell self-renewal. The involvement of STAT3 in the promotion of self-renewal has already been reported in several cancer types [43, 44], and its role as a promoter of stem-like cells and as a putative therapeutic target in GI cancers is well-described (reviewed in [45]). Indeed, the STAT3 inhibitor Napabucasin is undergoing clinical trials to target self-renewal and cancer stem cells [46]. Our results therefore suggest that LN521 may be a contributor to the elevated STAT3 signaling during colorectal tumor progression, and that blocking the activity of LN521 may represent an alternative to decrease STAT3 activation.

In human patient samples and in a preclinical mouse model, we found that most CRC liver metastases expressed laminin alpha 5, in areas where CK19-positive metastatic tumor cells also expressed cancer stem cell markers such as c-KIT and CD44v6. The detected laminin alpha 5 may not only be secreted by some tumor cells, as demonstrated in CPP19 metastatic tumor cells in our study, but also by surrounding hepatocytes. Indeed, small quantities of laminin alpha 5 are produced by the adult liver [47], possibly by hepatic progenitor cells [48]. However, since most of the detected laminin alpha 5 staining was detected in areas containing metastatic tumor cells rather than in the surrounding healthy liver, this could also suggest that presence of metastatic cells may promote local production of laminin alpha 5 in the liver. Incidentally enriched laminin alpha 5 deposits have been previously described to promote CK19 expression in hepatocellular carcinoma cells *in vitro* [49], which may contribute to the reported high expression of this cytokeratin isoform in liver metastases [26].

Altogether our results highlight the enriched expression of laminin alpha 5 in metastatic colorectal tumors and identify a significant correlation between high LN521 expression and poor survival in colorectal cancer patients. We also demonstrated the ability of LN521 to promote their self-renewal via the engagement of integrins α3β1 and α6β1 and the activation of STAT3 signaling and showed that LN521 enhance the invasive ability of colorectal tumor cells *in vitro*. These results suggest that targeting of LN521/integrin interactions may reduce the invasive ability of disseminating tumor cells and/or their metastasis-initiation ability in the liver. Thus, future preclinical studies will establish whether antibody or peptide-based targeting of LN521-integrin binding may provide therapeutic benefit in CRC patients, either directly and/or by sensitizing their tumor to other compounds as suggested for other LN-integrin isoform interactions [50, 51].

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Figure Legends

Figure 1: (A-B) Bar graph representing the comparative self-renewing ability of DLD-1 **(A)** and CPP19 **(B)** colorectal cancer cells pre-incubated on laminin 521, 111 or 332 as indicated, compared to cells pre-incubated without coating (CT). Data was obtained using Extreme Limiting Dilution Analysis and results are expressed as percentage of self-renewing cells compared to the total number of seeded cells. (**p<0.02 compared to non-coated cells, one-way ANOVA, n=3). **(C)** Left panel, experimental outline for cell invasion experiments and representative example of DAPI-stained cells having invaded through the Matrigel and Transwell membrane. Middle and Right Panels, bar graphs summarizing the quantification of cells detected via DAPI staining on the underside of the Transwell membrane after 6h invasion of DLD-1 and CPP19 colorectal cancer cells preincubated (grey bars) or not (Uncoated, black bars) on LN521-coated wells and invading through Matrigel mixed or not with LN521 as indicated. Data is expressed as a percentage of the average number of cells per field (+/- SEM) detected in uncoated controls across 3 independent experiments. *p<0.05, **p<0.02, ANOVA with Dunnett post-hoc test against uncoated control **(D)** Top panel: experimental outline for experiments enabling the independent quantification of self-renewing ability by invading and non-invading cells. Bottom panel: Left, graph representing the comparative self-renewing ability DLD-1 (black circles) and CPP19 cells (white squares) that did (Inv.) or did not (Non-Inv.) managed to invade through Matrigel mixed or not with LN521. Results for each cell line are expressed as % of the estimated self-renewing frequency in non-invading cells seeded in Matrigel only across three experiments (Mean + Confidence Interval); Right, table summarising the statistical analysis (Chi2 values and statistical significance for each comparison) of differences in self-renewal between DLD-1 (***a, b***) and CPP19 cells (***c, d***) seeded on Matrigel + LN521 and their matching controls seeded on Matrigel only.

Figure 2

(A-B) Correlation between self-renewal frequency (x-axis, % of total cell number) and laminin alpha 5 **(A)** or laminin alpha 3 **(B)** mRNA expression (y-axis, 1/DCp), quantified using quantitative RT-PCR. Correlation coefficient (R^2) and statistical significance (p) for each correlation analysis are provided. **(C)** Bar graph representing the self-renewal ability of DLD-1 and CPP19 colorectal cancer cells treated with LAMA5- (grey bars) or β-galactosidase-selective (black bars) siRNA. Results are expressed as percentage of self-renewing cells

compared to untransfected controls. (*p<0.05 compared to non-coated cells, unpaired t-test, n=3).

Figure 3

(A) Endogenous expression of integrin isoform mRNA in DLD-1 (left) and CPP19 (right) colorectal cancer cells, quantified using quantitative RT-PCR and expressed as 1/DCt. **(B)** Representative immunofluorescent staining for α3 integrin (Top) and β1 integrin (Bottom), respectively in DLD-1 and CPP19 cells incubated or not on LN521-coated plates with or without treatment with matching neutralizing integrin antibodies as indicated. Nuclei were detected using DAPI. Magnification bars represent 100 μm (left and middle) or 50 μm (right). **(C)** Self-renewing cell frequency (% of control) after treatment of DLD-1 (circles) and CPP19 cells (squares) pre-incubated with (grey) or without (black) LN521 and treated with neutralizing antibodies selective for integrin α3, α6 and/or β1 chains, in comparison with anti-IgG isotype controls. Results are expressed as percentage of the frequency in isotype control treated cells for each cell line + Confidence Interval (* and #, $p < 0.05$ compared to uncoated and LN521-coated isotype control cells, respectively; ##, p<0.02 compared to LN521-coated cells; ANOVA with Dunnett post-hoc test, n=3 experiments).

Figure 4

(A) Left, Representative Western blot picture of STAT3, phospho-STAT3 and β-actin detection in DLD-1 and CPP19 cells grown on LN521, 111 or 332, in comparison with control cells (CT) grown on non-coated wells; Right, Quantification of STAT3 (black bars) and phospho-STAT3 (grey bars) expression in DLD1 and CPP19 cells grown on LN521, 111, or 332, as detected using Western blot. All results are calibrated to account for housekeeping gene variation in each matching sample. Phospho-STAT3 expression is also calibrated to account for variations in total STAT3 expression. Results are expressed as % of expression in cells grown on non-coated wells (*, p<0.05, ANOVA with Dunnett post-hoc test). **(B)** Self-renewing cell frequency in colorectal cancer cells preincubated for 24h on control or LN521-precoated wells in the presence of vehicle or of the STAT3 inhibitors Napabucasin (1 μM) or Stattic (2 μM), as indicated. Results are expressed as % of total cell number (*, p<0.05; **p<0.02 compared to matching untreated controls, ANOVA with Bonferroni post-hoc test, n=3). **(C)** Self-renewing cell frequency in colorectal cancer cells treated with β-galactosidase (siCT) or LAMA5-selective siRNA (siLAMA5) in the presence of vehicle (black bars) or Napabucasin

(grey bars). Results are expressed as % of total cell number (*, $p<0.05$, ANOVA with Bonferroni post-hoc test, n=3). **(D)** Representative Western blot picture representing the expression of b-actin, STAT3 and phospho-STAT3 in CPP19 cells treated or not with 1 μM Napabucasin.

Figure 5

(A) Representative pictures of immunohistochemical (IHC) staining for laminin alpha 5 (LAMA5), cytokeratin 19 (CK19), c-KIT and CD44v6 in liver metastatic samples from 4 patients with stage IV colorectal cancer. Magnification bars = 200 μm. **(B)** Representative IHC staining for LAMA5, CK19, c-KIT and CD44v6 in a liver metastasis section collected following intra-splenic injection of CPP1 human colorectal cancer cells in BALBc/nude immunocompromised mice. Magnification bars = 100 μm.

Figure 6

Kaplan-Meier curves, Hazard ratio and p-values summarising the difference of overall **(A)** and disease-free survival **(B, C)** in colorectal cancer patients in relation with the high (red lines) or low (green lines) expression of laminin 521 (LN521, left panels), 332 (LN332, middle panels) or 111 (LN111, right panels) in samples collected from their primary tumor. (**A)** Overall survival data, GSE41258. **(B)** Disease-free survival (GSE24551). **(C)** Disease-free survival (GSE1433).

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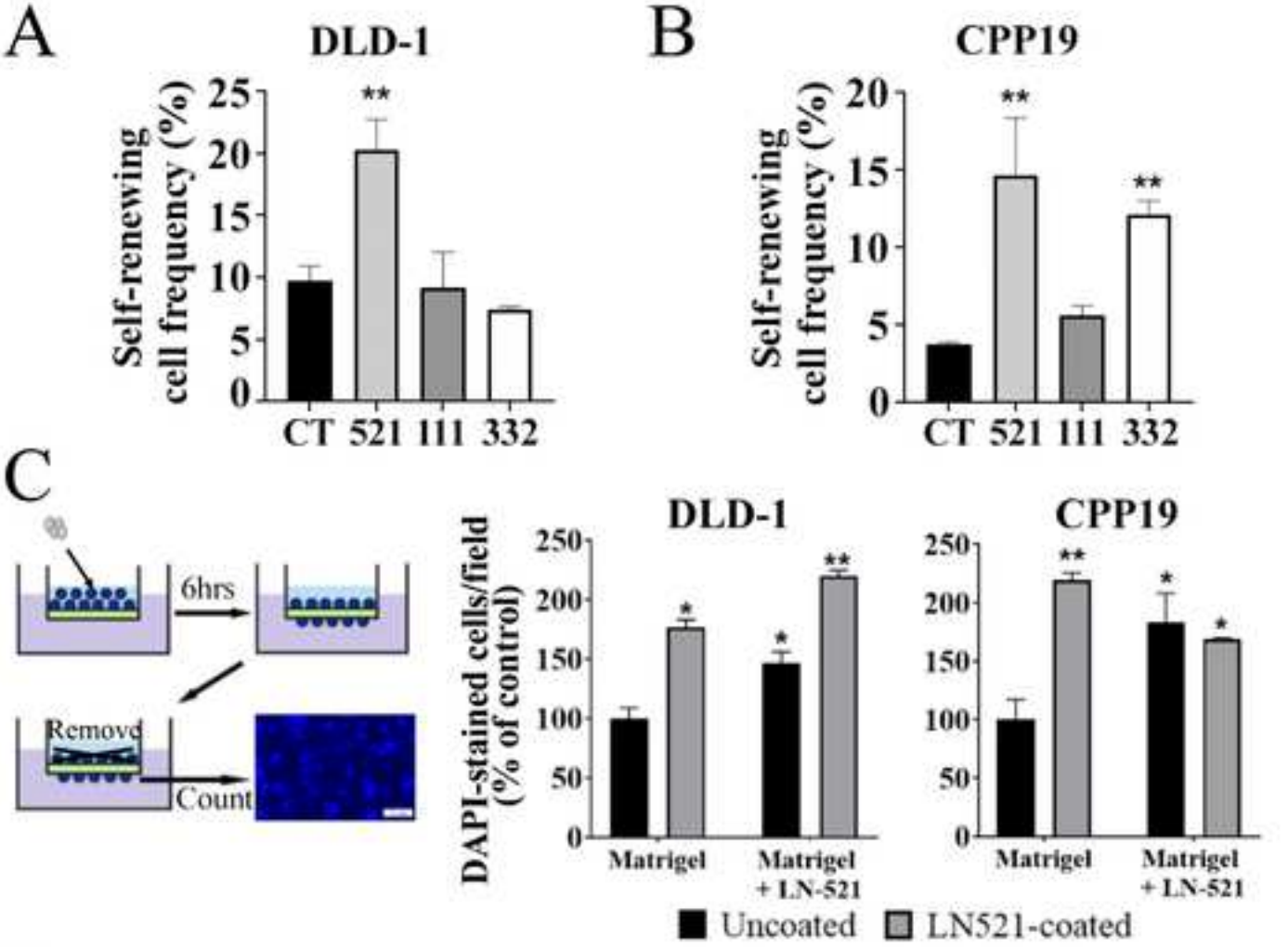
Qin et al., Figure 1



		Chi2 (LN521 vs Control)	Statistical significance
DLD-1	Non-Invading	10.5 *a*	0.0012
	Invading	20.6 *b*	5.71exp-6
CPP19	Non-Invading	0.113 *c*	0.737
	Invading	5.64 *d*	0.0276

Qin et al., Figure 2

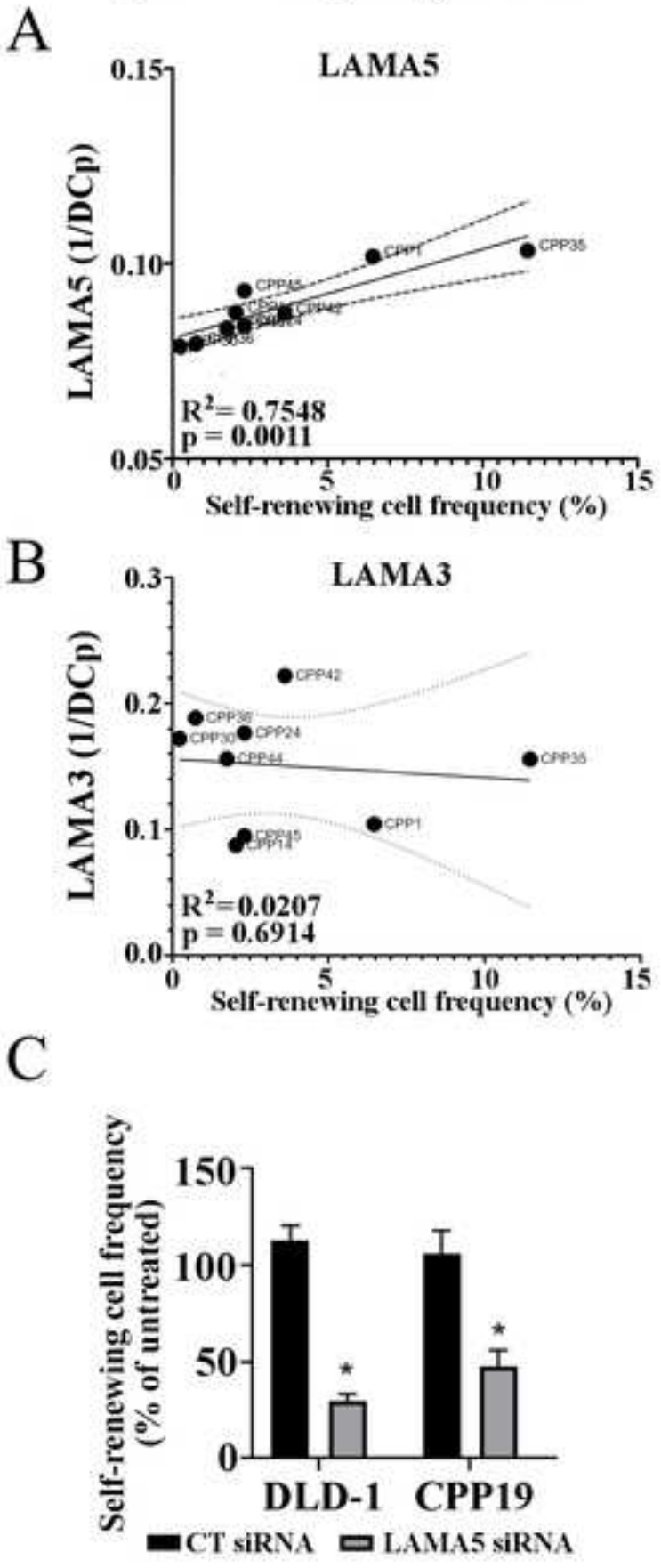


Figure 3

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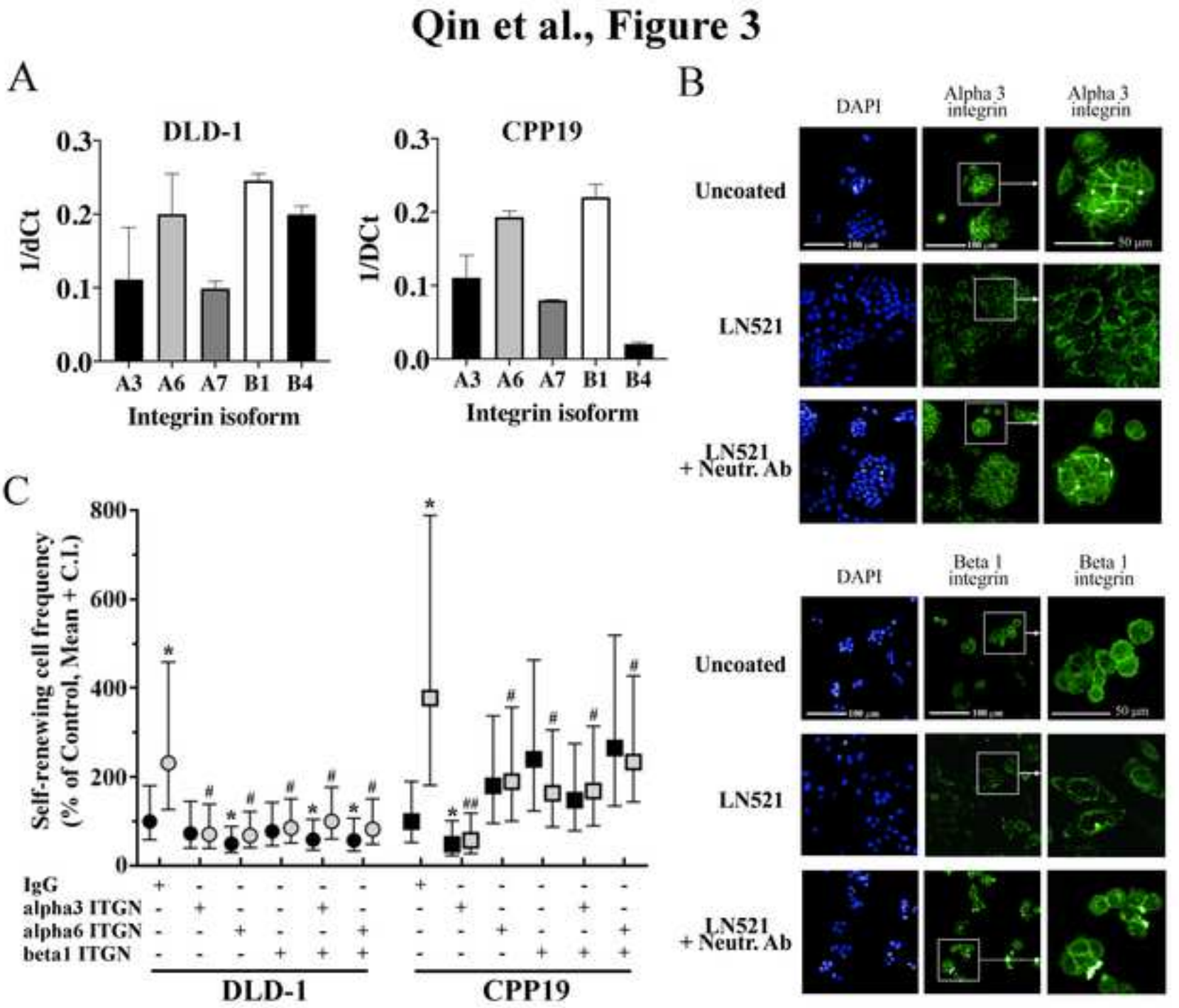

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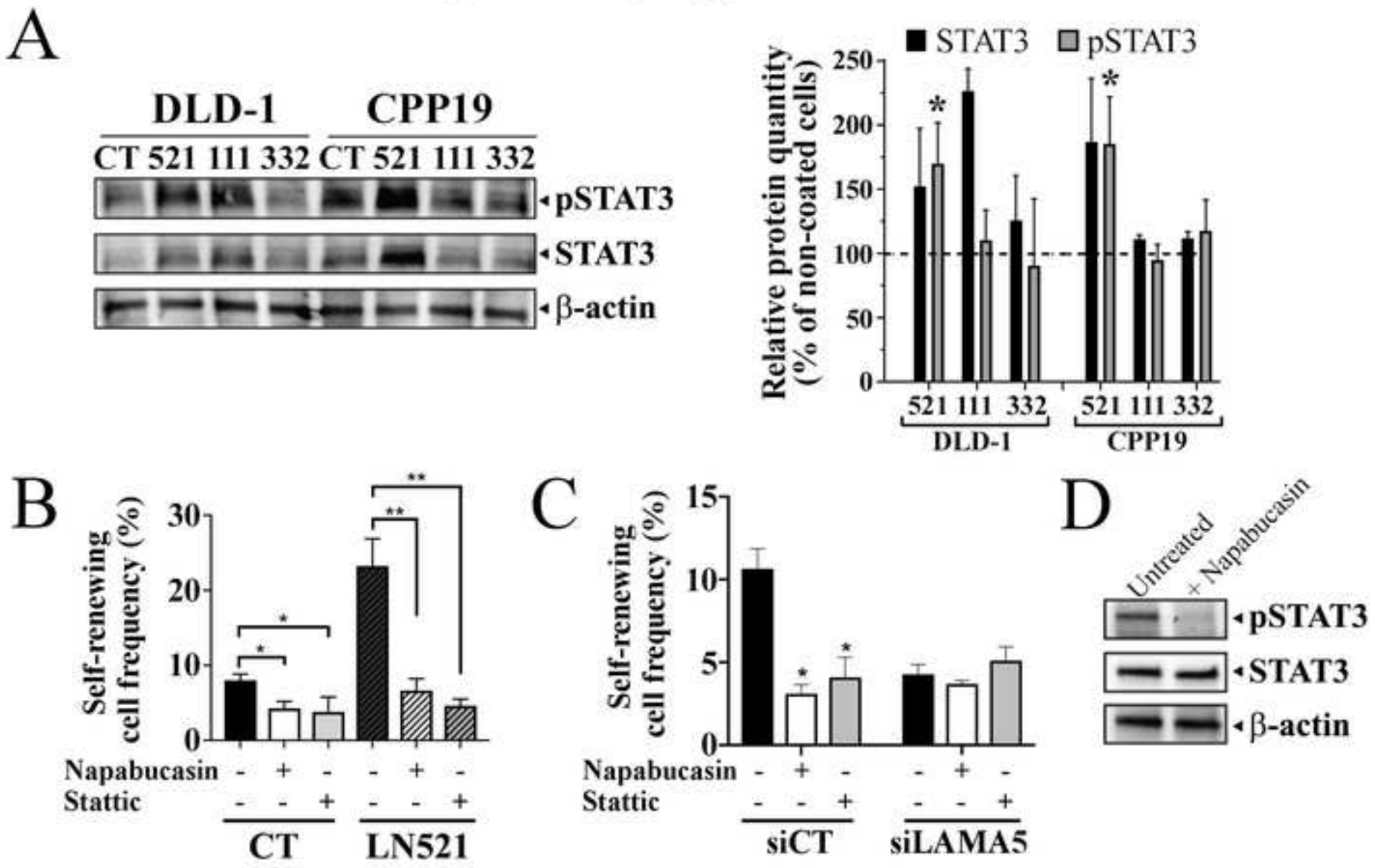

Figure 5

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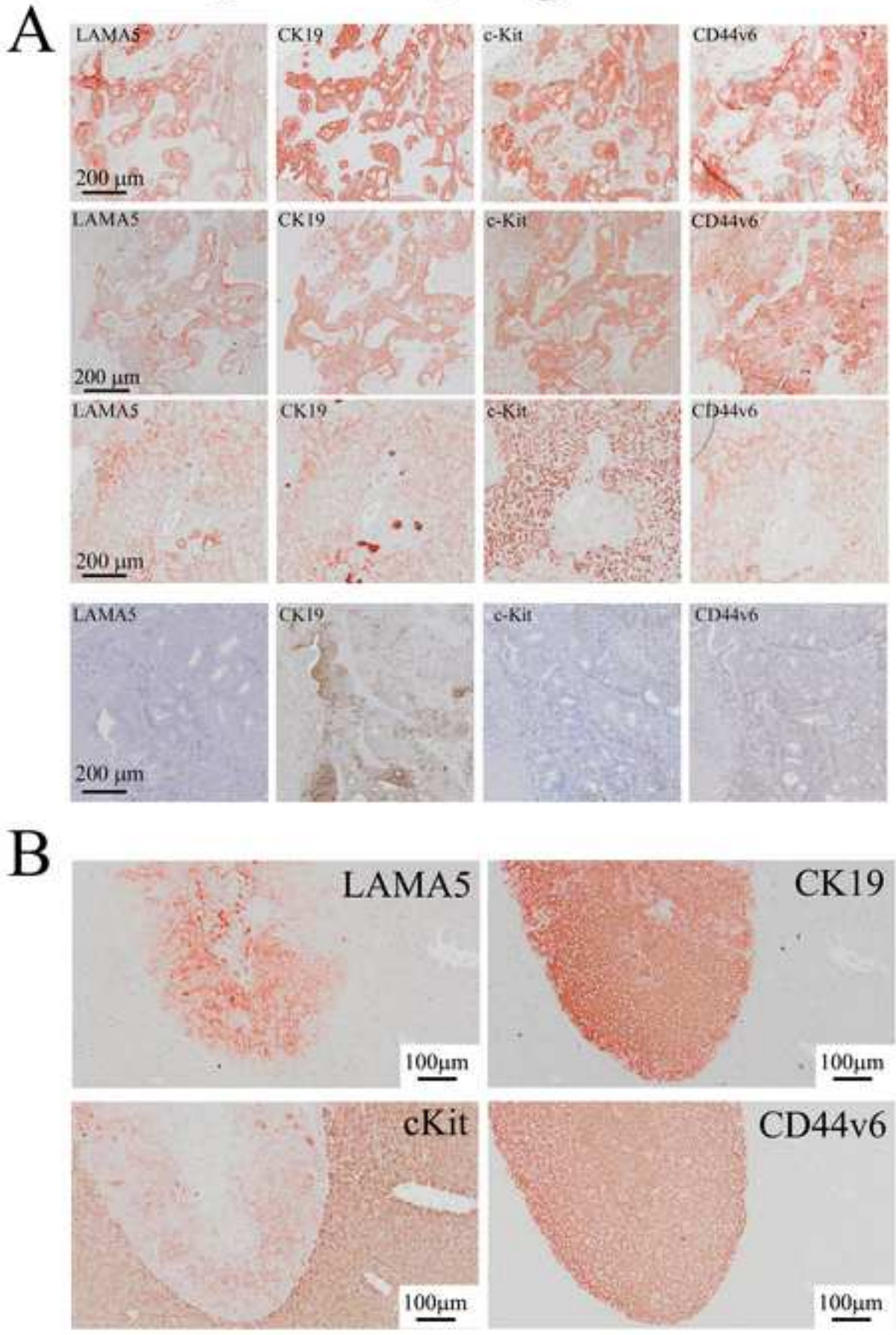
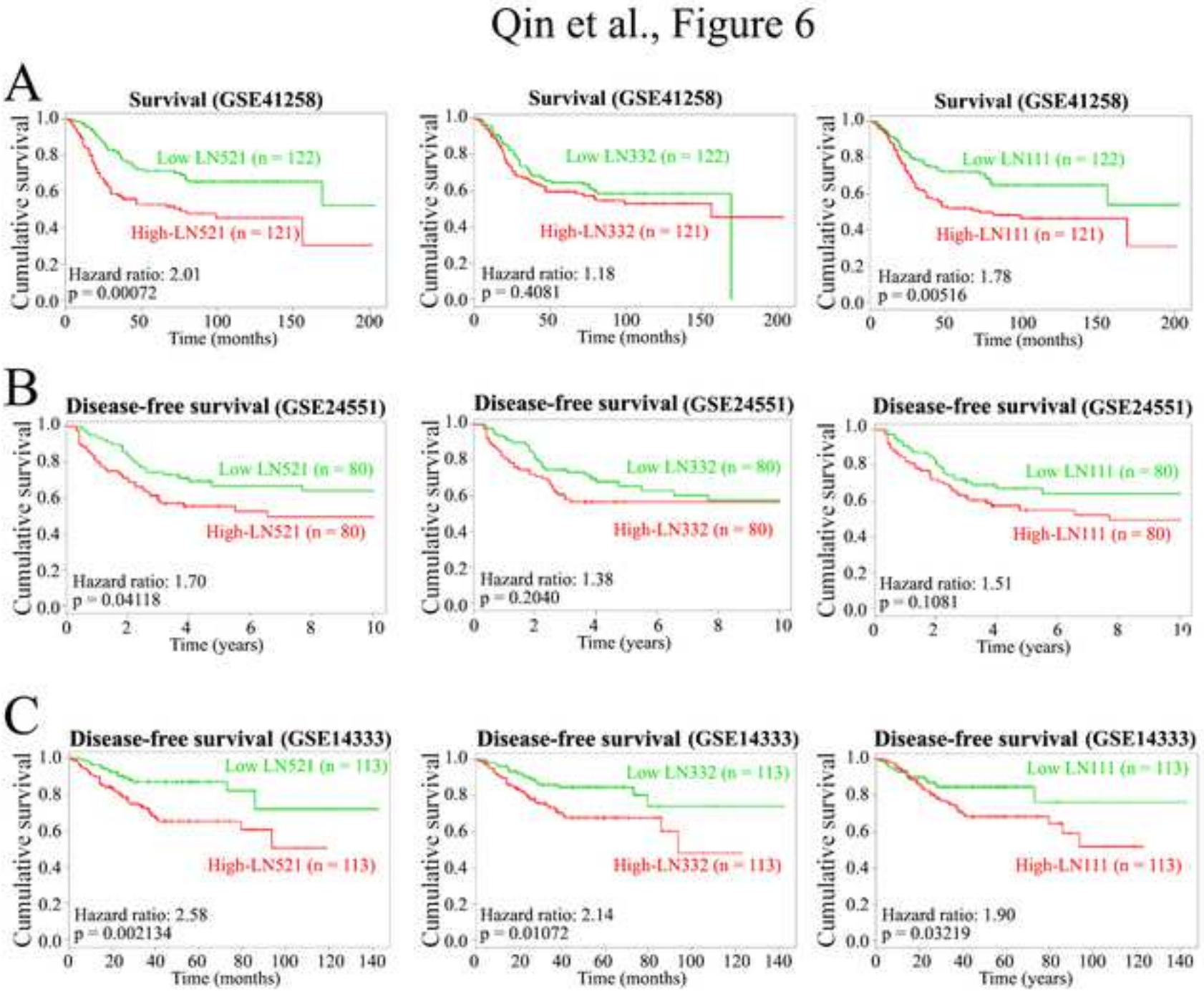


Figure 6



Supplementary Figures

Laminin 521 enhances self-renewal via Stat3 activation and promotes tumor progression in Colorectal Cancer

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Abstract

Remodeling of basement membrane proteins contributes to tumor progression towards the metastatic stage. One of these proteins, laminin 521 (LN521), sustains embryonic and induced pluripotent stem cell self-renewal, but its putative role in cancer is poorly described. In the present study we found that LN521 promotes colorectal cancer (CRC) cell self-renewal and invasion. siRNA-mediated knockdown of endogenously-produced laminin alpha 5, as well as treatment with neutralizing antibodies against integrin $\alpha3\beta1$ and $\alpha6\beta1$, were able to reverse the effect of LN521 on self-renewal. Exposure of CRC cells to LN521 enhanced STAT3 phosphorylation, and incubation with STAT3 inhibitors Napabucasin and Stattic was sufficient to block the LN521-driven self-renewal increase. Robust expression of laminin alpha 5 was detected in 7/10 liver metastases tissue sections collected from CRC patients as well as in mouse liver metastasis xenografts, in most cases within areas expressing metastasis cancer stem cell markers such as c-KIT and CD44v6. Finally, retrospective analysis of multiple CRC datasets highlighted the significant association between high LN521 mRNA expression and poor clinical outcome in colorectal cancer patients. Collectively our results indicate that high Laminin 521 expression is a frequent feature of metastatic dissemination in CRC and that it promotes cell invasion and self-renewal, the latter through engagement of integrin isoforms and activation of STAT3 signaling.

Keywords: colon and rectal carcinoma, metastasis, extracellular matrix, cancer-initiating cells.

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1. Introduction

Although clinical outcomes have steadily improved over the last few decades for patients with colorectal cancer, survival remains poor for those who have developed metastatic disease to distant sites such as the liver, lung or peritoneum [1]. Characterizing cellular and molecular processes that drive metastasis progression therefore represents an essential step to better prevent or target metastatic disease. Previous studies have demonstrated that the development of metastases requires tumor cells to display invasive properties, enabling their dissemination from primary sites and transit through the lymphatic and/or blood stream, as well as survival and self-renewing properties that will drive their ability to initiate metastasis growth and colonize the distant site [2, 3]. Invasion and self-renewal are both sensitive to microenvironmental cues provided by cellular and non-cellular actors of the microenvironment. Among the latter, extracellular matrix molecules have been described as instrumental modulators of cell motility, invasion, and stemness [4]. In particular, remodeling of basement membrane proteins such as laminins during cancer development is thought to alter interaction of these proteins with receptors located at the surface of cancer cells, thereby leading to phenotypic modifications [5].

Laminins are large multimeric basement membrane proteins, with functional units constituted through the assembly of one alpha, one beta and one gamma isoform [6]. In the present work we analyzed the contribution of laminin alpha 5 (LAMA5), and more specifically of the laminin-521 isoform (LN521, constituted by one alpha 5, one beta-2 and one gamma-1 chain), in the regulation of self-renewal and invasion of colorectal cancer cells. Laminin-521 (LN521) was previously shown to be important in the maintenance of embryonic and induced-pluripotent stem cells [7, 8], and we hypothesized that exposure to LN521 could also promote self-renewal in colorectal cancer cells. Since long-term self-renewal is a hallmark of cancer stem cells (CSCs) and contributes to their driving role in cancer progression and poor clinical outcomes [9, 10], we also investigated whether LN521 may play a role in regulating phenotypic and clinical features that are classically associated with metastatic development, such as cell migration, invasion and patient survival.

2- Materials and Methods

Immunohistochemical staining, Extreme Limiting Dilution Analysis (ELDA), Protein and RNA quantitation, in vivo liver metastasis induction in immunocompromised mice, as well as analysis of associations between laminin expression and CRC patient survival, were performed as previously described [11, 12]. Details for these procedures are provided under Supplemental Materials and Methods.

2.1- Laminin coating

Tissue culture plates (Corning) were coated with either LN521 (BioLamina, LN521-01), LN-332 (BioLamina, LN-332-0202) or LN-111 (Cultrex, 3400-010-02) as described [8]. Briefly, laminins were diluted to 10ug/ml in DPBS containing Ca2+ and Mg2+ (Gibco), added to tissue culture plates, and incubated overnight at 4 °C. Coating volume was adjusted to the plate/well size and final laminin density was 1.7μg/cm^2, lower than previously reported for the maintenance of embryonic stem cells (5μg/cm^2)[8]. Cells were seeded at approximately 20% confluency on laminin-coated wells for 48hrs to reach 70%-80% confluency at the time of harvest.

2.2- Inhibition of Integrin / Laminin binding.

Integrin neutralizing antibodies were included in cell suspension at the time seeding onto tissue culture plates coated or not with LN521 to inhibit the binding between laminin and integrin. A complete list of integrin neutralizing antibodies and their respective isotype controls is provided in Supplemental Table SI.

2.3- Invasion assays

For invasion assays, cells were preincubated or not with laminin as described above for 24h, then seeded onto 24-well culture plate inserts (Millipore, 8μm pore size) that had been coated for 2hrs at 37°C with growth factor-reduced Matrigel (Becton Dickinson) diluted to 200ug/ml with DMEM/HAM F-12 media and mixed or not with 10ug/ml LN521. Cells were seeded within the top compartment in DMEM/HAM F-12 medium and DMEM/HAM F-12 + 10% FBS was used as chemoattractant in the bottom compartment. Cells were allowed to travel through the insert for 6 hrs. Thereafter, cells that failed to invade were removed using sterile cotton buds. After fixing with 4% paraformaldehyde, the insert membrane was stained with 1ug/ml DAPI, washed three times with 1X DPBS and mounted under a coverslip using a slide

using mounting solution. Pictures of the bottom side of the insert were taken from 5 randomly selected fields for each condition. The average number of cells was quantified using Image J. For invasion experiments followed by self-renewal analysis, cells were left to invade as above. After removal of cells that failed to invade using a cotton bud, invading cells were resuspended for 3min using Accumax (Sigma-Aldrich), washed pelleted and seeded in Extreme Limiting Dilution Analyses. In alternate well, invading cells were removed using a cotton but and Accumax was applied to the upper Transwell chamber to resuspend the non-invading cells and seed them in suspension to perform an ELDA.

3- Results

3.1- Laminin-521 promotes self-renewal and invasion of CRC cells

To determine whether exposure to LN521 impacted on the self-renewal of colorectal cancer cells, we first exposed human CRC cells to various laminin isoforms and subsequently resuspended and seeded them into an extreme limiting dilution analysis (ELDA) in suspension. The ability of these cells to generate at least one colonosphere per well under all limiting dilution conditions was quantified and the frequency of self-renewing cells was calculated as described previously [11, 13] using the http://bioinf.wehi.edu.au/software/elda/ webtool [14]. We compared the ability of LN521 to regulate self-renewal with that of LN332, which was previously shown to promote cancer stem cell survival in primary hepatocellular carcinoma (HCC) samples [15] and to collaborate with collagen XVII in enabling survival of tumor-initiating cells [16], as well as with that of LN111, which is often used to support ES/iPS cell cultures [17] and constitutes the main component of Matrigel.

The DLD-1 colorectal cancer cell line and CPP19 patient-derived colorectal cancer liver metastasis cells [18] were grown on tissue culture plates pre-coated or not with LN111, LN332 and LN521 (Supplemental Figure S1) before seeding in a limiting dilution assay in ultra-low adherence plates. The frequency of cells displaying self-renewing activity was significantly enhanced after exposure to laminin 521 compared to cells preincubated on non-coated wells (20.22% +/- 4.97 vs 9.69% +/- 3.01, respectively, for DLD-1 cells; 11.93% +/- 6.12 vs 3.71% +/- 0.27 for CPP19 cells) (Figure 1A-B and Supplemental Figure S1). In contrast, self-renewal was not significantly enhanced by preincubation on LN111-coated plates, while LN-332 preincubation only increased self-renewal of CPP19 cells but not of DLD-1 (Figure 1A-B).

This suggests that LN521-induced signaling may promote self-renewal in colorectal cancer cells.

To further investigate the potential of LN521 to further promote tumor progression, we quantified the invasive capacity of DLD-1 and CPP19 cells through a Matrigel layer after and/or during exposure to LN521 (Figure 1C) and assessed the self-renewal ability of invading cells (Figure 1D). Invasion of CRC cells through Matrigel was significantly promoted by LN521 preincubation and/or by addition of LN521 to the Matrigel (Figure 1C). To ascertain whether LN521 can jointly promote invasion and self-renewal, two essential characteristics for metastasis formation, we collected cells having invaded or not through a Matrigel layer and quantified their self-renewal capacity. Cells preincubated on LN521 displayed significantly enhanced self-renewal compared to those preincubated on uncoated wells, with the exception of non-invading CPP19 cells (Figure 1D). Additionally, we found that the promoting impact of LN521 on self-renewal frequency was stronger in invading compared to non-invading cells (Figure 1D). Collectively our results suggest that that LN521 is able to concomitantly promote invasion and self-renewal in colorectal cancer cells.

3.2- Laminin alpha 5 contributes to the LN521-induced promotion of self-renewal.

In view of the essential role played by laminin alpha chains in the recognition of cellular laminin receptors [19, 20] we hypothesized that laminin alpha 5 (LAMA5) might be instrumental for the self-renewal increase induced by LN521 on CRC cells. First, we sought to determine whether CRC cells were able to produce the three laminin chains that make up LN521. Indeed, expression of *LAMA5, LAMB2* and *LAMC1* RNAs was readily detected using RT-qPCR in DLD-1 and CPP19 cells, along with those encoding several other laminin chains such as LAMA3, LAMB1, LAMB3, and LAMC2 (Supplemental Figure S2).

We also quantified LAMA5 and LAMA3 RNA expression and performed ELDA self-renewal assays *in vitro* on 9 additional early-passage primary cell lines (6 from primary tumors, 3 from liver metastases) that we recently generated from CRC patients [11, 13]. Variable expression of both laminin alpha chain RNAs was detected across all CPP lines. Expression of LAMA5 was positively correlated to the self-renewal potential in these cells ($R2 = 0.7548$, $p = 0.0011$) (Figure 2A), whereas no correlation was found between expression of LAMA3 and self-renewal potential ($R2 = 0.0207$, $p = 0.6914$) (Figure 2B).

To establish whether endogenous LAMA5 is indeed able to contribute to the promotion of self-renewal, selective siRNAs were then used to down-regulate *LAMA5* RNA expression (Supplemental Figure S2), and siRNA-transfected cells were seeded in an ELDA assay to quantify their sphere-forming efficiency, in comparison with cells transfected with a control siRNA targeting β-galactosidase. While transfection with β-Gal siRNA had no detectable effect on self-renewal, down-regulation of LAMA5 RNA expression resulted in a two to three-fold decrease in the sphere-forming ability of CPP19 and DLD-1 cells, (47.29 +/- 8.9% and 29.56 +/- 3.87 %, respectively, compared to untransfected cells) (Figure 2C).

Collectively these results suggest that endogenous production of laminin alpha 5 contributes to the maintenance of self-renewal in human colorectal cancer cells.

3.3- Role of integrins in the promotion of self-renewal by LN521.

Laminins are able to regulate cell signaling and alter cell phenotypes by interacting with specific receptors located at the cell surface, such as integrins. LN521 has been shown to primarily interact with two integrin isoforms, α6β1 and α3β1 [6]. mRNAs encoding integrin α3, α6 and β1 chains were readily detected in CRC cells, along with α7 and β4 chains (Figure 3A). To investigate if interaction with either or both of α6β1 and α3β1 integrins contributed to the identified role of LN521 in enhancing self-renewal capacity, we made use of neutralizing antibodies targeting the α6, α3, and β1 chain of integrins and thereby blocking their binding to laminins. DLD-1 cells were grown on LN521-coated or control plates for 24hrs, then treated with α6, α3, or β1 integrin neutralizing antibodies, either individually or in combination (α6β1 or α3β1), and grown under these conditions for another 24hrs before being resuspended and analyzed for self-renewal potential using ELDA.

Apoptotic cell death in DLD1 and CPP19 cells was monitored during these experiments via detection of cleaved caspase 3 expression (Supplemental Figure S3A). Apoptosis levels were low in all samples (1.92 to 5.56% in CPP19, 0.54 to 2.05% in DLD1). For both cell lines, we detected a non-significant trend towards lower apoptosis in cells pre-coated with LN521 and a slight but significant increase in apoptosis for cells treated with anti-α6β1 antibodies (Supplemental Figure S3A). The ability of these antibodies to inhibit the LN521-induced internalization of integrin isoforms was confirmed using immunofluorescent staining in DLD-1 and CPP19 cells, with strongest effects measured for α3 and β1 integrins (Figure 3B). In both cell lines, the robust increase in self-renewal frequency induced by exposure to LN521 was strongly and significantly inhibited by all three integrin neutralizing antibodies in comparison

with cells treated with isotype control IgG (Figure 3C). Control DLD-1 cells treated with all three neutralizing antibodies also exhibited a small reduction of sphere-forming frequency in comparison with their matching isotype antibody-treated controls, while a similar effect was only detected after α3 integrin antibody treatment in CPP19 cells. Altogether these results demonstrate that integrins α3, α6 and β1 all contribute to the promoting effect of LN521 on self-renewal in DLD-1 and CPP19 colorectal cancer cells. Overall, the similar level of neutralizing efficiency shown by α3, α6 and β1 antibodies in these experiments suggests that both α and β integrin chains are required for this process, consistent with previous findings that both integrin chains contribute to interaction with extracellular ligands such as laminins and for initiation of signaling [21, 22].

3.4- Activation of Stat3 signaling during the promotion of self-renewal by LN521

We then attempted to identify tumor cell signaling pathways involved in the regulation of self-renewal by LN521, acting via its interaction with α3β1 and α6β1. We elected to prioritize the analysis of AKT and STAT3 pathways based on their previous identification as LN521 and integrin targets and on their known role in cancer progression and/or stemness [8, 23]. Effector proteins of these pathways were quantified using Western blotting after exposing colorectal cancer cells to laminins for 48h.

Preincubation with LN521 was found to significantly enhance the expression and phosphorylation of STAT3 in both DLD-1 and CPP19 cells, while LN111 had a similar effect on DLD1 cells only and LN332 did not significantly alter STAT3 expression or phosphorylation (Figure 4A). In contrast, preincubation with LN332 and LN521 had similar effects on AKT levels in both cell lines (Supplemental Figure S3B), suggesting that the LN521-specific self-renewal activation in these cells was unlikely to be mediated by this pathway.

To further determine if the detected increase of STAT3 expression and activity contributed to the enhancement of self-renewal induced by LN521, we first quantified the colonosphere-forming frequency of DLD-1 cells pre-incubated or not with LN521 and treated respectively with DMSO or with the STAT3 inhibitors Napabucasin and Stattic (Figure 4B-D). Both inhibitors were able to reduce not only the baseline but also the LN521-enhanced self-renewal down to similar levels, suggesting that STAT3 activation may be required for the stimulatory effect of LN521 on self-renewal. To verify this finding, we used RNA silencing to determine the impact of STAT3 expression knockdowns on the ability of endogenous LAMA5 to

stimulate self-renewal in CRC cells. Both pharmacological STAT3 inhibitors as well as siRNA-mediated LAMA5 down-regulation inhibited self-renewal to similar extent, and no significant additive effect was detected when both types of treatment were performed simultaneously (Figure 4C).

These results strongly suggest that STAT3 signaling is an important contributor to the effect of LN521 on colorectal cancer cell self-renewal.

Finally, because of their well-characterized role as activators of STAT3 signaling and their suspected contribution to cancer stemness [24, 25], we then sought to establish whether interleukins such as IL-6 and IL-11 could act as mediators of the LN521-driven self-renewing frequency increase. Our results indicated that preincubation of colorectal cancer cells on LN521-coated plates did not modify the expression of IL-6, IL-11 and their respective receptors, suggesting that it is unlikely to be the case (Supplemental Figure S3C).

3.5- LAMA5 expression in human and mouse colorectal cancer liver metastases.

The promoting effect of LN521 and LAMA5 on self-renewal and/or invasion suggests that it may contribute to the metastatic process in colorectal cancer *in vivo*. This hypothesis implies that LN521, or at least LAMA5, may be expressed within colorectal metastases to support their initiation and long-term maintenance. To verify this assertion, we used immunohistochemical staining to establish whether LAMA5 is expressed in liver metastasis tissue sections from CRC patients, as well as in an experimental mouse model of liver metastasis.

Immunohistochemical staining for LAMA5 was performed on chemo-naïve liver metastasis samples collected during surgical resection from patients with stage IV disease (n = 10). Metastatic cells were sensitively detected via staining for cytokeratin 19 (CK19), a marker for the detection of carcinoma metastasis to the liver in several cancers including colorectal [26]. LAMA5 expression was detected in 7/10 samples, and its expression was prominent in areas that expressed the c-Kit receptor, recently identified as an essential contributor to the undifferentiated stem-like tumor cell phenotype in colorectal cancer [27], as well as CD44v6, a marker for metastatic CRC stem cells [28] (Figure 5A and Supplemental Figure S4A). One of 10 samples analyzed exhibited a distinct pattern of expression, with detectable c-Kit and CD44v6 staining despite little to no detection of LAMA5 (Supplemental Figure S4B). In

comparison, specific LAMA3 staining was not detected in our liver metastasis samples, while it was readily detectable in primary colorectal tumor sections (Supplemental Figure S4C).

To generate liver metastases in mice, we xenografted CPP1 patient-derived CRC cells in the caecum of BALBc/nude immunocompromised mice and liver metastases were collected 6-9 weeks after tumor cell injection and paraffin-embedded. Corroborating our results in patient samples, tissue sections from these liver metastases were also found to express LAMA5 in similar areas as cKit (Figure 5B). The latter was also found to be expressed by neighboring mouse hepatocytes, in contrast to our finding in human liver. Most cells in CPP1-derived metastases were found to express CD44v6 (Figure 5B), suggesting that CPP1-derived tumors may be enriched for cancer stem cells or that expression of this marker may not be restricted to cancer stem cells in this model.

Our results suggest that LAMA5 expression is frequent in liver metastases from colorectal cancer and that tumor cells expressing metastatic cancer stem cell markers are often detected in LAMA5-rich areas within these samples.

3.6- Laminin-521 expression is associated with poor disease-free survival in patients with colorectal cancer

Collectively, results obtained above suggest that expression of LAMA5, combined or not with expression of the LAMB2 and LAMC1 chains, may contribute to tumor progression and to the development of metastases in colorectal cancer. Because self-renewal and metastatic progression are linked to poor clinical outcome in colorectal and other solid cancers, we retrospectively analyzed previously published CRC datasets to test whether the expression of mRNA that encode LN521 chains was associated with disease outcome.

To do so, we first used the cBioPortal webtool (http://www.cbioportal.org) to extract and analyze the colorectal adenocarcinoma dataset (n = 592 samples) from the TCGA PanCancer Atlas [29]. Despite the highly heterogeneous nature of this patient cohort (multiple tumor stages, various tumor subtype and treatment regimen…), we found that High LN521 RNA expression was significantly associated with both disease-free survival (Logrank test, $p = 0.0353$) and overall survival ($p = 0.0127$) (Supplemental Figure S5). In contrast, no association was found in the case of LN332 (Logrank test, $p = 0.115$ for disease-free survival, $p = 0.215$ for overall survival) or LN111 (Logrank test, $p = 0.908$ for disease-free survival, $p = 0.447$ for

overall survival). We also used several previously published GSE14333 [30], GSE24551−GPL11028 [31] and GSE41258 [32] datasets, as they contained large cohorts (>150) of colon cancer patients. We analyzed these datasets using the SurvExpress bioresource [33], allocating patients to either high or low laminin chain expressing subgroups using median expression a cutoff for subgroup allocation. High expression levels of LN521-encoding mRNAs were significantly associated with poorer survival in all three datasets, with respective Hazard Ratios of 2.58 (n = 226 patients, p = 0.002134), 1.70 (n = 160 patients, p =0.04118) and 2.01 (n = 243 patients, p = 0.00072) (Figure 6 and Supplemental Figure S6). The association of LN521 mRNA expression with survival was consistently stronger (higher Hazard Ratio) and more statistically significant than for LN332 or LN111-encoding mRNAs (Figure 6 and Supplemental Figure S6), as well as for mRNAs encoding LN511 (Supplemental Figure S7), another LAMA5-containing laminin isoform shown to regulate metastasis development in breast and other cancer [34].

Collectively these results suggest that LN521 expression is strongly associated with disease-free and overall survival in multiple colorectal cancer patient cohorts.

4- Discussion

In the present study we characterized the promoting role of laminin-521 on colorectal cancer cell self-renewal and invasion and found that endogenous production of the laminin alpha 5 chain contributed to this effect. We demonstrated that integrins α3β1 and α6β1 are likely mediators of the self-renewal promotion induced by laminin alpha 5 and that downstream activation of STAT3 signaling is required for the promotion of self-renewal by LN521. Finally, we also found that colorectal cancer liver metastases frequently contain laminin alpha 5-rich areas and that high laminin-521 expression correlates with poorer disease-free and overall survival in several colorectal cancer patient datasets.

Laminin alpha 5 was recently shown to promote growth and angiogenesis of liver metastases from colorectal cancer, and to inhibit the Notch signaling pathway in metastasis endothelial cells [35]. It may do so by interacting with the Basal Cell Adhesion Molecule (BCAM) to mediate interactions between endothelial and metastatic tumor cells, a mechanism possibly restricted to K-RAS mutated colorectal tumors [36]. Here, our main findings were that laminin alpha 5, either endogenously secreted by tumor cells or exogenously provided to colorectal cancer cells, significantly enhanced the invasive ability as well as promoting self-renewal of

colorectal tumor cells. We also detected frequent expression of laminin alpha-5 within human colorectal cancer liver metastases, often in regions that also express metastatic cancer stem cell markers such as cKIT and CD44v6. Together these results suggest that laminin alpha 5 and LN521 may contribute to a local environmental niche that sustains self-renewing cells during progression of colorectal cancer to the metastatic stage and/or upon arrival at the metastatic site.

The potential role of specific laminin chains in the maintenance of cancer stem cells has been reported in prior studies (summarized in [5]). Thus, laminin gamma-2 was shown to contribute to a putative cancer stem cell niche in hepatic cancer [15], while laminin alpha 2 was shown to have similar attributes in glioblastoma multiforme [37]. In addition, exposure to multiple laminin isoforms (411, 421, 511 and 521) was reported to enhance the clonogenicity of glioma stem cells [38]. However, our study is the first to demonstrate that laminin-521 is able to concomitantly enhance the invasive capability and the self-renewal of some colorectal cancer cells, thus providing them with a competitive advantage for metastasis initiation. In addition, the present work is also the first to demonstrate that the effect of LN521 on self-renewal can be detected in patient-derived cancer cells, where endogenous LAMA5 expression also correlates with enhanced self-renewal abilities.

LN521 was also found to significantly promote CRC cell invasion. The invasive ability of CRC cells was highest when they were exposed to LN521 during preincubation as well as during invasion through Matrigel as a Matrigel-LN521 mix. This result, which highlights the role of LN521 in promoting the invasion of colorectal cancer cells, is in accordance with finding in other cancers such as melanoma, where cancer cells produce several laminins including 521 and use them as a substrate for migration [39].

In addition, our findings indicate that LN521 acts to promote self-renewal vie engaging with integrin $\alpha 3\beta 1$ and $\alpha 6\beta 1$ at the surface of tumor cells. This result is not unexpected as these integrin isoforms were reported previously as primary interactors for LN521 in other model systems [6]. While other integrins chains including $\alpha 5$ [40], $\alpha 2$ [41] or $\beta 3$ [42] contribute to the adhesion of colorectal cancer cells to their microenvironment in colorectal liver metastases, our results using neutralizing antibodies strongly suggest that the $\alpha 3$, $\alpha 6$ and $\beta 1$ integrin chains are instrumental to mediate the self-renewal promoting effect of LN521.

Exposure to LN521 was shown to increase STAT3 expression and phosphorylation in both DLD-1 and CPP19 CRC cells. In addition, the clinically-relevant pharmacological STAT3

inhibitor Napabucasin, as well as the STAT3 activation and dimerization inhibitor Stattic, were both able to reverse the LN521-driven increase in self-renewal. These results indicate that STAT3 signaling mediates the effect of LN521 on CRC cell self-renewal. The involvement of STAT3 in the promotion of self-renewal has already been reported in several cancer types [43, 44], and its role as a promoter of stem-like cells and as a putative therapeutic target in GI cancers is well-described (reviewed in [45]). Indeed, the STAT3 inhibitor Napabucasin is undergoing clinical trials to target self-renewal and cancer stem cells [46]. Our results therefore suggest that LN521 may be a contributor to the elevated STAT3 signaling during colorectal tumor progression, and that blocking the activity of LN521 may represent an alternative to decrease STAT3 activation.

In human patient samples and in a preclinical mouse model, we found that most CRC liver metastases expressed laminin alpha 5, in areas where CK19-positive metastatic tumor cells also expressed cancer stem cell markers such as c-KIT and CD44v6. The detected laminin alpha 5 may not only be secreted by some tumor cells, as demonstrated in CPP19 metastatic tumor cells in our study, but also by surrounding hepatocytes. Indeed, small quantities of laminin alpha 5 are produced by the adult liver [47], possibly by hepatic progenitor cells [48]. However, since most of the detected laminin alpha 5 staining was detected in areas containing metastatic tumor cells rather than in the surrounding healthy liver, this could also suggest that presence of metastatic cells may promote local production of laminin alpha 5 in the liver. Incidentally enriched laminin alpha 5 deposits have been previously described to promote CK19 expression in hepatocellular carcinoma cells *in vitro* [49], which may contribute to the reported high expression of this cytokeratin isoform in liver metastases [26].

Altogether our results highlight the enriched expression of laminin alpha 5 in metastatic colorectal tumors and identify a significant correlation between high LN521 expression and poor survival in colorectal cancer patients. We also demonstrated the ability of LN521 to promote their self-renewal via the engagement of integrins $\alpha3\beta1$ and $\alpha6\beta1$ and the activation of STAT3 signaling and showed that LN521 enhance the invasive ability of colorectal tumor cells *in vitro*. These results suggest that targeting of LN521/integrin interactions may reduce the invasive ability of disseminating tumor cells and/or their metastasis-initiation ability in the liver. Thus, future preclinical studies will establish whether antibody or peptide-based targeting of LN521-integrin binding may provide therapeutic benefit in CRC patients, either directly and/or by sensitizing their tumor to other compounds as suggested for other LN-integrin isoform interactions [50, 51].

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