

Functional interplay between collagen network and cell behavior within tumor microenvironment in colorectal cancer

Cuong Cao Le^{1, 2, 3}, Amar Bennasroune^{1, 2}, Benoit Langlois^{1, 2}, Stéphanie Salesse^{1, 2}, Camille Boulagnon-Rombi^{1, 2, 4}, Hamid Morjani^{1, 3}, Stéphane Dedieu^{1, 2*} & Aline Appert-Collin^{1, 2*}

1. Université de Reims Champagne-Ardenne, Reims, France.

2. CNRS UMR 7369, Matrice Extracellulaire et Dynamique Cellulaire, MEDyC, Reims, France.

3. Unité BioSpecT, EA7506, Reims, France.

4. Laboratoire de Biopathologie, Centre Hospitalier Universitaire et Faculté de Médecine, Reims, France.

* Co-senior authors

Email corresponding author: aline.bennasroune@univ-reims.fr

Colorectal cancer is the second most common cancer diagnosed in men and the third most commonly occurring in women worldwide. Interactions between cells and the surrounding extracellular matrix (ECM) are involved in tumors development and progression of in many types of cancer. The organization of the ECM molecules provides not only physical scaffoldings and dynamic network into which cells are embedded but also allows the control of many cellular behaviors including proliferation, migration, differentiation and survival leading to homeostasis, and morphogenesis regulation. Modifications of ECM composition and mechanical properties during carcinogenesis are critical for tumor initiation and progression. The core matrisome is constituted of five classes of macromolecules which are collagens, laminins, fibronectin, proteoglycans, and hyaluronans. In most tissues, fibrillar collagen is the major component of ECM. Cells embedded into fibrillar collagen interact with it through their surface receptors, such as integrins and discoidin domain receptors (DDR). On the one hand, cells incorporate signals from ECM that modify their functionalities and behaviors. On the other hand, all cells within tumor environment (cancer cells, cancer-associated fibroblasts, endothelial cells, immune cells) synthesize and secrete matrix macromolecules under the control of multiple extracellular signals. This cell-ECM dialog participates in a dynamic way in ECM formation and its biophysical and biochemical properties. Here we will review the functional interplay between cells and collagen network within the tumor microenvironment during colorectal cancer progression.

Keywords: Colorectal cancer, collagen, cancer-associated fibroblast, tumor cell, endothelial cell, in vitro model

37 **Collagen and colorectal cancer: state of play.**

38 In recent decades, several works have underlined the importance of the microenvironment in
39 colon cancer progression [1]. In the tumor microenvironment (TME), extracellular matrix
40 (ECM) plays a key role in this process. Among ECM adhesives components, type I collagen is
41 one of important factors regulating cancer-related events at different tumorigenesis stages [2].
42 After effacement of the basement membrane, paracrine signals from the nascent tumor lead to
43 profound reorganizations of submucosal ECM that include deposition of fibrillar collagens
44 together with growth factors and ECM modifying enzymes which stimulate active vascular
45 remodeling. Some recent studies based on global transcriptomic or proteomic approaches shed
46 new light on the specific markers that are dysregulated during early steps of colon
47 carcinogenesis, but also in locally advanced or metastatic colorectal cancer (CRC) [3-5].
48 Interestingly, proteomic analysis of detergent insoluble fractions of paired primary colon
49 tumors and liver metastasis compared with adjacent non tumorous tissues, illustrated the
50 pathological samples specific enrichment in core matrisome and several collagen modifying
51 enzymes such as MMPs, ADAMs and LOXL1 [5]. Desmoplasia and collagens deposition
52 constitute a hallmark of CRC and various collagens including type I, VI, VII, VIII, X, XI and
53 XVIII were found accumulated in CRC samples [6-12]. A recent study showed an increase of
54 type I collagen in tumor tissues compared to normal tissue [13]. Moreover, type I collagen
55 mRNA were also reported as increased in blood of CRC patients compared to healthy
56 individuals [13, 14]. Consistently, second harmonic generation imaging of fibrillar collagens
57 contents have shown clinical efficacy stratify high grade tumors and relevance to predict CRC
58 patient outcome [7, 15].

59 The most studied type I collagen receptors are integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$ [16].
60 These receptors can be activated by several ligands such as type I collagen after recognition of
61 its GFOGER sequence [17]. $\alpha 1\beta 1$ dimer was considered as the most expressed receptor in colon
62 carcinoma [18]. $\beta 1$ -integrin expression in tumors was correlated with reduced overall survival
63 and reduced disease-free survival in a large cohort of CRC patients [19]. Notably, $\beta 1$ integrin
64 is detected in CRC patients serum and its level of expression appears to correlate with
65 aggressiveness and presence of micrometastasis [20]. $\beta 1$ integrin overexpression is also
66 associated with CRC progression and colorectal liver metastasis [20, 21]. In vitro, $\beta 1$ integrin
67 expression is down-regulated in response to 3D type I collagen [22, 23]. However, although $\beta 1$
68 integrin seems to contribute to metastasis development, $\beta 1$ integrin targeted therapy is not
69 successful in CRC management. In fact, simultaneous inhibition of $\beta 1$ integrin and EGFR in
70 CRC does not improve radiotherapy efficiency [24].

71 Collagen also signals to cells through the receptor tyrosine kinases discoidin domain receptors
72 DDR1 and DDR2, both of them have also been reported to interact with type I collagen [25]
73 and to play a role in tumor progression [23]. These receptors, which harbor a tyrosine kinase
74 activity, recognize GVMGFO sequence of type I collagen [26] and exhibit a relatively late and
75 prolonged activation [27]. DDR1 is expressed in colon carcinoma and promote metastasis in
76 invasive colon carcinoma [28-30]. Concerning DDR2, a high expression was associated with
77 higher frequencies of T4, lymph node metastasis, peritoneal spread, and worse prognosis,
78 suggesting that DDR2 expression might be an effective therapeutic target [31].

79 This growing data set supports a key role of collagens and their partners during tumorigenesis
80 processes and as potential biomarkers of CRC. The following parts aim to highlight current

81 evidence regarding the functional interplay between cells within the TME and collagen network
82 during CRC progression. The main data are presented in Figure 1.

83 **The relationship between collagen and cancer cells**

84 Analysis of ECM signatures in patients colon tumors has revealed that type I collagen is highly
85 expressed [5]. Accordingly, high density of type I collagen constitutes a poor prognosis factor
86 in colon carcinoma and type I collagen-rich environment is able to induce mesenchymal gene
87 expression and invasion [32]. Beside the density, collagen topology (fiber alignment) and
88 elasticity (stiffness) appear to be also associated to colon tumorigenesis. Brauchle and co-
89 workers have demonstrated that the alignment of collagen fibers is increased in colon carcinoma
90 tissues when compared to normal tissues, and associated with increased stiffness [33].
91 Biophysical investigations have also shown different molecular fingerprints for collagen fibers
92 in colon carcinoma tissues when compared to normal tissues [33]. Another study has shown
93 that density and collagen fiber alignment were higher in tumor invasion front than in primary
94 tumor and normal tissue [34]. Of note, hypoxia, that is associated to collagen density and
95 organization, has an impact on colon cancer carcinoma migration and invasion through
96 promotion of epithelial to mesenchymal transition [35].

97 Concerning the role of type I collagen receptors in tumor progression, Roche's group has
98 elegantly recently shown that DDR1 plays a crucial role in the invasion function of metastatic
99 colon carcinoma [28, 29]. They have particularly demonstrated that pharmacological inhibition
100 of DDR1-BCR signaling axis decreased invasion and metastatic processes in colon carcinoma,
101 suggesting that DDR1 targeting could be an efficient co-treatment strategy in colon carcinoma
102 [28, 29]. More recently, NSD2 circular RNA has been shown to promote DDR1 expression and
103 colorectal cancer metastasis by targeting miR-199b-5p [36]. For integrins, Wu and co-workers
104 have lately reported that type I collagen is able to support colon carcinoma cell stemness,
105 invasion and metastasis through activation of $\alpha 2\beta 1$ integrin heterodimer and PI3K/AKT/Snail
106 signaling pathway [37].

107 Regarding the role of type I collagen in the cancer cell sensitivity to targeted therapies, a recent
108 study has demonstrated that 3D type I collagen may protect colon carcinoma against the anti-
109 EGFR cetuximab therapy by increasing tyrosine phosphorylation of MET and RON [32]. The
110 effect of 3D type I collagen on the sensitivity to vemurafenib of colon carcinoma, carrying the
111 $BRAF^{V600E}$ mutation has also been investigated. At the opposite of the general concept
112 describing type I collagen as a shield of colon carcinoma cells against therapies, authors have
113 demonstrated that cells seeded in 3D type I collagen were 10-fold more sensitive to the
114 vemurafenib targeted drug. On the contrary, 3D matrix was able to protect tumor cells against
115 the cytotoxic effect of the fluorouracil chemotherapeutic agent [38]. However, another carried
116 out on resistance to chemotherapy and the ATP binding cassette transporter P-glycoprotein
117 which is encoded by *ABCB1* gene, has shown that 3D ECM is able to increase sensitivity of
118 primary colon carcinoma cells to chemotherapy by affecting the cell polarity and consequently
119 the polarization of P-glycoprotein expression at the cell surface [39]. Interestingly, the
120 expression of *ABCB1* gene appears to be regulated in colon carcinoma. In fact, the
121 overexpression of the caudal-related homeobox transcription factor (Cdx2) has been reported
122 to upregulate the expression of *ABCB1*/gene and consequently P-glycoprotein in highly
123 resistant colon carcinoma to chemotherapy[40].

124 However, Cdx2 has been also described to play a role as a tumor suppressor [41]. In fact, Cdx2
125 expression has been shown to be lower in colon carcinoma with the highest grades [42]. In
126 agreement with the role of ECM in colon carcinoma progression, type I collagen has been
127 shown to promote tumorigenesis by downregulating Cdx2 expression [43]. Brummer's group
128 has demonstrated few years ago a positive correlation between BRAF mutation and low level
129 of Cdx2 expression in colon carcinoma. Type I collagen at high density has been also reported
130 to suppress HNF4 α when inducing mesenchymal gene expression in vitro and in patient-derived
131 colon tumors [32]. Consistently, invalidation or inhibition of HNF4 α promotes colon
132 carcinogenesis, whereas its enforced expression is able to inhibit cell growth in colon carcinoma
133 [44, 45].

134

135 **The relationship between collagen and cancer-associated fibroblasts**

136 The most abundant cell type in TME are cancer-associated fibroblasts (CAFs), an activated type
137 of fibroblasts that play a major role in tumorigenesis and metastatic processes [46]. CAFs
138 demonstrate a functional heterogeneity in colorectal cancer (CRC) that may arise from different
139 cellular origins and can affect the clinical course of colon cancer patients [47]. In CRC, an
140 abundance of CAFs in the TME has been associated with poor outcomes and transcriptomic
141 studies linked CAF signature with poor-prognosis and highly aggressive CRC molecular
142 subtypes. CAFs are not only associated with advanced CRC but also found in early stages [48].
143 Several studies identified CAFs as potential prognosis and recurrence markers in patients with
144 colon cancer [49-52]. Histologic evaluations of CRC patient samples and organotypic 3D co-
145 culture models demonstrated that CAFs are the primary drivers of collagen synthesis and
146 remodeling in the highly desmoplastic environment found in CRC [53, 54]. Interestingly, a
147 significant heterogeneity was observed within CAF population related to collagen remodeling
148 [55]. Transcriptome and Proteome profiling identified CRC CAFs as the main source for
149 connective tissue components of the ECM, such as collagens, thus altering the molecular
150 composition of the matrix by increasing the deposition of new matrix components [56, 57].
151 Another way for CAFs to remodel ECM is to degrade it by using MMPs and formation of
152 degradative protrusions. Genes induced in CRC CAFs, compared to normal colonic fibroblasts,
153 include several tumor-promoting MMPs and TGF- β 1 increased Collagen I and various
154 proteases expression by CAFs [56-58]. In CAFs, formation of invadopodia that remodel
155 collagen fibers, is dependent on twist1 and palladin (isoform 4). Twist1-expressing fibroblasts
156 acquired CAFs properties such as collagen contraction, and alignment and Palladin and
157 collagen α 1(VI) were identified as two major mediators of these Twist1 effects. Interestingly,
158 Twist1, palladin and collagen α 1(VI) are overexpressed in purified colon CAFs as compared
159 with their normal counterparts and associate with poor prognosis in CRC [59]. In addition to
160 MMPs, CAFs also express other proteases such as the fibroblast activation protein (FAP), a
161 collagenase and gelatinase [60]. Stromal FAP expression in human colon cancer samples is a
162 marker of early stage in cancer development and correlated with poor patient outcome [61].
163 FAP α activity has a strong impact on fibroblasts secretome composition, including matrix
164 processing enzymes, and influence morphology and collagen contraction capacity of
165 immortalized CRC CAFs. Recent studies established a direct link between CAFs and the
166 modifications of ECM organization and stiffness described in colon cancer. LOXL2, a collagen
167 cross-linker was reported as highly expressed in CAFs and is associated with poor CRC survival
168 [62]. Hic-5, a non-enzymatic adaptor protein, was described as a novel factor responsible for

the development of CRC, by promoting in CAFs the production of collagen I and LOX that lead to stiffness of cancer tissues [63]. More recently, in a collagen gel co-culture system, with fibroblasts and CRC cells, Delaine-Smith's group demonstrated that fibroblast-derived TG2 (transglutaminase-2), a protein cross-linking enzyme, induced gel stiffening by formation of thicker collagen fibers and proposed a regulatory link between TG2 and LOX. In addition, stiffness is further increased by fibroblast/CRC crosstalk and a potential role for extracellular vesicles in mediating this tumor-driven fibroblast response is suggested by authors [64]. Another study reported that fibroblasts activated by late-stage CRC cell-derived exosomes, became specialized in type I collagen and physical remodeling of ECM through cytoskeletal re-organization, membrane protrusion formation and secretion of matrix-remodeling proteins [65].

The relationship between collagen and endothelial cells

Angiogenesis exerts crucial functions during major steps of CRC progression [1, 3, 66]. Stimulation of CRC cells by oncogenic drivers such as EGF or stabilization of hypoxia inducible factors (HIFs) were involved in the secretion of angiogenic diffusible factors and ECM structural compounds in the TME [67]. Moreover, collagen supports nascent vascular structures during intussusceptive angiogenesis in CRC [68]. A nine genes signature including collagen I, X and XI was specifically enriched in angiogenic and hypoxic CRC genesets [4]. Another study identified a matrisomal signature of 110 genes induced during the angiogenic switch of the standard RIP1-Tag2 murine model of tumor angiogenesis [3]. The expression of this set of genes, which includes collagens I, VI, VIII, X and various ECM regulators was positively correlated with that of endothelial cell markers and increased with CRC progression. This signature was also specifically induced in hepatic metastasis suggesting a functional contribution to both early events and metastatic cascade. It is now well-established that tumor and stromal cells synergize to activate pro-angiogenic signals in the TME [3, 61, 66, 69, 70]. CAFs and tumor-associated macrophages (TAM) are both involved in TGF- β signaling activation during the angiogenic switch [3]. Stromal activation of this pathway promotes both tumor initiation and early metastatic events [66] and was specifically associated to consensus molecular subtypes CMS4 of CRC that express various angiogenesis markers and present the worst overall survival [71]. Several reports illustrated the contributions of tumor resident or infiltrated stromal cells to ECM modifying events that accommodate endothelial cells fitness and provide angiogenic cues [61, 69, 72]. Although a clear scenario is sometimes difficult to draw on the angiogenic consequences of collagen deposition, emerging angio-active parameters include types of collagens (network, fibrils-anchoring or fibrillar collagens that convey different angiogenic signals), topology and stiffness. Post-translational modifications such as proteolytic degradation or cross-linking can modulate the biophysical properties of collagen rich scaffolds [11, 70, 72, 73]. FAP- α expression and activity was linked to the secretion of pro-angiogenic factors such as angiopoietin-1 and VEGF-C by colon patient-derived CAFs [61]. Gain and loss of function experiments illustrated that FAP- α -dependent CAF secretome can stimulate 3D endothelial spheroids sprouting. In vivo, targeting of FAP- α into an immune competent murine model of colon cancer decreased blood vessel density and induced fibrillar collagen accumulation [69]. The activity of SNAI1 and PDGFBB contributes to CAF ability to assemble aligned collagen fibers that promote endothelial cell proliferation and morphogenesis in a 3D model of CAF-derived matrices [72]. SNAI1 expression by fibroblasts was also associated with the abundance of CD34 positive endothelial cells in an in vivo model of CRC. A pro-tumoral action of TAM could be explained by their ability to assemble collagenous ECM enriched with

type 1, 6 and 14 collagens [74]. These fibers were deposited, cross-linked and linearized at areas of tumor invasiveness demonstrating the crucial importance of TAM in organizing collagenous ECM niches. Co-culture of TAM with CRC cells can potentiate the production of tumor-derived MMP2 and MMP9 [75]. Recruitment of collagenolytic enzymes-expressing immune cells in the CRC TME might influence the bioavailability of ECM-immobilized angiogenic factors such as VEGF, as reported in other tumor context [76, 77]. Collagen-enriched niches emerge as biomarkers of desmoplastic and angiogenic CRC microenvironment [78, 79]. High expression of collagens I and IV, with tumor endothelial marker-1 (TEM-1, endosialin), especially when distributed around tumor vessels, allows stratification of CRC patients according to their poor prognosis [78]. Collagen-enriched niches might also account for the adaptive response of the TME to anti-angiogenic therapies [79, 80]. Collagen IV empty sleeves resulting from tumor vessel pruning triggered by VEGFR2 can promote a rapid vascular regrowth after treatment withdrawal [80]. Although, VEGFR2 blocking in a CRC model normalized tumor vessels, decreasing diameter while ameliorating collagen IV perivascular coverage [79]. Endostatin, a collagen XVIII-derived fragment, is an inflammatory marker detected around blood vessels and in the plasma of advanced CRC patients [12]. This molecule, efficient to inhibit both lymphangiogenesis and hemangiogenesis [81], is considered as a valuable tool to control metastatic CRC growth since several studies reported its moderate toxicity without observing the increased metastatic dissemination encountered in response to the anti-VEGF antibody bevacizumab [30].

In vitro tumor microenvironment models using collagen

Two-dimensional (2D) collagen-coated systems routine use has largely shown their limitations to summarize the complexity of tumor initiation and progression processes. It is absolutely necessary to include some major extracellular components to mimic properties of the TME such as the spatial configuration [82] and the addition of supporting materials with mechanical properties close to the ECM encountered during disease progression [83]. The use of in vitro 3D models should fill the gap between traditional 2D cell culture and animal models, by mimicking the cancer micro- and macro-environment potentially able to integrate multiple cell types in a controlled environment and should allow to better characterize CRC drivers and develop new therapeutic strategies in constantly upgraded models of growing complexity. One possible approach is to develop spheroids of cancer cells seeded on low-attachment tissue culture plates. Whereas this type of culture allows cancer cells to communicate with one another and to release low levels of intrinsic collagen [84], substantial aspects of TME are still missing. More complex models using biological scaffolds such as collagen are therefore added to create an ECM to obtain biomimicry and study cancer progression by recreating the tumor microenvironment. Patient-derived xenograft models are an important tool for preclinical and clinical research, especially when orthotopically transplanted. However, in this model, the principal limit is that TME cannot be properly reconstituted owing to important stromal cells such as cancer-associated fibroblasts and endothelial cells which are not derived from the tumor samples and can be late recruited [85]. New 3D models of cancer using a collagen matrix can promote the crosstalk between cancer and stromal cell. Co-cultures of different colorectal cancer cell lines with fibroblasts and endothelial cells in 3D spheroids have been elaborated to test drug dose-response and compared with results in 2D and homotypic 3D cultures. The results suggest that 3D co-cultures are more relevant, providing a higher level of translational information that should help to define patient-specific treatment options [86].

Pape and colleagues developed a CRC model using high-density type I monomeric collagen, termed as tumoroids [87]. This model consists of a central cancer mass containing either the highly invasive HCT116 or less invasive HT29 cells embedded in collagen type I hydrogels to mimic the TME in situ [88]. The stromal compartment in this model is easily manipulated and ECM components and stromal cell types can be added accordingly. Furthermore, on-chip biomimetic microenvironments using microfluidic technologies are being developed to better reproduce the complexity of in vivo restrictions. In this model, human colonic microvascular endothelial cells cultivated in a 3D vessel-mimetic device are attached to the wall of the lateral channels of the microfluidic chip whereas HCT-116 cells are embedded in collagen IV-enriched Matrigel in the central chamber [89].

Considering the feature of tumor heterogeneity, the main limitation of these models is the presence of a single CRC cell type exhibiting a unique genetical pattern. The development of more realistic preclinical models is absolutely required and is a major challenge for the coming years, especially for improving drug screening. The use of patients-derived 3D tumor models may provide a solution to overcome the oversimplified 2D cell cultures and the limitations of in vivo models [90]. These new designs are not intended to completely supplant but rather complete and expand the currently available techniques.

Figure 1. Relationship between type I collagen network and cells in tumor microenvironment. On the one hand, cancer cells, cancer-associated fibroblasts and endothelial cells influence collagen structure and composition. On the other hand, type I collagen participates to tumor progression. DDR1: discoidin domain receptor I, FAP: fibroblast activation protein, LOX: lysyl oxidase, MMP: metalloproteinase, PDGFBB: Platelet-derived growth factor BB, TG2: Transglutaminase 2, TGF- β : Transforming growth factor- β ,

Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

CAF: cancer-associated fibroblast, CRC: colorectal cancer, DDR: discoidin domain receptor, ECM: extracellular matrix, FAP: fibroblast activation protein, SHG: second harmonic generation, TME: tumor microenvironment

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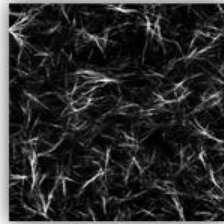
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Collagen network



- DDR1 activation (invasion)
- $\alpha 2\beta 1$ (invasion, metastasis)
- Chemotherapy/targeted therapy resistance

- Support nascent vascular structures
- Pro-angiogenic niches
- Stock of angio-active signals

Collagen degradation Collagen synthesis and deposition Collagen stiffness

MMPs/FAP secretion

LOX, TG2 exosomes

Tumor Cells

- TGF- β signaling
- Exosomes (contractility and invasion)

Endothelial Cells

- TGF- β and PDGFBB signaling
- Angiogenic alignment of collagen fibers
- Secretion of pro-angiogenic factors



CAFs