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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertação de Candidatura ao grau de **Mestre em Oncologia** –
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“The price of success is hard work, dedication to the job at hand, and the determination that whether we win or lose, we have applied the best of ourselves to the task at hand.”

Vince Lombardi

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Abstract

Colorectal cancer (CRC) is one of the most common types of cancer worldwide and the second leading cause of cancer-related deaths. To improve conventional diagnosis and treatment, not only cancer cells but also other elements of the tumor microenvironment (extracellular matrix (ECM), immune cells, epithelial cells, cancer stem cells (CSC), fibroblasts) need to be considered. The ECM, the most abundant element at this microenvironment in CRC, is a dynamic macromolecular structure that provides mechanical support to tissues, serves as storage of several growth factors and has also been recognized as a crucial modulator of cell behaviour. In CRC, the ECM is remodelled and reorganized, presenting biochemical and biomechanical modifications which increase its stiffness and cause an impact on cellular differentiation, invasion and sustaining of the CSC niche. CSC represent a small subpopulation of cells with the ability of self-renewal and clonal tumor initiation. Although it is accepted that the ECM has an important role on the stemness of cancer cells, its role on CRC stem cells remains to be elucidated.

Therefore, the main objective of this work was to evaluate the impact of normal and tumor ECM on CRC cell stem-like properties and to disclose potential mechanisms involved in this process. For that, normal and tumor tissues derived from CRC patients were decellularized and repopulated with CRC cell lines (HCT-15 or HT-29). Cells were then analysed regarding their stemness surface markers, genetic expression of EMT- and stemness-related genes and production of stem-associated factors.

Decellularization and repopulation of the matrices were successful and evaluated using different staining's. Although the tumor matrices produced only slight variations on the expression of EMT and stemness-related genes by the cancer cells, it induced a significant increase in the expression of the currently used markers CD44, CD166 and CD133, evidencing a more stem phenotype. Furthermore, this work showed that cells cultured in tumor ECM present higher levels of TGF-β and lower levels of TNF-α on the conditioned media. The activity of MMP was also evaluated and we demonstrated that cells cultured in tumor ECM have a higher MMP activity compared to cells cultured in normal ECM.

Overall, this work established that tumor ECM, through its mechanical and biochemical properties, is capable of modulating CRC differentiation phenotype towards stemness.

Key words: cancer stem cells; extracellular matrix; colorectal cancer; decellularization

Resumo

O cancro colorretal (CCR) é um dos tipos mais comuns de cancro no mundo e a segunda principal causa de morte relacionada com cancro. Para melhorar o diagnóstico e tratamento convencionais, devem ser considerados não apenas as células cancerígenas, mas também outros elementos do microambiente tumoral (matriz extracelular (ECM), células imunes, células epiteliais, células estaminais do cancro (CSCs), fibroblastos). A ECM, o elemento mais abundante neste microambiente do CCR, é uma estrutura macromolecular dinâmica que fornece suporte mecânico aos tecidos, serve como armazenamento de vários fatores de crescimento e é também reconhecida como um modulador crucial do comportamento celular. No CCR, a ECM encontra-se remodelada e reorganizada, apresentando alterações bioquímicas e biomecânicas que aumentam a sua rigidez e causam impacto na diferenciação celular, invasão e manutenção do nicho das CSCs. As CSCs representam uma pequena subpopulação de células com a capacidade de autorrenovação e iniciação tumoral. Embora se saiba que a ECM tem um papel importante na estaminalidade das células cancerígenas, a sua influência nas CSCs do CCR ainda é desconhecida.

Assim, o principal objetivo deste trabalho foi avaliar o impacto da ECM normal e tumoral nas propriedades estaminais das células de CCR e revelar possíveis mecanismos envolvidos nesse processo. Para isso, tecidos normais e tumorais provenientes de pacientes com CCR foram descelularizados e repopulados com linhas celulares de CCR (HCT-15 ou HT-29). As células foram de seguida analisadas relativamente aos seus marcadores de estaminalidade, expressão genética de genes relacionados com a EMT e pluripotência e produção de fatores associados à estaminalidade.

A descelularização e repopulação das matrizes foram bem-sucedidas e avaliadas usando diferentes colorações. Embora as matrizes tumorais tenham mostrado apenas pequenas variações relativamente à expressão de genes associados à EMT e estaminalidade, estas induziram a expressão dos marcadores CD44, CD166 e CD133, evidenciando um fenótipo mais estaminal. Além disso, este estudo mostrou que as células em cultura na ECM tumoral apresentam maiores níveis de TGF-β e menores níveis de TNF-α no seu meio condicionado. A atividade das MMP também foi avaliada e os resultados mostram que as células em cultura na ECM tumoral têm maior atividade de MMP comparativamente às células cultivadas na ECM normal.

No geral, este trabalho demonstrou que a ECM tumoral, através das suas propriedades mecânicas e químicas, é capaz de modular as células de CRC no sentido de um fenótipo estaminal.

Palavras-chave: cancer stem cells; extracellular matrix; colorectal cancer; decellularization

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List of abbreviations

5-FU - 5-fluorouracil

ALCAM - Activated Leucocyte Cell Adhesion Molecule

APC - Adenomatous Polyposis Coli

ATCC – American Type Culture Collection

bFGF – Basic Fibroblast Growth Factor

BSA – Bovine Serum Albumin

CAF – Cancer-associated Fibroblast

CD133 – Cluster of Differentiation 133

CD166 - Cluster of Differentiation 166

CD44 - Cluster of Differentiation 44

CHSJ – Centro Hospitalar São João

CIMP - CpG island methylator phenotype

CIN - chromosomal instability

CM – Conditioned Media

CMS – Consensus Molecular Subtypes

CRC – Colorectal Cancer

CRCSC – Colorectal Cancer Stem Cell

CSC – Cancer Stem Cell

DDR - Discoidin Domain Receptors

ECM – Extracellular Matrix

EGFR – Epidermal Growth Factor Receptor

ELISA – Enzyme-Linked Immunosorbent Assay

EMT – Epithelial-to-Mesenchymal Transition

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

GAG - Glycosaminoglycans

H&E – Haematoxylin and Eosin

HA – Hyaluronan / Hyaluronic Acid

HBSS – Hank's Balanced Salt Solution

hEGF – Human Epidermal Growth Factor

HNPPC - Hereditary Nonpolyposis Colorectal Cancer

I3S – Institute for Investigation and Innovation in Health

ILK - Integrin Linked Kinase

KRAS - Kirsten rat sarcoma viral oncogene homolog

LOX - Lysyl-Oxidase

MDSC - Myeloid-Derived Suppressor Cells

MMP - Matrix metalloproteinase

MSI - microsatellite instability

NF-kB – Nuclear Factor-kappa B

PBS – Phosphate Buffered Saline

Pen/Strep – Penicillin-Streptomycin

PFA – Paraformaldehyde

PG - Proteoglycans

Rpm – Rotations per minute

RT – Room Temperature

SDS – Sodium Dodecyl Sulphate

TGF-β – Transforming Growth Factor β

TME - Tumor Microenvironment

TNF-α – Tumor necrosis factor α

TP53 - tumor protein p53

Chapter I - Introduction

1.1 Cancer: a heterogeneous disease

Cancer continues to be a major public health problem and it is currently the second leading cause of death in the world, following cardiovascular diseases (1, 2). The majority of cancers are considered spontaneous, which means there is no specific genetic predisposition associated with it. However, about 5-10% of the cases may be caused by a genetic predisposition. The most common genetic alterations occur in tumor suppressor genes and oncogenes (3). Environmental factors are responsible for 95% of the cancer cases (4) and include the consumption of alcohol, tobacco, obesity, infections, among others (2).

Normal cells can acquire certain biological abilities that give them the potential to become neoplastic, and eventually, malignant. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. In 2011, the same authors proposed two additional emerging hallmarks: the capacity of the cells to deregulate cellular energetics, which allows a most effective support of neoplastic proliferation, and avoiding the immune destruction, particularly by T and B lymphocytes, macrophages and natural killer cells. Adding to the two emerging hallmarks, the authors also suggested two enabling characteristics that facilitate the acquisition of the previously mentioned hallmarks: genomic instability and tumor-promoting inflammation (5) (Figure 1).

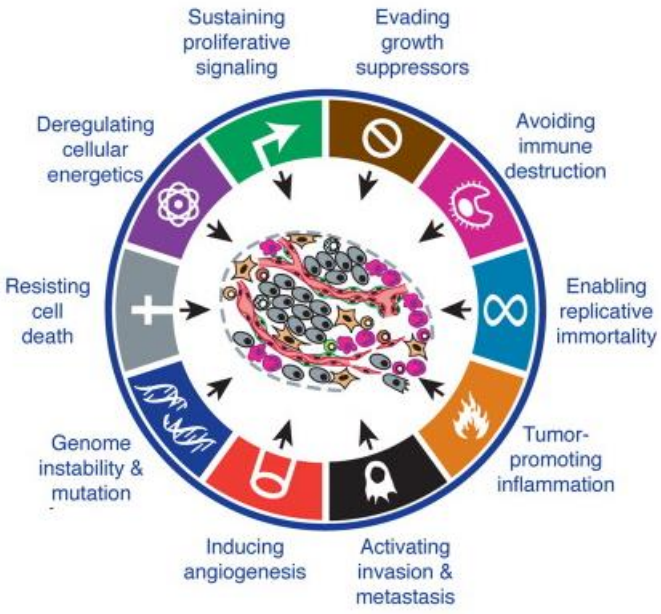


Figure 1. The hallmarks of cancer | It is accepted that cancer cells need to acquire biologic abilities in order to grow, proliferate and become neoplastic. These are entitled the hallmarks of cancer (Hanahan and Weinberg, 2011).

1.2 Colorectal cancer

1.2.1 Epidemiology

Colorectal cancer (CRC) remains a serious health problem since it is the third leading type of cancer in man and the second most commonly occurring in women, affecting approximately one million individuals per year (6). Currently, CRC is the second leading cause of cancer-related mortality worldwide. In 2018, CRC was responsible for 880792 deaths worldwide, in both sexes (GLOBOCAN, 2018).

The incidence rates of CRC vary tremendously among different parts of the globe. The highest incidence rates belong to the developed countries in Europe, North America and Asia and the lowest in Oceania, Latin America and Africa (Figure 2) (GLOBOCAN 2018).

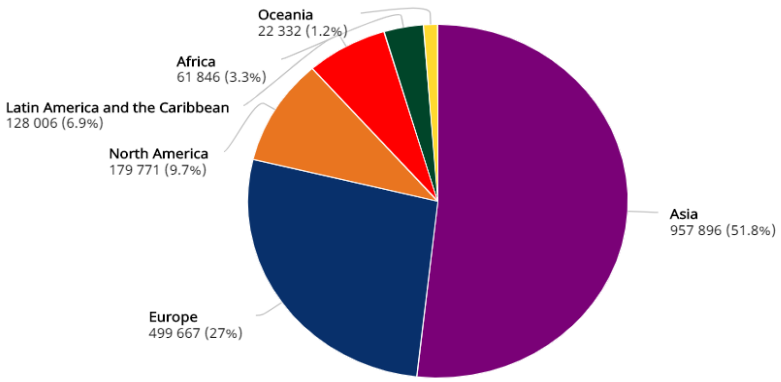


Figure 2. Estimated number of new cases in 2018, colorectum, both sexes, all ages | Percentage of cases relative to the number of total cases in the world. Data source: GLOBOCAN 2018. Graph production: Global Cancer Observatory.

1.2.2 Causes of CRC

As all solid cancers, CRC is a highly heterogeneous disease that results from the interaction between genetic and environmental factors. Most CRC cases are sporadic (70%-80%) and only a small proportion of cases have inherited causes, as familial adenomatous polyposis (FAP), non-polyposis hereditary CRC (HNPPC) (Lynch Syndrome) or MYH-gene associated polyposis. An additional 20%-25% of cases are thought to have an association with hereditary factors not well established yet and these cases are known as familial CRC(7).

Among the environmental risk factors, age is the most relevant. Others are the excessive intake of red meat and saturated fat, alcohol consumption, a diet low in fruit and vegetables, a sedentary lifestyle, overweight, and tobacco (7, 8).

1.2.3 Carcinogenesis of CRC

Ever since the association between genetic alterations and the colon carcinogenesis was proved, there have been countless studies and investigations leading to the understanding of the role of genomic and epigenomic alterations on CRC origin (9). CRC follows the "adenoma-carcinoma" sequence proposed by Fearon *et al* in 1990. It derives from a sequential gaining of genetic and epigenetic modifications that lead to the initiation and progression from an adenoma to a carcinoma (10). The formation of this type of cancer

starts generally with a mutation on colorectal epithelial cells that leads to the inactivation of the adenomatous polyposis coli (APC) gene (11). The acquisition of subsequent mutations that activate constitutively the Kirsten rat sarcoma viral oncogene homolog (KRAS) and the v-Raf murine sarcoma viral oncogene homolog B (BRAF) pathways allows the formation of an intermediate adenoma. These cells start to accumulate mutations in genes that regulate other pathways, ultimately leading to the formation of invasive tumours (Figure 3) (11-13).

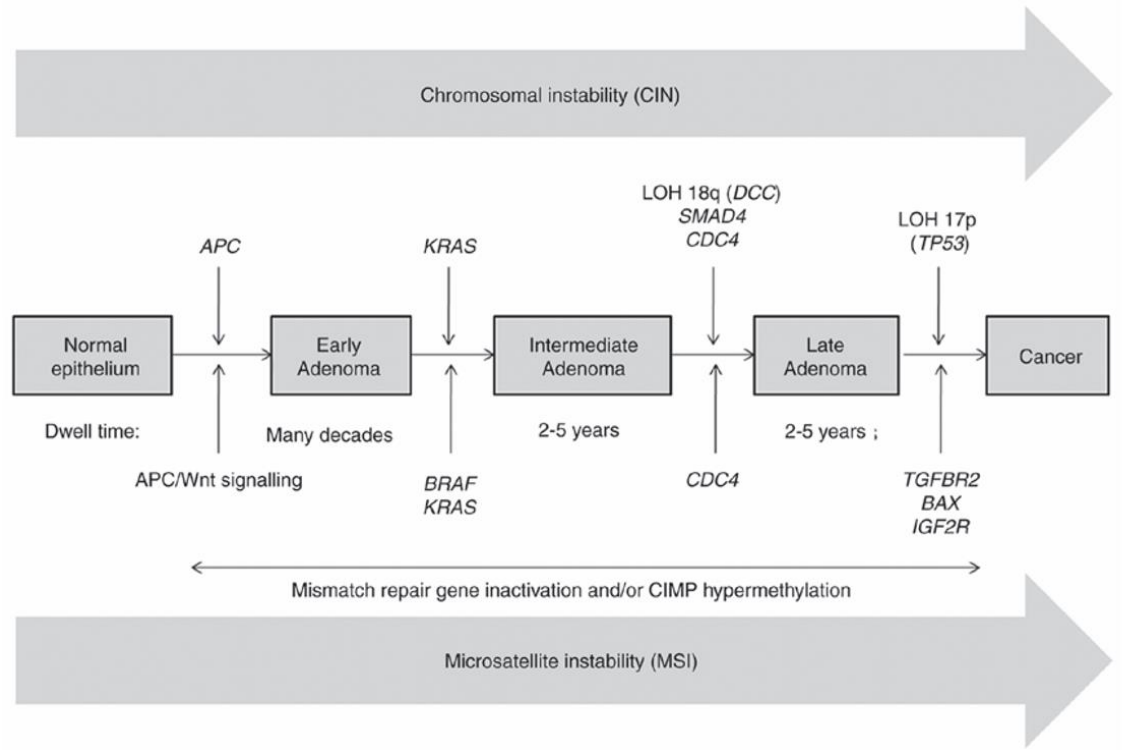


Figure 3. The colorectal adenoma-carcinoma sequence | Colorectal cancer derives from a sequence of genomic and epigenomic alterations that are caused by three major pathways: CIN pathway, MSI pathway and CIMP pathway. Image extracted from: Nguyen *et al.*, 2017.

Currently, there are three major pathways associated with CRC carcinogenesis: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) (14).

The CIN pathway is the most common genomic instability found in CRC (65%-70% of sporadic CRCs). It results of a high rate of gain or loss of a part or of the whole chromosome that leads to a karyotypic variability between cells. The CIN tumours are characterized by the accumulation of mutations on oncogenes such as KRAS and BRAF and also on tumor suppressor genes as the APC gene or the tumor protein p53 (TP53) gene (15, 16).

The MSI pathway consists of repetitive DNA sequences (usually between one to five base pairs) that are called microsatellites. This pathway occurs in 15%-20% of sporadic CRC

cases and in >95% of HNPPC cases (14). In this pathway, there is a progressive insertion or deletion of nucleotides within the microsatellite region that leads to a longer or shorter allele compared to normal cells of the same individual (14, 17).

The CIMP pathway occurs when DNA hypermethylation of the promoter of the CpG islands of tumor suppressor genes leads to a transcription inactivation, resulting in gene silencing (18, 19). It was reported by Ogino *et al* that the clinical features of CIMP colorectal tumours were similar to those of MSI-associated CRC cases (20).

1.2.4 CRC stages

The histopathological classification of CRC is performed based on three important factors. The disease is classified based on the local invasion depth and size of the tumor (T stage), the involvement or not of lymph nodes (N stage) and the presence/absence of distant metastases (M stage)(21) (table 1). Depending on the TNM staging, each tumor can be divided in stage 0, 1, 2, 3 or 4 (Figure 4). The final CRC stage is then determined taking into consideration the TNM grading and can vary from Stage 0 to Stage IVB.

Table 1. Colorectal cancer stages. | Colorectal cancer can be divided into 5 stages: 0, I, II (IIA, IIB, IIC), III (IIIA, IIIB, IIIC) and IV (IVA, IVB). They differentiate on the depth of the tumor and on the infiltration of the tumor into lymph nodes, nearby organs and distant organs.

	T	N	M
Stage 0	Tis	N0	
Stage I	T1, T2	N0	
Stage II	T3, T4	N0	
Stage IIA	T3	N0	
Stage IIB	T4a	N0	
Stage IIC	T4b	N0	
Stage III	Any T	N1-2	
Stage IIIA	T1, T2 T1	N1 N2a	
Stage IIIB	T3, T4a T2-T3 T1-T2	N1 N2a N2b	
Stage IIIC	T4a T3-T4a T4b	N2a N2b N1-2	
Stage IV	Any T	Any N	M1
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

The choice of treatment for each patient is partially based on the stage of the cancer, considering the TNM staging and also the individual medical history. However, the response to the treatment varies between patients (21).

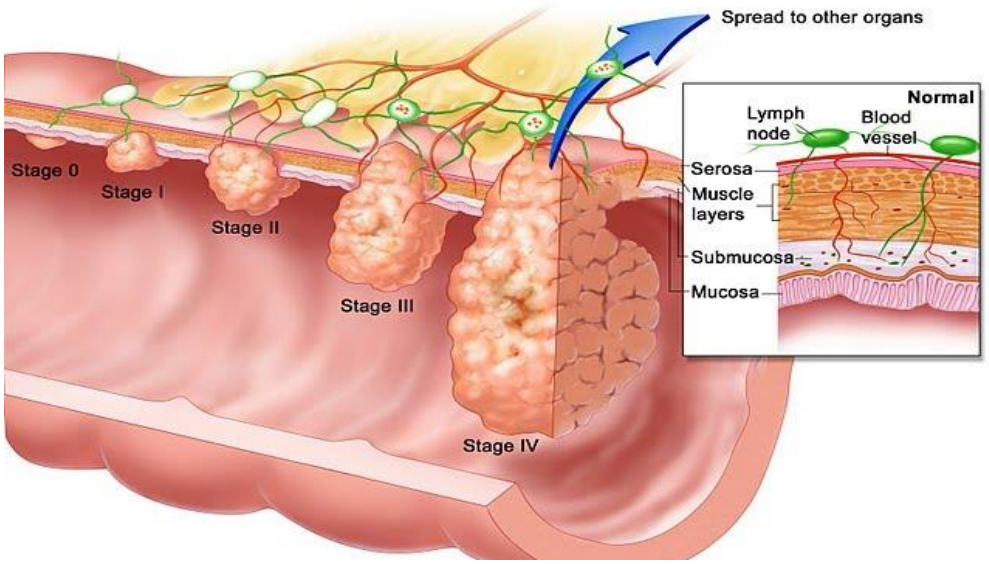


Figure 4. The colorectal cancer stages | Colorectal cancer can be classified in five stages. Stage 0 comprehends the tumours that are confined to the mucosa. Stage I colorectal cancer exists when the tumor invades the submucosa and/or the muscularis propria. When the tumor invades the subserosa and/or the adjacent organs, the colorectal cancer is stage II. Stage III colorectal cancer comprehends the tumours that are metastasized to the lymph nodes. Lastly, stage IV colorectal cancer implies the existence of metastasis in distant sites of the body. Image extracted from: Terese Winslow, 2005.

1.2.5 Therapeutics in CRC

The treatment decisions for CRC are made according to several factors: the existence of other medical conditions, the patient´s overall health, the potential side effects of the treatment plan, among others. Importantly, the decision is based on the tumor-specific molecular features, staging and location of the tumor. Surgery is the most common treatment for CRC, but it is unlikely to cure cancers that have already spread to distant organs. In fact, approximately 25% of the cases of CRC present metastases at initial diagnosis (22). In these cases, chemotherapy is the main treatment, being 5-fluorouracil (5-FU) and oxaliplatin the most commonly used (7). Although the treatments in CRC are improving, *Mejri et al*. have shown that approximately 38% of the patients relapse after primary therapy (23).

1.3 The tumor microenvironment

For a long time, it was assumed that the tumor was a relatively homogenous mass of cancer cells. Nowadays, it is known that tumours are not only composed of cancer cells, but also of multiple cell types that can contribute to its growth. This combination of these cellular and non-cellular components is called the tumor microenvironment (TME) (5, 24) (Figure 5).

Neoplastic cells are capable of transmitting certain signals to the TME, making it pro-tumorigenic. The functional interactions between cancer cells and the tumor microenvironment revealed that this component has an important role on proliferation, invasion, survival and motility of cells. Nowadays, the TME is already seen as a crucial driver of the malignant process and cancer progression (5, 25, 26).

The composition of the TME can vary among tumours. In CRC, the TME is composed of epithelial derived cells (normal cells and pre-cancerous cells), transformed cells (cancer cells and cancer stem cells), myeloid cells (dendritic cells, macrophages, myeloid-derived suppressor cells (MDSC), mast cells, neutrophils, eosinophils and basophils), lymphoid cells, fibroblasts, endothelial cells and pericytes. In addition, the non-cellular component of the TME, called the extracellular matrix (ECM) (24, 27), is probably the most abundant and less studied element of the TME. Additionally, the TME has in its composition several factors released by the cells or entrapped in the ECM as it is the case of growth factors, hormones, cytokines/chemokines and matrix metalloproteinases (MMPs).

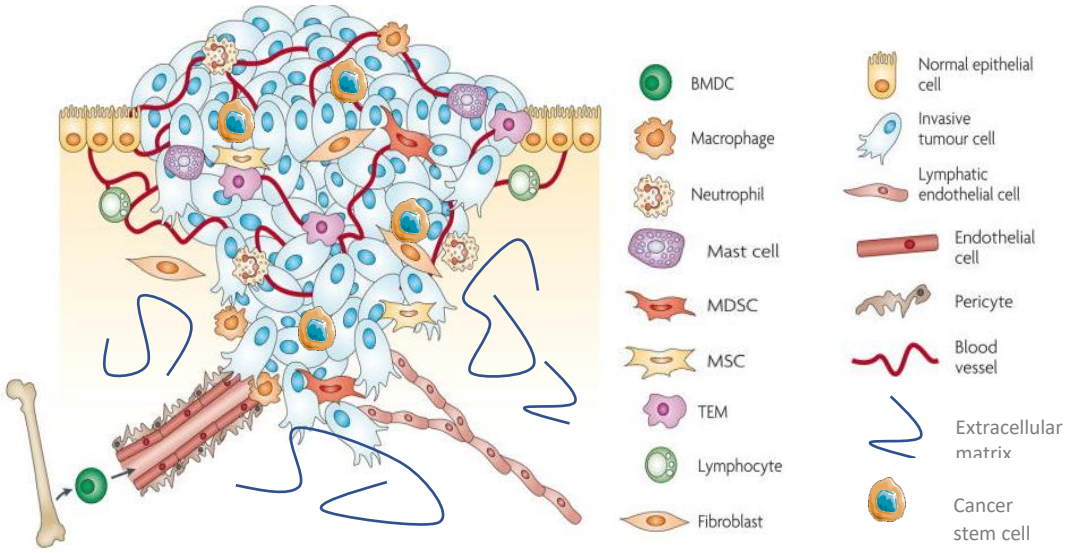


Figure 5. The tumor microenvironment | The tumor microenvironment is composed of several cellular and non-cellular components. Among the cellular components, there are immune cells (macrophages, lymphocytes, neutrophils), endothelial cells, fibroblasts, blood vessels, and the cancer cells themselves. The non-cellular component of the tumor microenvironment is the extracellular matrix (ECM), which confers mechanical stability to tissues in normal conditions. Image adapted from Joyce, J. A. *et al.,* 2009.

Along with all the cell types present in the TME, macrophages play an important role on tumor progression. These cells have plasticity, which allows them to intercalate their phenotype between anti-inflammatory (M2 phenotype) or pro-inflammatory (M1 phenotype), depending on the signals present in the surrounding microenvironment. If a macrophage polarizes into an M2 state, it promotes an anti-inflammatory response, which can lead to tumor progression, angiogenesis and metastases (28, 29). Along with these immune cells, the MMPs also play an important role on tumor invasiveness and metastases. In cancer, these proteases have an enhanced proteolytic activity which promotes the ECM extensive remodelling, affecting cancer aggressiveness and metastatic spreading (30).

The role of the TME in CRC progression has been subject of more intense study, resulting in a new microenvironment-based CRC classification. Accordingly, CRC was divided in four consensus molecular subtypes (CMSs) based not only on the tumor cells and their genetic/epigenetic alterations, but also on the TME (24, 31, 32) (Figure 6).

The CMS1 is mainly composed of MSI tumours, having an overexpression of DNA damage repair genes/proteins and a defective DNA mismatch repair. Also, it is characterized by a hypermethylated DNA and a considerable percentage of cases have BRAF mutations. This subtype is also characterized by an immune infiltrate. The CMS1 can also be named CMS1 MSI immune (24, 31). Regarding CMS2, these tumours have a strong CIN phenotype and present frequent copy number gains in oncogenes and copy number losses in tumor suppressor genes. Their Wnt and MYC pathways are typically activated, they have an overexpression of the epidermal growth factor receptor (EGFR) and a mutant TP53. The CMS2 can also be called CMS2 canonical (24, 33). The CMS3 has an enrichment of multiple metabolic signatures and a presence of KRAS activation, which correlates with a prominent metabolic adaptation. For this reason, CMS3 can also be named CMS3 metabolic. Lastly, CMS4 is characterized by an upregulation of genes and proteins implicated in the epithelial to mesenchymal transition (EMT), TGFβ signaling, angiogenesis and complement-dependent inflammatory system. CMS4 tumours also present a higher immune infiltration and ECM remodelling. These tumours present a more mesenchymal phenotype and consequently can also be named CMS4 mesenchymal (24, 31, 33).

CMS1 MSI Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGFβ activation, angiogenesis

Figure 6. The consensus molecular subtypes of CRC | As the TME is now seen as a crucial intervenient in tumor progression, a new microenvironment-based classification of CRC tumours has been established. Image extracted from: Guinney *et al.*, 2015.

Cells present within the TME can interact with each other through autocrine/paracrine mechanisms or through gap junction channels (24). Besides these two models of interaction, cells can also communicate through extracellular vesicles, either exosomes or tumor-derived microvesicles (34, 35). Lastly, the communication between the cells may occur through the ECM, which is a major component of the CRC TME. The ECM not only supports the architecture of tissues with roles on rigidity, porosity, spatial arrangement and orientation of cells, but it is also highly dynamic and involved directly and indirectly in cell signalling mechanisms . As such, the ECM is thought to be a crucial player in the progression of cancer, namely CRC (24) .

1.4 The extracellular matrix

1.4.1 The ECM in physiological conditions

The ECM is a complex macromolecular three-dimensional network of proteins that has a highly dynamic structure and is present in all tissues and organs (30, 36). This non-cellular component of the TME is composed of approximately 300 proteins, known as the core matrisome (37). There are two main forms of ECM that differ in location and also in composition: the interstitial connective tissue matrix, which surrounds cells and provides structural support for tissues; and the pericellular matrix (as the basement membranes), which is a specialized form of ECM that separates the epithelium from the surrounding stroma (30, 36).

The ECM is composed of several types of structural proteins, with collagen as the major constituent(36). Other proteins present are proteoglycans such as versican, perlecan and decorin (37); glycoproteins such as elastin, fibronectin and laminins; and glycosaminoglycans (GAGs) such as hyaluronan (HA), heparan sulphate and chondroitin sulphate (30, 36).

Along with these proteins, there are also other ECM-associated proteins that are not a structural part of the ECM but are important to its regulation and cellular interaction. These can be growth factors, cytokines and ECM-modifying enzymes, such as MMPs or the enzyme lysyl-oxidase (LOX) (30). MMPs are able to degrade the components of the ECM, contributing to its remodelling and having a crucial impact on physiological (organogenesis, for example) and pathological conditions (30).

In addition to providing physical scaffolds into which cells are embedded, the ECM is responsible for providing structural support to tissues and organs and allowing tissue integrity and elasticity (30, 36, 38). Additionally, the ECM has a role on cell adhesion, signalling, differentiation, proliferation, survival, polarization and migration (Figure 7). This component does so by providing signals to cells that can regulate their behaviour and trigger biological activities essential for normal development (36). Furthermore, the ECM functions as a reservoir for growth factors or signalling molecules (39). Along with the biochemical, the biomechanical properties of the ECM (stiffness and deformability) can provide inputs into cell behaviour (30, 38, 40).

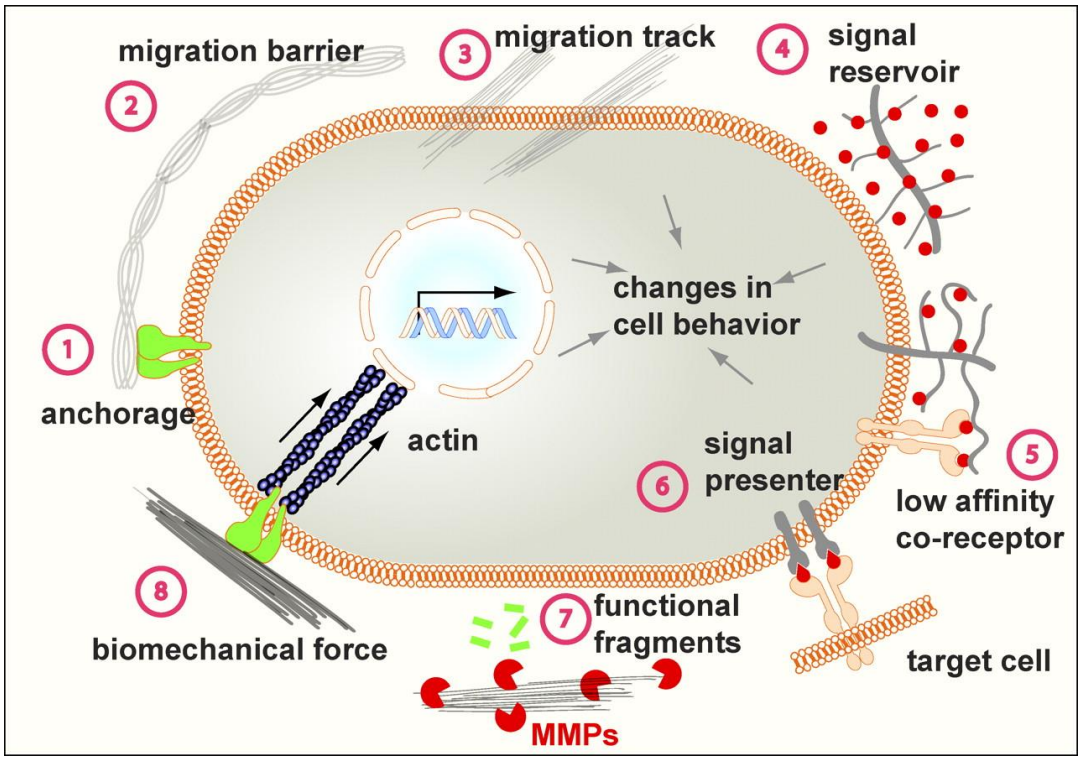


Figure 7. Functions of the ECM. | The ECM has several functions that can dictate the behaviour of its surrounding cells: it serves as an anchor to cells, can function as a migration barrier and a migration track to cells, serves as a signal reservoir, it can be a low affinity co-receptor and a signal presenter. Additionally, it has ECM-related proteins that modulate its behaviour (as the MMPs) and also influences the surrounding cells through its mechanical properties. Image extracted from Lu *et al.,* 2012.

1.4.2 Interactions between the ECM and cells

The interaction between the ECM components and the surrounding cells is established through the binding of ECM molecules to cell surface receptors. This binding activates intracellular signaling pathways, which influences cells' behaviour (41).

Several receptors allow cellular communication with the ECM: integrins, discoidin domain receptors (DDRs), the hyaluronan receptor CD44, syndecans and the 67kDa laminin receptor (30, 36, 41). Integrins are receptors that can establish the link between the actin cytoskeleton and ECM proteins (collagen, fibronectin and laminin), transmitting signals that can regulate cell adhesion, migration, proliferation, apoptosis, survival and differentiation (30, 42). The DDRs are proteins that bind to collagen and are involved in organogenesis and on ECM remodelling. CD44 is a cell surface receptor that mainly binds to HA but can also bind to other ligands such as collagen or MMPs (36). The syndecans are proteins that bind to the fibronectin present in the ECM and the 67kDa laminin receptor binds to laminin (41).

Along with the interactions through cell surface receptors, the communication between the cells and the ECM can be established through the release of factors between these two components of the TME (41). For example, TGF-β can be secreted by stromal cells of the TME in a latent complex, which can be sequestered by the ECM. This factor can then be released by the degradation of the ECM in its active state, and playing then a role on cancer cells behaviour modulation (43). The ECM present in the TME can also act as a reservoir for angiogenic factors that are released upon its degradation (41).

1.4.3 The ECM as part of the CRC microenvironment

The ECM can be considered a central part of the tumor in several types of cancer, contributing to its histopathology, behaviour and progression (44). This non-cellular component supports tumor cells and can be responsible for their survival; it contributes to the formation and sustainment of the cancer stem cell niche; it supports primary tumor growth and the formation of a pro-invasive microenvironment, thus contributing to metastization (45-47). The fibrotic ECM can also act as a barrier to drug delivery, which makes it partially responsible for resistance to therapy (48, 49). Additionally, the ECM is capable of promoting EMT, which is a process related with invasion, dissemination and metastases in epithelial carcinomas (39, 50).

Specifically in CRC, the ECM has a different architecture and composition when compared to the normal colon ECM (28, 51). The CRC ECM presents a disrupted organization, with a degraded basement membrane and a disorganized structure. The ECM in this type of cancer is stiffer, mainly due to the accumulation of collagen, which is increased in CRC matrices when compared to normal colon ECM. The collagen in the CRC ECM is thickened and the fibers exhibit a parallel orientation. This change in the orientation of the collagen fibers can function as a migration track for tumor cells during their migration (39, 51) and has been correlated with tumor progression and enhanced cell migration in breast cancer (52). A mediator of collagen crosslinking, LOX, has been found to be up-regulated in several cancers, including CRC (30, 53). Accordingly, tumor ECM stiffening can be caused partially by this enzyme, which binds collagen and elastin fibers (54). Adding to the collagen's role on CRC progression, increased levels of GAGs have also been found in CRC comparing to normal colon tissue. These non-fibrillar ECM component can play an important role on collagen remodelling (51). As well as their role on collagen remodelling, GAGs can interact with growth factors and cytokines present in the TME, regulating cancer progression.

Additionally, these molecules can be involved in the regulation of angiogenesis and in the invasion and metastasis of cancer cells (55).

Although it is accepted that the tumor ECM can modulate the hallmarks of cancer and interact with the cells of the TME (41), there is not much knowledge on its influence on cancer stem cells (CSC) in CRC.

1.5 Cancer Stem Cells

The tumor is a mass composed of differently genetic modified cells and with different biological characteristics. One subpopulation of cells within the tumor mass is the cancer stem cell (CSC) subpopulation.

CSC are a special small subpopulation of cancer cells that have very specific characteristics, such as the ability to self-renew, multilineage differentiation and tumorigenic potential. Self-renewal is the capacity of a cell to undergo asymmetric division, producing one undifferentiated cell with stem characteristics and another differentiated cell. The multilineage differentiation refers to the capability of a single cell to give rise to different tissue cell types. Lastly, the tumorigenic potential means that the cell is capable of invading a tissue and produce a tumor upon transplantation (57, 58). Adding to these characteristics, CSC are relatively quiescent and have a slow cycling rate, which can provide advantage in resistance to therapies that target rapidly cycling cells (59). In fact, these cells are considered to confer resistance to certain therapies currently used, which can also be attributed to an overexpression of efflux pumps, to specific signaling pathways, to the epithelial to mesenchymal transition (EMT) or to an efficient DNA damage repair machinery (60-62).

There are currently three theories that can explain the origin of CSC. Firstly, these cells can originate from the malignant transformation of a normal stem cell. Since these normal stem cells have the ability of self-renewal and proliferation, mutations can accumulate during their long survival, making them more prone to malignant transformation. Secondly, a CSC can be derived from the dedifferentiation of a common cancer cell by, for example, inducing the expression of several factors such as *Sox2* and *c-Myc* (63) . Other theory states that they may originate from the malignant transformation through the influence of the TME (57). In fact, it has been shown that non-cancer stem cells can give rise to CSC by the influence of TGF-β signalling, which means this growth factor can play a role on the stemness of cancer cells (64). The question whether such induction occurs from transformation or from the

selection of a small niche of resident transformed stem cells still persist. In fact, the EMT process has also been implicated in the acquisition of CSC traits, since induction of this process may induce several features of stem cells such as self-renewal ability (57, 65, 66).

The concept of CSC has been extremely important in establishing explanations for the existence of intra-tumor heterogeneity. In fact, two models have been proposed in order to justify the heterogenous potential of tumor cells (Figure 8). Firstly, the stochastic model, which defends that a population of tumor cells acquires the appropriate set of somatic mutations, developing metastatic skills (67). In this model, all cells in a tumor have an equal potential to drive tumor growth and metastases (68). The second model is called the CSC model and it suggests that tumours are initiated by the rare population of CSC (69, 70). In this model, certain cancer cells retain embryonic characteristics that enable them to give rise to morphologically distinct tumor cell populations (71).

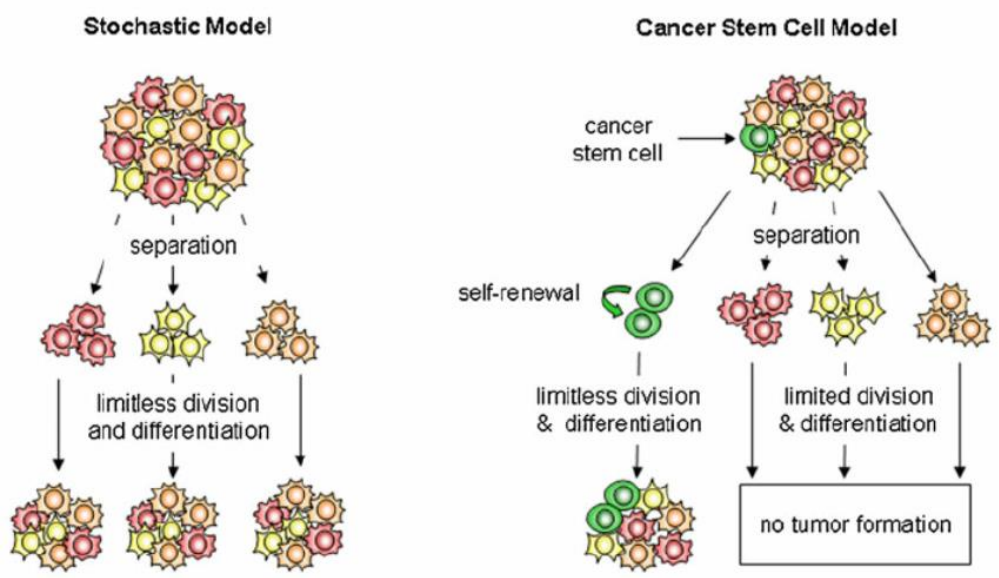


Figure 8. The two models of tumorigenesis. | The heterogeneity of cancer cells can be explained by two currently accepted models. The stochastic model asserts that every cell within a tumor has an equal ability to proliferate and form a new tumor when transplanted. The CSC model states that only a small population of cancer cells, the CSC, has the capability to form new tumours when transplanted due to its self-renewal capacity, multilineage differentiation and tumorigenic potential. By their asymmetric division, CSC can give rise to different clones, originating cells with different tumorigenic and immune escape capacities. Also, as the tumor grows and therapies are administrated, CSC can have an advantage over the non-CSC by their resistance to therapies. Image extracted from E Van Vlerken *et al.,* 2012.

Although the presence of CSC has already been established in some cancers, as leukaemia (71) and breast cancer (72), the existence of CSC in other types of tumours is still being

confirmed. However, these cells have already been isolated in colon, brain, head and neck, melanoma, hepatic, pancreatic, lung, prostate and ovarian tumours (73-81).

CSC need a specific niche in order to maintain their stemness (Figure 9). The CSC' niche is composed of all surrounding cells (stromal, immune cells and its vasculature) and also of secreted factors that contribute to the maintenance of a microenvironment suitable for the CSC features (82, 83). There is evidence of the role of several cell types on the CSC niche in different types of cancer. Notably, cancer-associated fibroblasts (CAFs) have been proven to promote CSC self-renewal and also *in vivo* tumor growth (84).

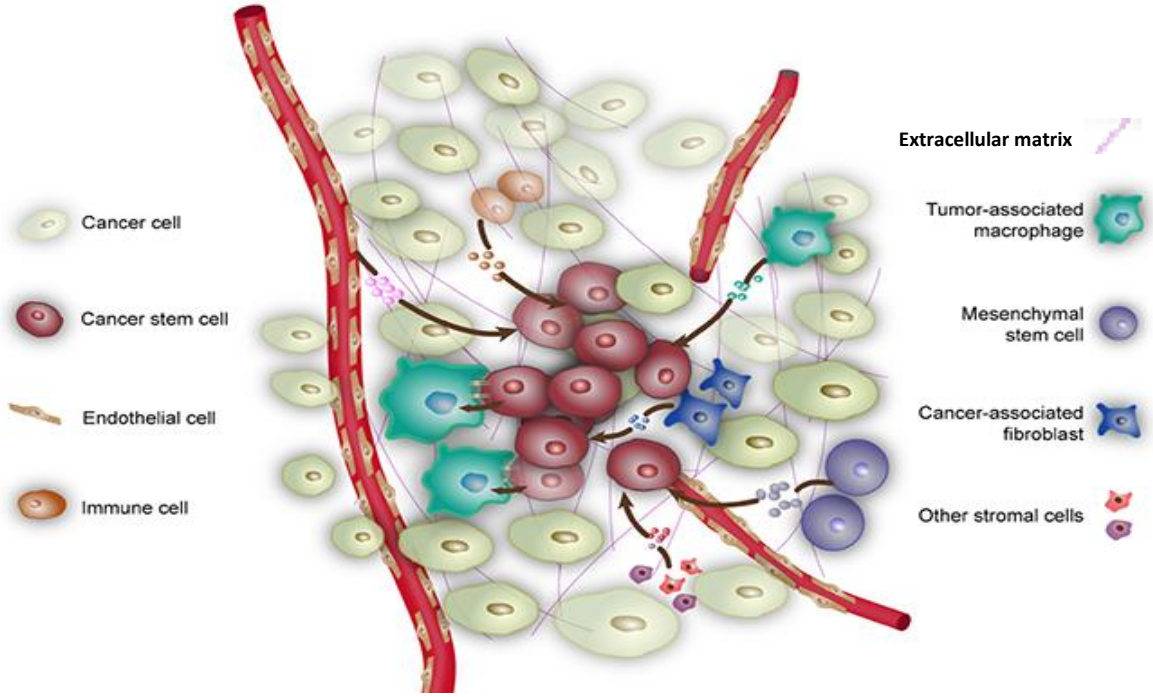


Figure 9. The cancer stem cells niche. | Cancer cell stemness is maintained due to the niche surrounding these cells. Their properties depend not only on their intrinsic characteristic but also on the signals provided by other members of the niche. Several cellular and non-cellular components contribute to the sustainment of the niche such as cancer-associated fibroblasts, mesenchymal stem cells, inflammatory cells, hypoxia, angiogenesis and lastly, the interactions with the extracellular matrix. Image adapted from: Plaks, V. *et al.*, 2015.

Also within the niche of CSC, myofibroblast-secreted factors, as hepatocyte growth factor, is able to activate Wnt signalling and restore CSC phenotype in differentiated tumor cells (85). Hypoxia, which is a common feature of tumours, is involved in the maintenance of self-renewal and undifferentiated state of CSC (86). Endothelial cells are able to promote the stem cells phenotype in CRC through the activation of Notch signalling (87). Mesenchymal cells present in the TME have been proposed to contribute to the CSC phenotype by the secretion of prostaglandin E2 (88). Lastly, immune cells, specifically $CD4^+$ T cells, can influence the CSC self-renewal through the secretion of IL-22 (89).

1.5.1 Cancer stem cells in CRC

As described before, CSC are part of the CRC microenvironment. They form a small population of cells that are part of the heterogenous population of CRC cells. There are several possible origins of colorectal cancer stem cells (CRCSC) documented (Figure 10). Normal intestinal stem cells are located at the bottom of the crypts in the colonic mucosa and are responsible for generating all epithelial cell types along the crypt-villus axis. These cells may be a source of CRCSC because of their inherent stem features, long lifetime and rapid turnover (90). However, it is also believed that CRCSC may derive from dedifferentiation of normal gastrointestinal stem cells (91) .

Another possible source of the CRCSC is the dedifferentiation and subsequent malignant transformation, possibly by the nuclear factor-kappa B (NF-kB) modulation of the Wnt signalling (90). Additionally, CRCSC can derive from bone marrow-derived progenitor cells that go through metaplasia and dysplasia, leading to cancer (92).

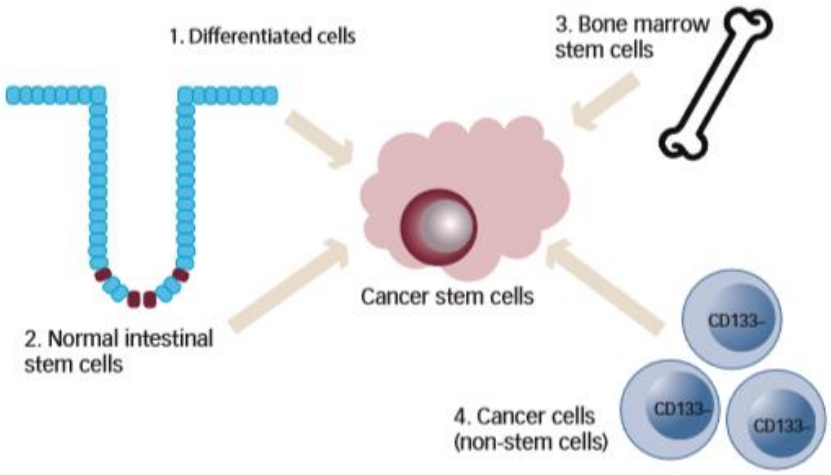


Figure 10: The possible origins of CRCSC | CRCSC are thought to derive from multiple mechanisms. They can originate through the dedifferentiation of normal differentiated intestinal cells (1) or of non-stem cancer cells (4). They can also derive from bone marrow stem cells (3) or from the malignant transformation of normal intestinal stem cells (2). Image extracted from: Hubbard *et al., 2016.*

Although the existence of CSC in CRC is widely accepted, there are no specific markers of CSC in CRC. In fact, one of the main problems regarding CSC is that the number and identity of these cells can vary tremendously among tumours, which increases the difficulty in finding specific markers (56). However, according to the literature, some of the most

commonly used markers for the identification and isolation of cancer stem cells in CRC are the surface markers CD133, CD44 and CD166. There are also several transcription and growth factors that can be associated with stemness in CRC (71).

CD133 is one of the most frequently used proteins for the identification of CSC in CRC. Also known as Prominin-1, CD133 is a five-transmembrane protein located at the membrane protrusions of embryonal epithelial structures (93). Although the function of this protein still remains uncertain, there is a hypothesis that CD133 can have a role on inhibiting cell differentiation (94). In 2007, CD133-positive cells isolated from CRC tumours demonstrated self-renewal capacity and high tumorigenic potential (95). CD133 can also contribute as a prognostic marker for CRC. Several studies demonstrate that high levels of CD133 are associated with shorter overall survival and relapse-free interval (96).

CD44 has also emerged as an important marker for colorectal CSC. It is a transmembrane glycoprotein that functions as a receptor for constituents of the ECM, such as hyaluronic acid. This receptor is capable of regulating cell adhesion, proliferation, growth, differentiation, survival and migration, being an important player of tumorigenesis (97-99).

CD166 is a multidomain transmembrane type 1 glycoprotein of the immunoglobulin superfamily. This molecule can also be called ALCAM (activated leucocyte cell adhesion molecule) (22, 100) and its expression is correlated with aggressive diseases such as melanoma, prostate, breast and ovarian cancers (100-103). A study from Dalerba *et al.* confirmed that, when xenografted in low numbers into immunodeficient mice, human $CD166^{+}$ CRC cells are indeed capable of inducing tumorigenesis (104).

Along with these surface markers, other factors have been implicated in CRC stem cells such as the transcription factor NANOG (105, 106) and the growth factor TGF-β (107). NANOG is a key regulator of pluripotency of embryonic stem cells by the formation of a complex with the transcription factor OCT4. NANOG also induces the expression of *SLUG* and *SNAIL* in CRC, which are EMT genes (108). Also, this transcription factor has been shown to promote CSC phenotype in CRC (109). TGF-β is a growth factor that, in normal conditions, regulates several cellular processes as immune and inflammatory responses. Its activity is frequently deregulated in CRC cases. Furthermore, it has been proven that this factor has an important role on the regulation of CRCSC by the regulation of SNAI1, CD44, CD133 and SOX2 (110).

Regarding other types of cancer, INTEGRIN α6, SNAI2, ZEB1 and OCT4 have also been studied as important mediators of the CSC niche (111-115).

1.6 Cancer Stem Cells and the ECM

The ECM is an essential component of the adult stem cell niche (116). Increasing evidence suggests that the ECM serves as a niche for CSC, as well as for all other cell types in the TME. This non-cellular component can provide both structural and biochemical support to CSC but still regulate proliferation, self-renewal and differentiation of these cells (117). In order to understand how the ECM can regulate these mechanisms of stemness, it can be advantageous to comprehend the role of different ECM components on CSC maintenance (117).

As stated before, collagens are the major constituent of the ECM. Studies have shown that several collagens are overexpressed in CSC from different types of cancer (118-120). Also, collagen I has been shown to preserve stemness by the activation of signalling programs that induce self-renewal (121). These fibrous proteins can also promote EMT and maintain CSC characteristics in lung cancer (120).

Interestingly, ECM glycoproteins, as fibulin-1 seems to promote resistance to certain therapies in cancer cells (122) in contrast to, fibulin-3 which can inhibit self-renewal of lung CSC and also pancreatic CSC (123). Laminins on their turn, seem also to play a role on the maintenance of the CSC niche. Accordingly, Laminin 511 has been described to support of breast CSC self-renewal (124) while Laminin 332 is engaged in maintaining CSC characteristics and EMT phenotype in lung cancer cells (120).

Proteoglycans of the ECM play a crucial role in ECM assembling and cell signaling. These components are highly expressed by CSC. In particular, Syndecan-1 is capable of inducing CSC phenotype and Wnt signaling pathways in breast cancer cells (125) while Glypican-3 promotes self-renewal of hepatocellular CSC (126).

Lastly, polysaccharides, as Hyaluronic acid (HA), which can be produced by tumor cells, binds to the receptor CD44, activating several pathways that promote cell survival and cancer stem features (117). Breast CSC produce high levels of HA and this overproduction promotes stemness characteristics in this type of cancer (127). Also, the HA-CD44 interaction is reported to induce multidrug resistance in breast and ovarian cancer and to stimulate stem cells markers expression and stemness properties in head and neck CSC (128, 129).

As the tumor ECM is mechanically distinct from the normal ECM, its increase in stiffness can also modulate CSC' phenotype. In melanoma CSC, soft matrices seemed to increase *SOX2* expression and promote self-renewal (130). On the contrary, a stiff matrix increased

breast cancer CSC markers (CD44, Nanog and CD49f) expression through integrin linked kinase (ILK) signaling (131).

The ECM seems to have multiple effects on CSC depending on the type of cancer. However, knowledge in this field is still extremely scarce, especially in the case of CRC. Therefore, there is an urgent need to understand if and how the ECM biochemical and biomechanical properties can modulate the CRCSC niche.

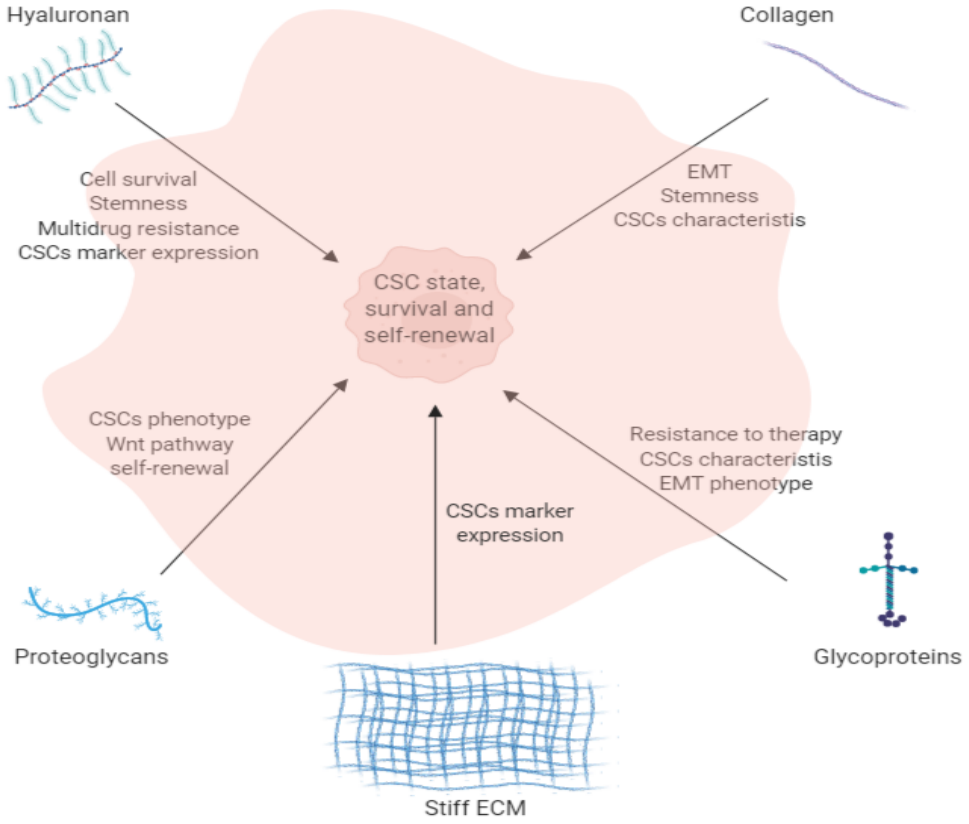


Figure 11: The ECM-CSC interactions | The ECM can play multiple roles on the CSC' niche. This non-cellular component can regulate the CSC not only through biochemical signaling but also through biophysical interactions.

Chapter II - Aims

As previously mentioned, knowledge on how the ECM impacts the CSC niche is still scarce, especially in the case of CRC. It is currently known that this non-cellular component can be an essential constituent of the adult stem cell niche (116). Also, the ECM has a crucial importance on cancer since it is capable of modulating the different cell's behaviour within the TME. Additionally, changes in the ECM composition and architecture may favour tumor cell survival and resistance to certain therapies (132).

Considering this, the **main aim** of this dissertation was to evaluate the impact of the tumor ECM on stem-like properties of CRC cells.

Our group has previously shown that decellularized colorectal tissues, derived from cancer patients surgical resections, are useful 3D-organotypic models to dissect the ECM-cell interactions, retaining bioactivity and allowing the comparison between normal and tumor matrices from the same patient (28). Therefore, considering the advantages of this model, the access to normal and tumor tissue specimens from CRC patients, and the expertise in the research team, this was the selected strategy to accomplish the main aim of this work.

Accordingly, this research plan was divided in two sections:

1. To confirm the efficiency of the decellularization and recellularization.

2. To evaluate different stemness characteristics on CRC cells cultured within normal or tumor decellularized matrices, focusing on the:

- expression of several pluripotency, EMT and stemness related genes;
- expression of cell surface markers related with stemness on CRC cells;
- production of TGF-β and other factors relevant for cancer cell stem-like properties;
- activity of MMP-2 and MMP-9, as important elements in ECM remodelling, cancer cell invasion and bioavailability of growth factors entrapped within the ECM.

Chapter III – Materials and Methods

3.1 Clinical samples and Ethics Statement

Fresh surgical specimens from colorectal cancer patients were collected from the Pathology Department at Centro Hospitalar Universitário São João (CHUSJ). Matched specimens from tumor and normal adjacent mucosa were transported in cold Hank's Balanced Salt Solution Medium (HBSS) (Gibco, Invitrogen) to the Institute for Investigation and Innovation in Health (i3S), where they were cut in smaller fragments to be cryopreserved. Immediately, tissue fragments were covered in mounting medium for cryotomy (OCT compound, Thermofisher) and rapidly frozen in 2-methylbutane (VWR chemicals) cooled with liquid nitrogen. After, they were stored at -80ºC until further use. The biological material was obtained according to a protocol established between i3S and CHUSJ, approved by the Ethics Committee (/2011) following strictly the Helsinki declaration. All subjects signed an informed consent prior to the collection of the material.

3.2 Cell lines and culture conditions

In this work, two different colorectal adenocarcinoma cell lines were used. HCT-15 (ATCC® CCL-225™) and HT-29 (ATCC® HTB-38™) cells, derived from human primary colon adenocarcinomas, were purchased from the American Type Culture Collection (ATCC, Massanas, VA, USA).

Both HCT-15 and HT-29 cell lines were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 1% penicillin-streptomycin (Pen/Strep) (Biowest, 100x, cat no. L0022-100) and 10 % fetal bovine serum (FBS) (Biowest, cat. Nº S181BH-500). The cell lines were maintained at 37ºC in a humidified atmosphere of 5% CO_2. The cells were detached with 0.05% trypsin (Sigma-Aldrich, T4799) for 5-10 minutes at 37ºC and its action was neutralized using RPMI-1640 medium containing 1% Pen/Strep (Biowest) and 10% FBS (Biowest). The cells were used for assays when >80% confluence was reached.

3.3 Decellularization

In order to obtain 3D biomimetic scaffolds, normal and tumor fragments derived from colorectal cancer patients surgical resections, were exposed to a specific decellularization protocol, that we have previously optimized to remove all cellular components from the specimens(28). Therefore, normal and tumor frozen colorectal specimens, from the same patient, were washed in Phosphate Buffered Saline (PBS), cut in similar sizes using a 4 mm biopsy disposable punch (Kai medical) and placed in a 24-well plate. The decellularization protocol lasts 3 days and involves chemical, enzymatic and physical processes. The entire protocol was performed under agitation at 165 rpm and at room temperature (RT). The fragments were firstly incubated in Hypotonic Buffer A (10mM Tris; 0,1% EDTA; pH 7.8) for 18h. Then, the fragments were washed three times in PBS (one hour per wash) and incubated with 0,2% Sodium Dodecyl Sulfate (SDS) for 24 hours. After this incubation, the fragments were washed three times with Hypotonic Buffer B (10 mM Tris, pH 7.8) (20 minutes per wash) and incubated for three hours with a DNAse solution (50 U/mL DNASE (ref. A3778, Applichem); 100 mM Tris, 20 mM $MgCl_2$, pH 7.8) at 37ºC. Three final washes with PBS were performed (one hour per wash) to remove any residual detergent or DNAse. The decellularized matrices were kept at 4ºC in PBS complemented with gentamicin (Gibco, ref: 15710-049) (10 µg/mL) and fungizone (Capricorn Scientific, ref: AMP-C) (1%), for a maximum of 7 days, until further use.

3.4 Recellularization

After the biological material was decellularized, the matrices were recellularized with the different colon adenocarcinoma cell lines, HCT-15 and HT-29. Therefore, previously decellularized matrices were washed with PBS twice and incubated with RPMI-1640 supplemented with 1% Pen/Strep (Biowest) and 10 % FBS (Biowest) for at least 30 minutes. Normal and tumor matrices were then transferred to a 96-well plate with a U-bottom. Afterwards, $25x10^4$ HCT-15 or HT-29 cells were resuspended in 10 µL of culture medium (RPMI-1640 supplemented with 1% Pen/Strep and 10 % FBS (Biowest)) and placed on top of each matrix (normal and tumor) and incubated for 18 hours at 37ºC in a humidified atmosphere of 5% CO_2. After the incubation, the matrices were transferred to new wells and 200 µL of culture medium were added to each matrix, which was changed every 2/3 days. The cells were cultured in this 3D model under these conditions for 7 days. After this period, conditioned media

were collected for further analysis and the cells were detached from the matrices using Accutase (GRISP, ref. GTC01). To each matrix, 200 µL of Accutase was added and a further incubation of 30-40 minutes at 37ºC in a humidified atmosphere of 5% CO_2 was performed. At the end of this incubation period, Accutase action was inhibited by the addition of RPMI-1640 with 10% FBS or diluted in PBS. Then, the cells were counted with a haemocytometer and used in the distinct experimental assays.

3.5 Histological analyses – Decellularization assessment

3 µm-thick sections from paraffin-embedded samples (native and decellularized normal and tumor matrices) were processed and stained with Haematoxylin and Eosin (H&E), Masson's Trichrome or Vectashield containing DAPI for nucleic acids fluorescent staining.

3.5.1 H&E staining

Native and decellularized normal and tumor tissues were fixed using formalin with 0.02% eosin. Fixed fragments were then paraffin-embedded and cut in 3 µm-thick sections to perform H&E staining. Briefly, slides were immersed in the following solutions: Clear-rite (Thermo, ref. 6915) for 15 minutes (twice), 100% Ethanol for 5 minutes (twice), 70% Ethanol for 5 minutes, tap water for 5 minutes, Haematoxylin Gill II (Thermo, ref. 6765008) for 2 minutes, running tap water for 6 minutes, 95% Ethanol for 1 minute, Eosin (Thermo Fisher Scientific, ref. 6766008) for 4 minutes, 95% Ethanol for 1 minute, 100% Ethanol for 2 minutes (twice) and Clear-rite (Thermo Fisher Scientific, ref. 6915) for 2 minutes (twice). After staining, the slides were mounted with Thermo Scientific Richard-Allan Scientific Mounting Medium (Fisher Scientific, ref. 12657746). The slides were left to dry overnight in a fume hood and visualized in an Axiovert 200M inverted fluorescent microscope from Zeiss.

3.5.2 Masson's Trichrome staining

Native and decellularized normal and tumor tissues were fixed using formalin with 0.02% eosin. Then, they were paraffin-embedded and cut into 3 µm-thick sections to perform Masson's Trichrome, using a kit (Sigma HT15-1KT) staining. All reagents

used were from the kit except the Bouin's solution and the Mounting medium. The slides were immersed in the following solutions: Clear-rite for 15 minutes (twice), 100% Ethanol for 5 minutes (twice), 70% Ethanol for 5 minutes, tap water for 5 minutes, Bouin's solution (VWR chemicals, ref. 7000.1000) for approximately 2 hours, running tap water until removal of yellow colour, deionized water for 2 minutes, Working iron haematoxylin solution for 10 minutes, running tap water for 10 minutes, deionized water for 2 minutes, Biebrich Scarlet-Acid Fuchsin solution for 5 minutes, deionized water for 2 minutes. The slides were placed in phosphomolybdic-phosphotungstic acid solution for 5-10 minutes and immersed in aniline blue solution for 5 minutes. The slides were washed in distilled water and placed in 1% acetic acid solution for 3-5 minutes. The dehydration of the slides was performed by the immersion of the slides in the following solutions: 95% Ethanol for 3 minutes (twice), 100% Ethanol for 3 minutes (twice) and Clear-rite for 5 minutes (thrice). After the staining, the slides were mounted with Thermo Scientific Richard-Allan Scientific Mounting Medium (Fisher Scientific). The slides were left to dry overnight in a fume hood and visualized in an Axiovert 200M, inverted fluorescent microscope from Zeiss.

3.5.3 DAPI staining

Native and decellularized normal and tumor tissues were fixed using formalin with 0.02% eosin. Then, they were paraffin-embedded and cut on 3 µm-thick sections to perform DAPI staining. The slides were immersed in the following solutions: Clear-rite for 10 minutes (twice), 100% Ethanol for 5 minutes (twice), 70% Ethanol for 5 minutes. The slides were washed with deionized water for 5 minutes (twice) and mounted with Vectashield containing DAPI (Vectashield, Vector Laboratories, CA, USA). The slides were left to dry overnight and visualized with the Axiovert 200M inverted fluorescent microscope from Zeiss.

3.6 Recellularization assessment

The efficiency of recellularization was evaluated by histological analyses and by immunocytochemistry (F-actin/α-tubulin staining).

3 μm-thick sections from paraffin-embedded recellularized matrices were processed and stained with H&E and Masson's Trichrome.

The H&E staining was performed as described in 3.6.1 and the Masson's Trichrome staining was performed as described in 3.6.2.

The efficiency of the recellularization process was also evaluated by immunocytochemistry staining for F-actin/α-tubulin. After the 7-day incubation with HCT-15 or HT-29, the matrices were washed 3 times with PBS and fixed using 4% PFA for 20 minutes at RT. After washing with PBS, the samples were incubated for 10 minutes with 50 mM NH_4Cl to block free aldehyde groups. After three washes with PBS (5 minutes each), the cells within the matrices were permeabilized by incubation with 0,5 % Triton X-100 for exactly 5 minutes. The samples were then washed three times with PBS and incubated with 5% bovine serum albumin (BSA) (Sigma-Aldrich) for 30 minutes to block general proteins and increase the specific antibody/antigen binding. The matrices were then incubated with the primary antibody, a monoclonal anti-α-tubulin (1:4000) antibody (Sigma-Aldrich, T9026), for approximately 1 hour, and washed with PBS. Anti-mouse Alexa-Fluor-488-conjugated (1:1000) secondary antibody (Invitrogen, Molecular Probes) was incubated for one hour. At the end, the samples were washed with PBS three times for 5 minutes and incubated with Alexa-Fluor-568-Phalloidin (1:40) (Invitrogen, Molecular Probes) for 20 minutes at RT for F-actin staining. The samples were washed three times with PBS for 5 minutes, mounted on Vectashield Mounting Medium with DAPI (Vectashield, Vector Laboratories, CA, USA), and visualized with the high-resolution Leica TCS-SP5 AOBS confocal microscope.

3.7 Flow cytometry

To evaluate the presence of different stem cell markers, the cell surface receptors CD133, CD166, CD44, CD24, CD49f and PD-L1 were analysed by fluorescence-activated cell sorting (FACS) (Table 1). HCT-15 and HT-29 cells cultured in normal and tumor matrices were detached from the matrices by incubation with Accutase for 30 minutes at 37º C. They were then harvested and centrifuged at 1500 rpm for 5 minutes at 4º C. Pellets were resuspended in flow cytometry buffer (PBS, 2% FBS, 0,01 % sodium azide) and stained with PE anti-human CD166 (BioLegend), FITC anti-human CD44 (BioLegend), APC anti-human CD133 (BioLegend), FITC anti-human PD-L1 (BD Biosciences), PE-Cy5 anti-human CD49f (BD Biosciences) and PE anti-human CD24 (Miltenyi Biotec). As isotypic controls, we used mouse IgG1 control PE (ImmunoTools), mouse IgG1 control FITC (ImmunoTools) and mouse IgG1 control APC (ImmunoTools). After additional washes with flow cytometry buffer, cells were fixed with 1 % paraformaldehyde (PFA) for 10 minutes at 4º C. After 3 additional washes, samples were filtered, and cells were acquired on a FACSCanto flow cytometer (BD Biosciences) using FACSDiva software. Data analysis was performed with FlowJo software.

Table 2: Antibodies used for evaluation of cells stemness by FACS

Antigen	Format	Clone	Isotype	Stain	Manufacturer	Cat no.
CD44	FITC	BJ18	Mouse IgG1	Surface	BioLegend	338804
CD133	APC	Clone 7	Mouse IgG1	Surface	BioLegend	372806
CD166	PE	3A6	Mouse IgG1	Surface	BioLegend	343903
PD-L1	FITC	MIH1	Mouse BALB/c IgG_1	Surface	BD Biosciences	558065
CD24	PE	32D12	mouse IgG1κ	Surface	Miltenyi Biotec	130-098-861
CD49f	PE-Cy5	GoH3	Rat SD	Surface	BD Biosciences	551129

3.8 Matrigel Invasion assay

In order to access the invasion capacity of the HCT-15 and HT-29 cells cultured in normal and tumor matrices, invasion assays were performed using Corning BioCoat Matrigel Invasion Chambers (cat. n. 354481). The matrigel-coated inserts of 8 µm-pore-size, which mimic the basement membrane, were placed in 24-well plates. The filters were rehydrated with RPMI-1640 supplemented with 1% Pen/Strep (Biowest) and 10 % FBS (Biowest) for 1 hour at 37ºC in a 5% CO_2 humidified atmosphere. After rehydration of the filters, HCT-15 and HT-29 cells were detached from the normal and tumor matrices with Accutase (GRISP, ref. GTC01), as described above, counted and $5x10^4$ cells were placed in the upper compartment of the filters. The wells contained previously added 750 µL under and 500 µL on top of the filters of RPMI-1640 supplemented with 1% Pen/Strep and 10 % FBS (Biowest) per well. Afterwards, the filters were incubated for 24 hours at 37ºC under a 5% CO_2 humidified atmosphere. After 24 hours, the medium was discarded, the inserts were washed in PBS and the cells were fixed with 4% PFA. Non-invasive cells, present on the top of the insert, were removed with a cotton swab. The filters were removed and mounted (with the invading cells upwards) onto a microscope slide using Vectashield Mounting Medium with DAPI (Vector Laboratories). The nuclei of invading cells were counted using the Leica DM2000 fluorescence microscope (Leica Microsystems).

3.9 Enzyme-Linked immunosorbent assay (ELISA)

TNF-α and TGF-β, present in conditioned media from matrices repopulated with HCT-15 or HT-29 cells, were measured by Enzyme-Linked Immunosorbent Assay (ELISA) (BioLegend, LEGEND MAX; TGF-β1 cat: 436707; TNF-α cat: 430207) according to the manufacturer's instructions. Briefly, samples and standards were incubated for two hours in 96-well plates with a capture antibody specific for each cytokine. After washing, a detection antibody, specific for the cytokine in analysis, was added to the wells and incubated for 1 hour with agitation. Then, the wells were washed and incubated with Avidin HRP D solution for 30 minutes with agitation. The wells were washed again and incubated with substrate solution F for 10 minutes in the dark. In order to stop the reaction, Stop Solution was added. The absorbance was measured in a Synergy fluorescence microplate reader (BioTek, Winooski, VT, USA) and correlated with cytokine concentration (pg/mL).

3.10 RNA extraction

In order to evaluate the expression of several pluripotency and epithelial-to-mesenchymal transition (EMT) related genes, total RNA was extracted from the cells previously cultured in normal and tumor matrices. After detachment from the matrices, cells were centrifuged at 1200 rpm for 5 minutes at 4ºC, resuspended in 500 µL of TriPure Isolation Reagent (Roche, ref. 11667165001) in a fume hood and according to the manufacturer´s instructions and transferred into 1,5 mL eppendorf tubes. The cells were then stored at -80º C until further use. For the RNA extraction, the cells were incubated for 5 minutes at RT to ensure the complete dissociation of nucleoprotein complexes. After incubation, 100 µL of chloroform were added to each sample and the tubes were shaken vigorously for approximately 15 seconds. The tubes were then incubated at RT for 15 minutes and centrifuged at 12000g for 15 minutes at 4ºC in order to separate the solution into three phases. After the centrifugation, the colorless upper part of the solution was transferred into new tubes. In order to precipitate the RNA, isopropanol was added (250 µL per tube) and the tubes were gently inverted manually, followed by an incubation at RT for 10 minutes. Afterwards, the tubes were centrifuged at 12000g for 10 minutes at 4ºC and the supernatant was discarded. Ethanol was added (500 µL per tube) and the RNA was washed by vortexing, and then centrifuged at 7500g for 5 minutes at 4ºC. The supernatant was discarded and the excess ethanol from the RNA pellet was removed by air drying for at least one hour. Subsequently, each pellet was resuspended in 20 µL of RNAse-free water and incubated for one hour at 4ºC. RNA concentration and purity were analysed using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). The RNA samples were stored at -80ºC until further use.

3.11 Complementary DNA (cDNA) synthesis

The synthesis of cDNA was performed using 1 µg of RNA. Two independent mixes were prepared. The first mix (annealing mix) was prepared with 1 µg of the previously extracted RNA, 1 µL of random primers and 9 µL of RNAse/DNase free water. The mix was then incubated for 10 minutes at 70 ºC using a thermocycler (BIOMETRA T1 Thermoblock Thermal Cycler). The second mix was composed of 4 µL of 5x first-strand buffer (Invitrogen), 1 µL of 10 mM dNTPs Mix (Invitrogen) 2 µL of 0,1 mM DTT (Invitrogen), 0,2 µL of NZY Ribonuclease Inhibitor (nzytech), 0,5 µL

of Reverse Transcriptase (nzytech) and 1 µL of RNAse/DNase free water (Invitrogen). After the incubation of the first mix, 8 µL of the second mix was added to the annealing mix and incubated for 1 hour at 37ºC using the previously mentioned thermocycler. The obtained cDNA was stored at -20º until further use.

3.12 Quantitative Real-Time PCR

After the cDNA synthesis, qRT-PCR was carried out using a TaqMan Universal PCR Master Mix (KAPA PROBE FAST qPCR Master Mix, lKAPA Biosystems) and probes for *NANOG, SNAI1, SNAI2, ITGA6A, ITGA6B, VIMENTIN, FIBRONECTIN, SOX2, OCT4*, *ZEB1, TGF-B* and *GAPDH* (used as housekeeping gene) (table 2). The qRT-PCR was performed in a 7500 Real Time PCR system (Applied Biosystems). The program was 1 step of 95 ºC for 20 seconds followed by 40 cycles of 95ºC for 3 seconds and 60 ºC for 30 seconds. Each reaction was executed in triplicate and relative mRNA expression of the target genes was normalized to the levels of the housekeeping gene (*GAPDH)* using the comparative ΔΔCT method.

Table 3: Genes measured using TaqMan Universal PCR Master Mix and corresponding assay reference. All probes used were supplied by Integrated DNA Technologies (IDT)

GENE	Assay Reference
NANOG	Hs.PT.58.21480849
SNAI1	Hs.PT.58.2984401
SNAI2	Hs.PT.58.1772559
ITGA6 A	Hs.PT.58.71647180
ITGA6 B	Hs.PT.58.71647184
VIMENTIN	Hs.PT.58.38906895
FIBRONECTIN	Hs.PT.58.40005963
SOX2	Hs.PT.58.237897.g
OCT4 (POU5F1)	Hs.PT.58.14648152.g
ZEB1	Hs.PT.58.39178574
TGF-B1	Hs.PT.58.39813975
GAPDH	Hs.PT.39a.22214836

3.13 Gelatin Zymography

Conditioned media from normal and tumour matrices repopulated with HCT-15 and HT-29 were analysed by gelatin zymography. The medium was collected and centrifuged at 1300 rpm for 3 minutes at 4ºC to eliminate possible cellular debris and the supernatant was transferred to a new eppendorf. Protein content was determined using the DC protein assay kit (BioRad), and 15 µg of protein were mixed with a non-reducing sample buffer (10% SDS, 4% sucrose, and 0,03% bromophenol blue in 0.5 M Tris-HCl pH 6.8) and PBS. Separating (10% polyacrylamide, 0.1% gelatin (Bovine skin type B, Sigma G9391) and stacking (2,5% polyacrylamide) gels were prepared. The samples were loaded and separated for approximately 4 hours at 80V. After electrophoresis, gels were washed twice with 2% Triton X-100 for protein renaturation. The gels were incubated for 16 hours at 37 ºC in MMP substrate buffer (50 mM Tris-HCl; pH 7.5, 10 mM $CaCl_2$ and 0,5 % NaN_3). After the incubation, gels were stained with Coomassie Blue solution (Sigma Aldrich) for approximately 30 minutes followed by a wash with deionized water. White bands of proteolytic degradation appeared on a blue background. Finally, the gels were scanned and the MMPs molecular weight and activity was estimated by densitometric analysis (QuantityOne, BioRad).

3.14 Tumorsphere forming assay

One of the gold standard methods to identify cancer stem cells is the *in vitro* formation of spheres. In this method, cells are grown in non-adherent, serum-free conditions. In these conditions, only cancer stem/progenitor cells can survive and proliferate. To perform this assay, 6-well plates are coated with a hydrophobic polymer. Poly-HEMA solution was prepared by dissolving 12g of Poly(2-hydroxyethyl methacrylate) (Sigma, ref. P3932) into 1 L of 95% ethanol (Aga, ref. 4.000.16.00.00) in a 37ºC incubator. After the solution was prepared, 2 mL of Poly-HEMA were added to each well of the 6-well plate, in a laminar air flow cabinet. The 6-well plates were incubated overnight at 54 ºC in a non-CO_2 incubator for the complete evaporation of the solvent. For the preparation of the tumorspheres' medium the following supplements were added to 500 mL of phenol red-free RPMI (Gibco, Invitrogen, ref. 11835-030): 10 mL of B27 supplement (Gibco, Invitrogen, ref. 12587-010), 5 mL of N2 supplement (Gibco, Invitrogen, ref. 17502-048), 50 µL of bFGF (Gibco, Invitrogen, ref. 13256-029), 100 µL of hEGF (Sigma, E4269) and 5

mL of Pen/Strep (Biowest). The prepared medium was filtered through a 0,45 µm filter. HCT-15 and HT-29 cells cultured in normal and tumor matrices were detached from the matrices using Accutase (GRISP) and centrifuged at 1200 rpm for 5 minutes at 4 ºC. The supernatant was discarded, and the pellet was resuspended in 2 mL of cold sterile PBS. The solution was passed through a 25-gauge needle using a syringe 3 times, to separate the cells into a single cell suspension. The cells were counted using a haemocytometer. The tumorspheres' medium previously prepared was added to each well (2 mL per well) as well as 5000 cells/well. Cell were incubated in a humidified atmosphere at 37ºC and 5% CO_2 for approximately 10 days without replenishing the media. Tumorspheres were observed in an Axiovert ZOOM microscope from Zeiss.

3.15 Statistical analysis

All statistical analyses and graphs presented in this work were performed using the GraphPad Prism Software 6.0 (GraphPad-trial version). Data were analysed for Gaussian distribution using D'Agostino-Pearson or Shapiro-Wilk normality tests. For non-parametric samples, Mann-Whitney tests were used. For parametric samples, *t*-tests were used (either paired *t*-test or unpaired *t*-test). The experiments were treated as means ± SD. Statistical significance was achieved when $p\ value < 0.05$.

Chapter IV - Results

4.1 The decellularization method efficiently removes cells from normal and tumor CRC tissues

In order to obtain normal and tumor extracellular matrices as tissue scaffolds for cancer cell colonization, we performed a decellularization protocol recently optimized by our group (28). Previously, quantification methods confirmed efficient DNA removal upon use of the same decellularization protocol, in similar colorectal cancer resections (28). In this work, the methods selected to evaluate decellularization efficiency were H&E, Masson's Trichrome (Figure 12) and DAPI staining (Figure 13).

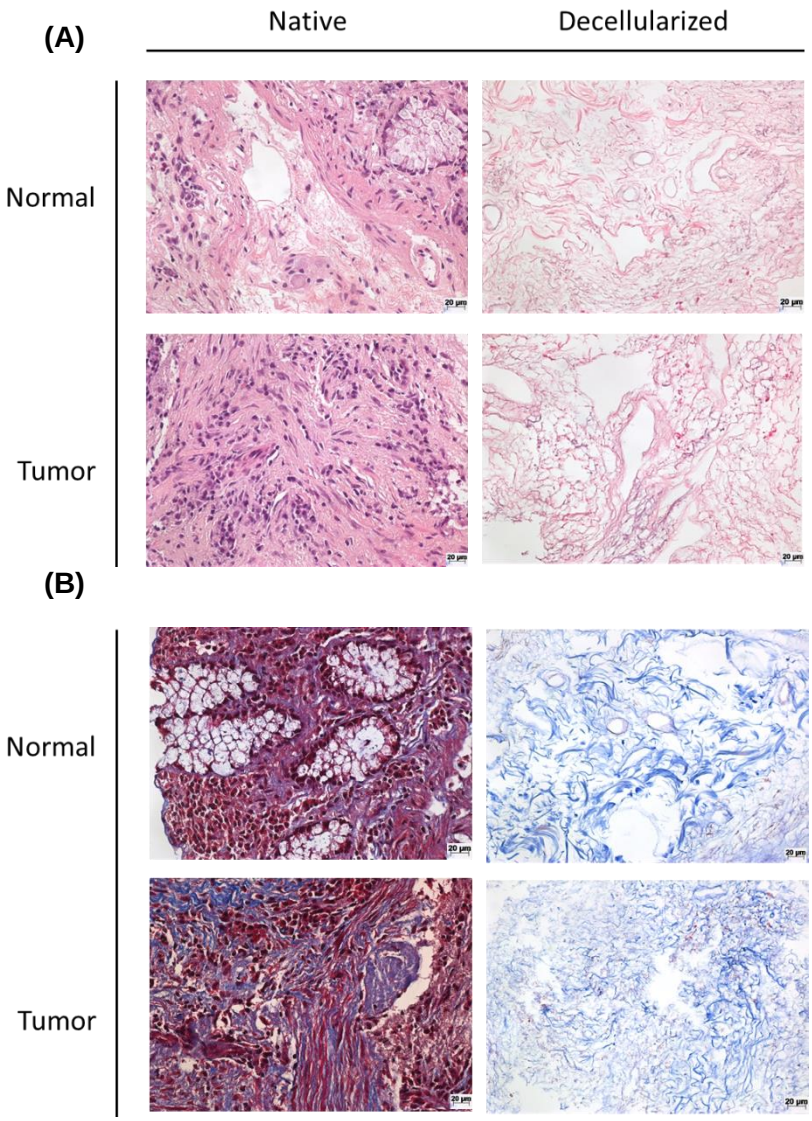


Figure 12. H&E (A) and Masson´s Trichrome (B) staining of native and decellularized normal and tumor tissues derived from colorectal cancer patients' surgical resections. | Representative images of native and decellularized matrices from normal and tumor samples stained with H&E (A) and Masson´s Trichrome (B). Scale bar=20μm

On H&E staining (Figure 12.A), native tissues display a pattern of dark spots, which indicates the presence of cell nuclei (stained blue by haematoxylin). The ECM and cell cytoplasm are stained with varying degrees of pink, stained by eosin, which explain the pink pattern obtained in the decellularized tissues. The lack of blue staining on decellularized tissues H&E (Figure 12.A) and nuclear DAPI staining (Figure 13) indicate absence of cells, proving the efficiency of the decellularization process applied.

Regarding the Masson´s Trichrome staining (Figure 12.B), the colours red, pink and/or dark brown indicate the presence of cells, while collagen fibers and other ECM components are stained in blue. Notably, native tissues display cellular components, while decellularized tissues only exhibit the ECM components, indicating the success of the decellularization on removing cells from normal and tumor tissues.

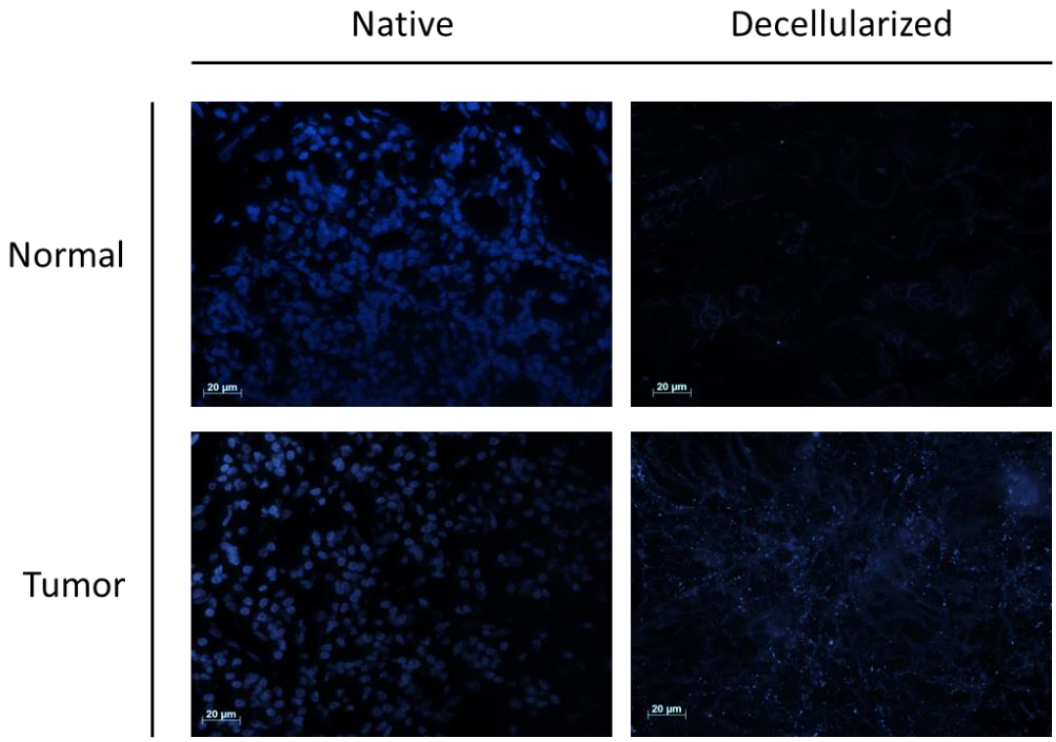


Figure 13. DAPI staining of native and decellularized normal and tumor tissues. | Native and decellularized matrices were stained with DAPI, which allows the staining of the nucleic acids. It is notable that with the decellularization protocol the nuclei are removed from the matrices both in normal and in tumor tissues. The images are representative of all decellularized matrices. Scale bar=20μm

Altogether, H&E, Masson Trichome and DAPI staining's confirmed that the decellularization protocol efficiently removes cells from normal and tumor tissues, preserving the architecture of the ECM.

4.2 Normal and tumor ECM is repopulated by CRC cells

After decellularization, normal and tumor matrices were repopulated with two different colorectal adenocarcinoma cell lines (HCT-15 or HT-29).

To evaluate if the CRC cells were efficiently repopulating the decellularized ECM, we performed immunohistochemistry (for α-tubulin and F-actin) (Figure 14), H&E (Figure 15) and Masson's Trichrome staining (Figure 16).

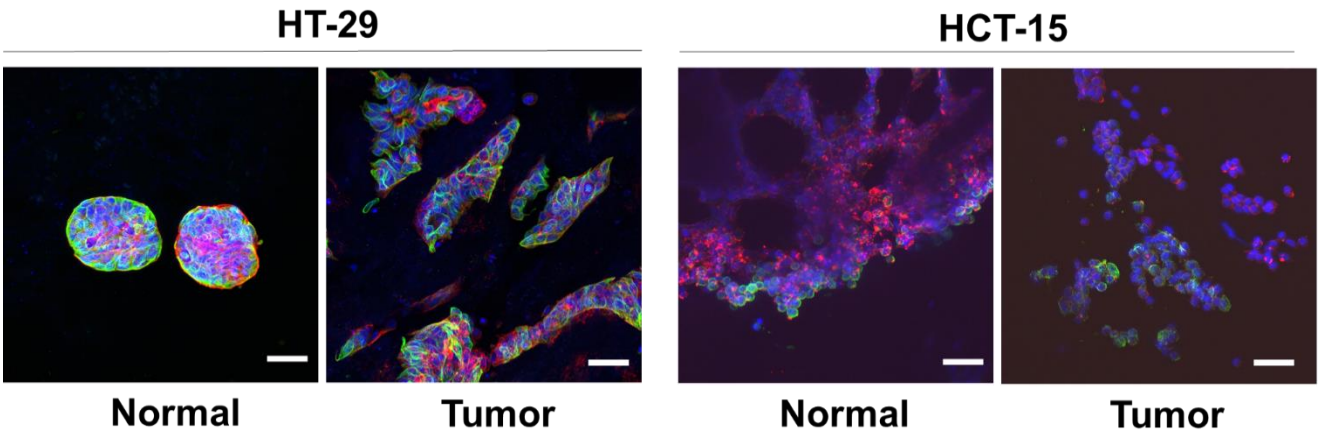


Figure 14. Confocal microscopy of normal and tumor repopulated matrices. | Representative z-stack images of F-actin and α-tubulin staining of HT-29 (left) and HCT-15 (right) cells embedded within normal and tumor repopulated matrices. Blue represents the nucleus of the cells (counterstained with DAPI), red represents α-tubulin and green represents F-actin. Scale bar: 50 µm

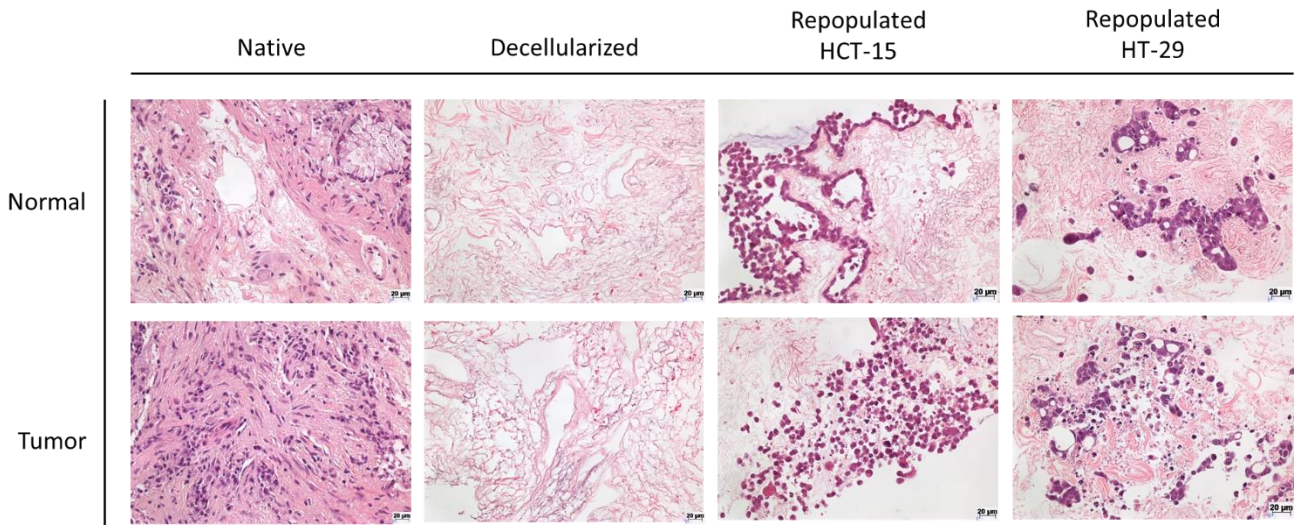


Figure 15. H&E staining. | Representative images of H&E staining of native, decellularized and repopulated normal and tumor tissues. Scale bar=50µm

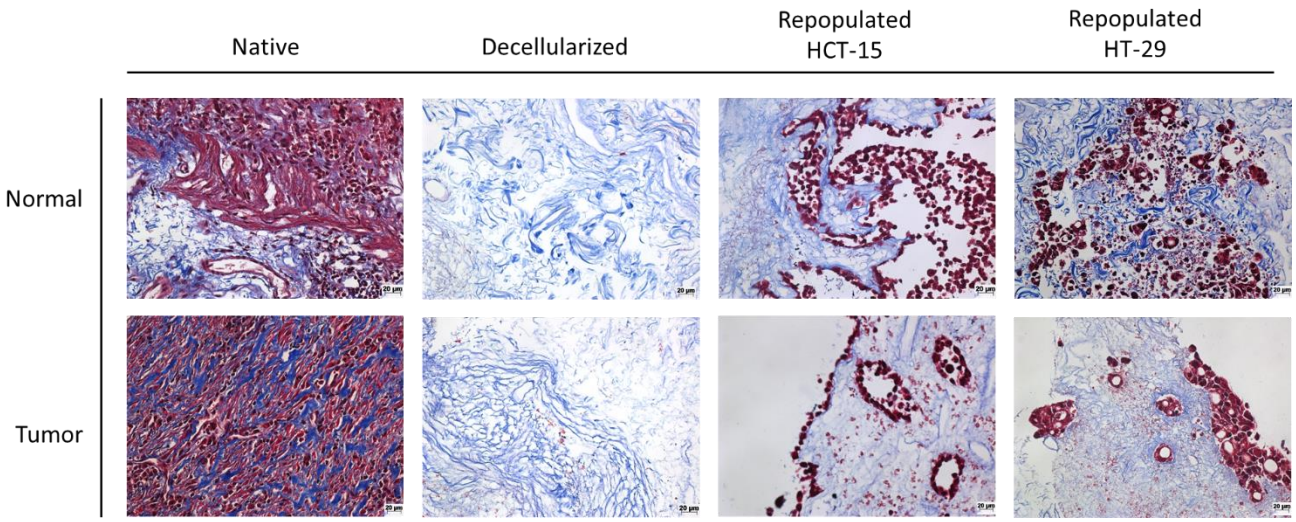


Figure 16. Masson´s Trichrome staining. | Representative images of Masson's Trichrome staining of native, decellularized and repopulated normal and tumor tissues. Scale bar=20μm

F-actin and α-tubulin are major constituents of the cytoskeleton. Immunofluorescence staining of these proteins and confocal microscopy image acquisition of whole matrices allowed the visualization of the cells within the 3D structure of the decellularized ECM. (Figure 15), confirming in depth recellularization of both normal and tumor matrices. Interestingly, the cellular distribution pattern differs according to the cell and the repopulated matrices. HT-29 cells grow in normal matrices as small and apparently cohesive round spheres, while in tumor matrices, these cells are organized in longer but still adhesive structures. HCT-15 cells grow in normal matrices as a less cohesive monolayer while in tumor matrices display a more individualized pattern.

Importantly, native and decellularized normal and tumor tissues revealed similar patterns as the ones previously described, confirming that the matrices used for recellularization have been efficiently decellularized (Figures 16 and 17). Regarding the H&E staining (Figure 16), it is possible to see dark stained nuclei on the matrices repopulated with HCT-15 or HT-29 cells, which indicates a successful matrices colonization.

On repopulated matrices Masson´s Trichrome staining (Figure 17), is also possible to observe dark red spots of nuclei and cytoplasmic components, which indicates the presence of HCT-15 or HT-29 cells within these matrices.

Therefore, we conclude that HCT-15 and HT-29 cells successfully repopulate normal and tumor decellularized matrices.

4.3 Tumor matrices modulate stem-like features of colorectal cancer cells

After the colorectal cancer cells (HCT-15 and HT-29) were cultured in both normal and tumor ECM for seven days, several analyses were performed to understand if the different matrices had an impact on cell stemness. The expression of EMT- and stemness-associated genes were evaluated, as well as the expression of CRC stemness-associated cell surface markers.

4.3.1 Tumor matrices alter the expression of stemness-associated genes

We started by analysing the expression of several genes associated with stemness, pluripotency and epithelial-to-mesenchymal transition (EMT) by Real-Time quantitative PCR. In order to perform this analysis, we extracted RNA from cells cultured, for one week, over normal and tumor decellularized matrices (Table 4).

Table 4. Concentration of RNA extracted from the cells.

RNA extraction (ng/µL)				
Patient nr.	HCT-15 Normal	HCT-15 Tumor	HT-29 Normal	HT-29 Tumor
1	771,6	592,9	422,4	243,1
2	127,6	336,5	286,3	270,9
3	700	242,1	419,3	411,7
4	242,3	157,9	127,6	268,9
5	179,4	560	170,4	781,4
6	148,3	123	273,4	75
7	632,7	822,2	958,9	890,6
8	385,2	250	529	504,1

Expression of EMT-related genes such as *SNAIL1, SLUG, VIMENTIN* and *ZEB*1, as well as pluripotency-associated genes like NANOG, OCT4, SOX2 were evaluated. *TGFB1* expression was also analysed, considering the relevance of this growth factor in regulating cancer stemness (110).

Regarding the HCT-15 cell line (Figure 17.A), tumor ECM tends to induce increased expression of EMT-related genes as *VIM, ZEB1 and especially SLUG.* Overall, expression of pluripotency-associated genes does not alter upon influence of normal or tumor ECM. However, *OCT4,* which is involved in maintaining self-renewal, and *TGFB1* expression appear to increase when the cells are cultured within the tumor matrices.

Regarding the HT-29 cell line (Figure 17.B), most of the analysed genes do not alter their expression when the cells are cultured in a tumor ECM, as opposed to normal ECM from the same patient. However, there is a trend to increase *SOX2* and *OCT4* and to decrease *SLUG* and *TGFB1* expression.

ITGA6 gene is the encoding gene for the integrin α6 protein, an important molecule of interaction with the ECM and a possible marker for CSCs (133). This gene has two splice variants that are expressed by cells with a different compartmentalization within the crypts of the colon. Integrin α6A is found in the proliferative cells of the glands while Integrin α6B is restricted to the quiescent/differentiated cells located on the villus and surface epithelium (134). Studies have supported that the ratio of Integrin α6 variants could be an important indicator of whether the cells undergo differentiation or remain differentiated (135, 136). Considering this, the ratio between the expression of these two splice variants, *ITGA6A* and *ITGA6B*, was determined (Figure 17.C) and the results show no association between the mRNA expression of the variants and the influence of the ECM. However, both in normal and tumor matrices, HT-29 cells display higher *ITGA6A* than *ITGA6B* whereas HCT-15 cells show no prevalence of any of these isoforms.

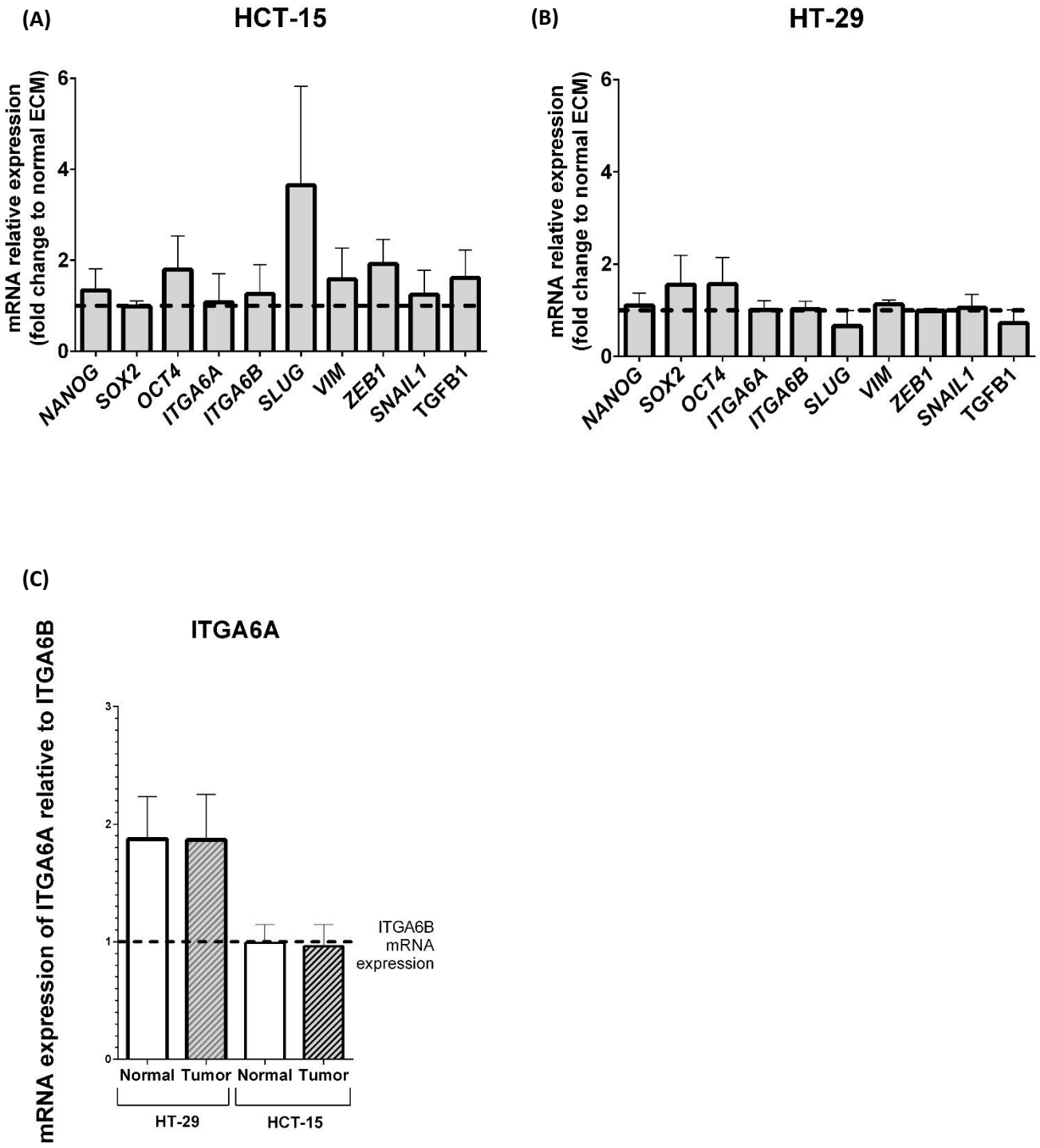


Figure 17. | mRNA relative expression of stem- and EMT-related genes. | Several genes were evaluated through qRT-PCR and the mRNA expression levels were determined. Values of the mRNA expression of HCT-15 (A) and HT-29 (B) cells in normal ECM are represented as 1. (C) Ratio between the mRNA expression of *ITGA6A* relative to *ITGA6B* on both cell lines (HCT-15 and HT-29), upon culture within normal and tumor matrices. Data was obtained using matrices from at least 8 different patients and different cell line passage numbers. Bars represent mean values and error bars represent standard error mean (SEM).

4.3.2 Tumor ECM induces an increase in stem cell-associated surface markers

Although there is still no consensual set of markers to identify CRCSC, a panel of the most described in the literature for colorectal cancer was used (71). Therefore, after seven days of culture within normal or tumor matrices, the expression of the following cancer cell surface markers was analysed: CD44, CD133, CD166, CD49f and PD-L1. As the identification of cancer stem cells often requires conjugation of several markers, we performed not only single but also double staining ($CD44^+CD166^+$, $CD44^+CD133^+$ and $CD133^+CD166^+$) for each cell line. In order to reduce inter-patient variability, we decided to compare fold-change values considering the % of positive cells within tumor relative to the % of positive cells within normal matrices.

Regarding HCT-15 cells (Figure 18), tumor matrices induced an increase in the % of CD133 positive cells of ~4-fold. In the same cells, there was also a ~5-fold increase in the amount of $CD44^+/CD133^+$ cells, as well as more than 2-fold increase in $CD133^+/CD166^+$ cells (Figure 18.B). As indicated by mean fluorescence intensity (MFI) values, we could also observe that tumor ECM induced a higher amount of CD133 molecules per cell (Figure 18.C). Although not statistically significant, these tendencies are clearly reproducible and should not be disregarded.

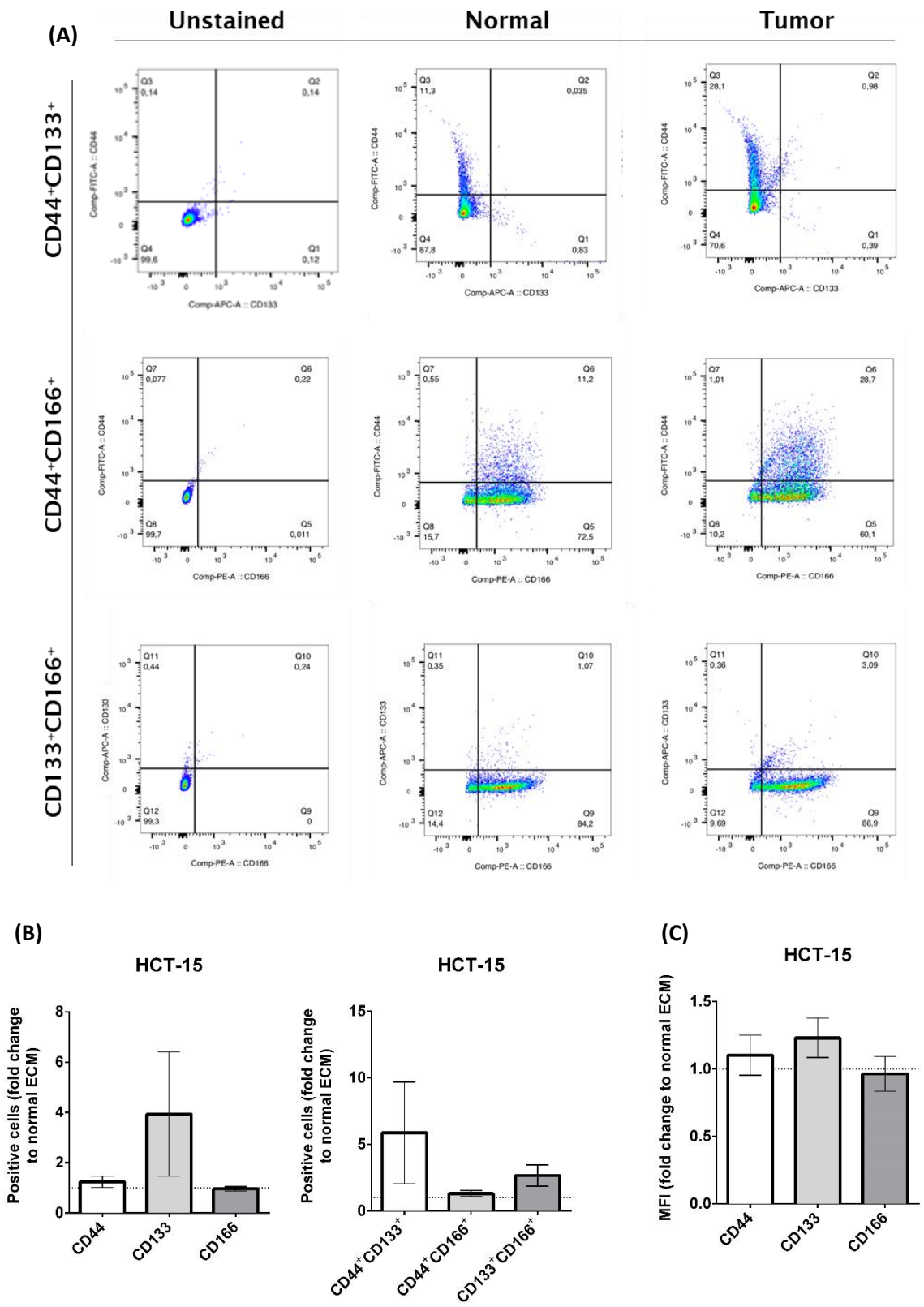


Figure 18. | Expression of surface markers related with CRCSC in HCT-15 cells. | (A) Representative dot plots of unstained and double positive staining of cells differentiated over normal or tumor matrices. (B) Fold-change of the percentage of positive single cells and double positive cells from tumor ECM relative to normal ECM, as represented by the line at Y=1. (C) Fold-change of mean fluorescence intensity (MFI) values of cells from tumor matrices relative to cells from normal matrices, as represented by the line at Y=1. Data represents experiments performed with matrices from at least 8 different patients. Bars represent mean values and error bars represent standard error mean (SEM).

Concerning HT-29 cells, tumor ECM induced an increase in all cell surface markers analysed (Figure 19.A), especially on $CD44^+CD133^+$, $CD44^+CD166^+$ and $CD133^+CD166^+$ double-positive cells, which were significantly augmented (Figure 19.B). Tumor ECM also induced an increase in the number of molecules per cell concerning all markers, especially CD133, as concluded by the significant increase in MFI values relative to those obtained in cells from normal ECM (Figure 19.C).

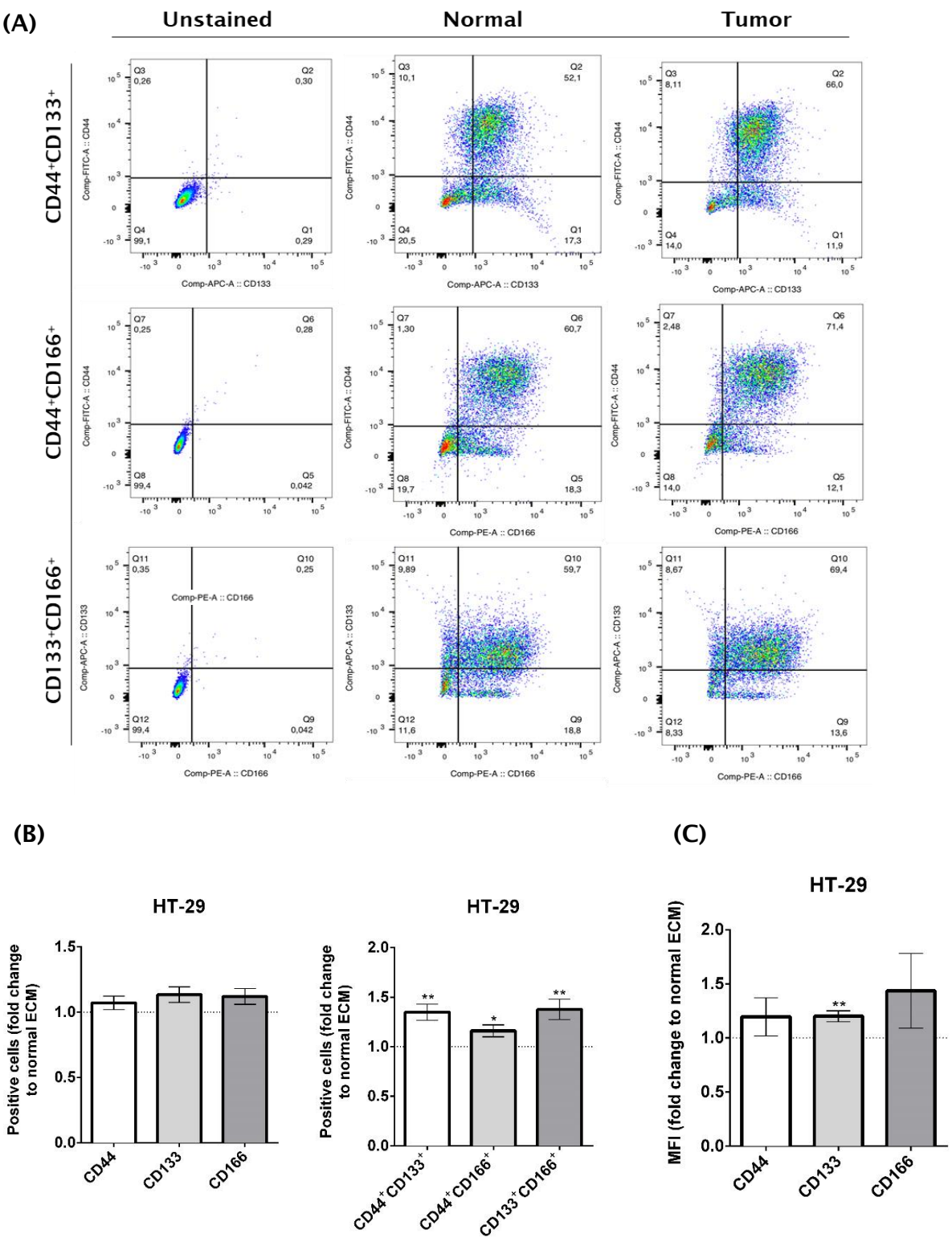


Figure 19. | Expression of surface markers related with CRCSC in HT-29 cells. | (A) Representative dot plots of unstained and double positive staining of cells differentiated over normal or tumor matrices. (B) Fold-change of the percentage of positive single cells and double positive cells from tumor ECM relative to normal ECM, as represented by the line at Y=1. (C) Fold-change of mean fluorescence intensity (MFI) values of cells from tumor matrices relative to cells from normal matrices, as represented by the line at Y=1. Data represents experiments performed with matrices from at least 8 different patients. Bars represent mean values and error bars represent standard error mean (SEM). * significantly different at P<0,05.

Along with the previously referred CSC markers, we also evaluated the expression of integrin α6, also designated CD49f (Figure 20), a previously described marker for CSC in CRC. The percentage of CD49f positive cells was close to 100% in both cell lines and it seems to be maintained independently of the matrices in which the cells were cultured. Considering the high percentage of expression, further analysis of this marker was discontinued after experiments with paired matrices from 3 different patients.

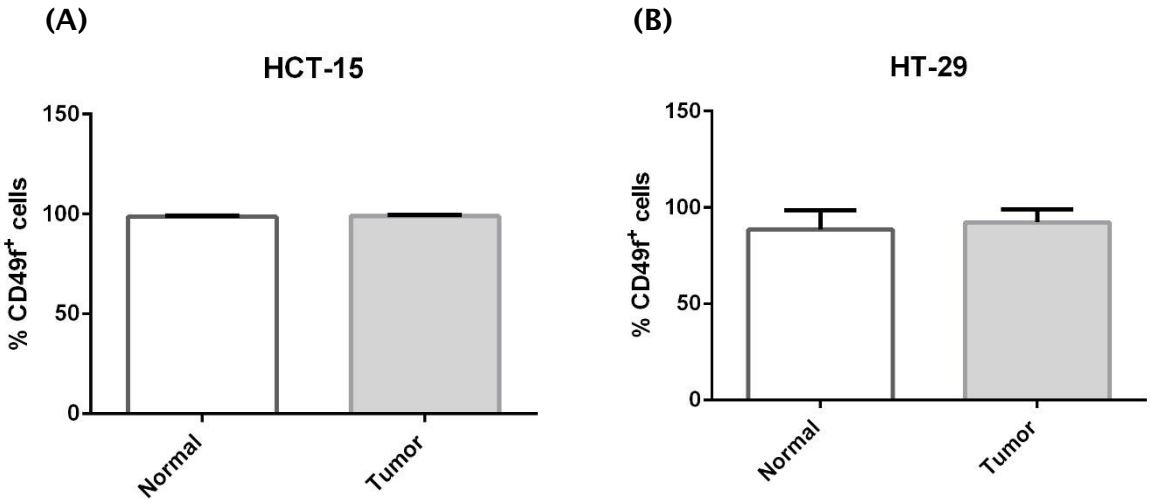


Figure 20. CD49f expression in HCT-15 and HT-29 cells. | Flow cytometry analysis of CD49f in HCT-15 (A) and HT-29 (B) cells from normal ant tumor matrices. Data represents percentage of CD49f-positive cells obtained using matrices from 3 different patients and different cell line passage number.

Besides its major role in suppressing the anti-tumor immune response, PD-L1 has also been positively correlated with stemness markers in breast cancer(137) and to modulate the CSC-like phenotype(138). Keeping this in mind, expression of PD-L1 was analysed by flow cytometry, although performed with cells cultured within paired normal and tumor matrices from only 2 different patients, due to biological material limitations (Figure 21). In HCT-15 cells no conclusions could be drawn, since tumor ECM from one patient induced a considerable increase in PD-L1 expression but tumor ECM from another patient resulted in an opposite effect, with a slight decrease (Figure 21.A). Regarding HT-29 cells, tumor ECM seemed to stimulate PD-L1 expression since the number of positive cells increases in comparison with the effect from normal ECM with matrices from both patients. Nevertheless, this increase was more striking when matrices from the first patient were used (Figure 21.B).

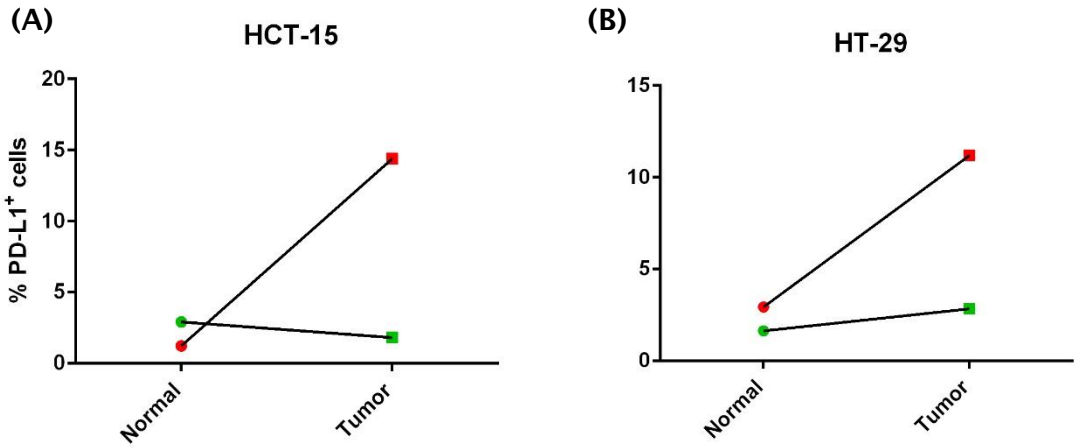


Figure 21. PD-L1 expression in HCT-15 and HT-29 cells. Flow cytometry analysis of PD-L1 in HCT-15 (A) and HT-29 (B) cells differentiated over normal ant tumor matrices. Data represents percentage of PD-L1-positive cells obtained using matrices from 2 different patients and different cell line passage number.

4.4 Tumor ECM alter secreted growth factors

TGF-β is a known regulator of cancer cell stemness(139) and simultaneously has an important role in CRC progression. Therefore, the amount of this cytokine in the supernatants of repopulated normal and tumor matrices was quantified by ELISA. In the case of HCT-15-repopulated matrices, results evidenced no differences regarding the levels of TGF-β in the presence of tumor ECM (Figure 22.A). Concerning HT-29, however, this increase was significant, with tumor matrices inducing the release of 34,28±7,83 pg of TGF-β/mL as opposed to normal matrices, which had the secretion of 13,50±2,62 pg of TGF-β/mL (Figure 22.B). To assess the basal levels of TGF-β on not repopulated decellularized normal and tumor matrices, these were maintained under the same conditions as the repopulated ones, but without cells. Notably, both types of ECM present similar TGF-β levels (Figure 22.C). However, this basal level analysis was performed using paired matrices from only 2 patients.

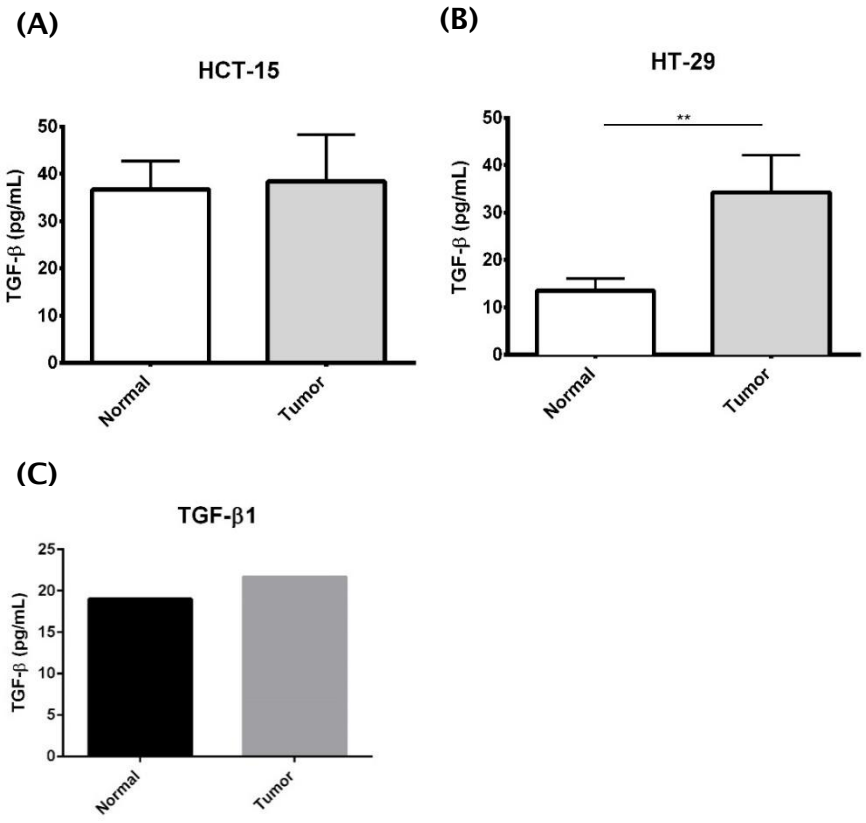


Figure 22. Levels of TGF-β in the supernatants of repopulated matrices. | The levels of TGF-β in conditioned media from HCT-15 and HT-29 cells cultured in normal and tumor ECM were analysed by ELISA (A) TGF-β levels of normal and tumor ECM repopulated with HCT-15. (B) TGF-β levels of normal and tumor ECM repopulated with HT-29. (D) TGF-β levels of normal and tumor ECM without cells. Data represent experiments performed with matrices from 20 different patients (A and B) and 2 different patients (C). Bars represent mean values and error bars represent standard error mean (SEM). * significantly different at P<0,05.

Due to some reports stating that TNF-α and TGF-β synergize and increase CSC properties (140) and that increased TNF-α levels are correlated with more advanced stages of CRC (141), the levels of this cytokine were also determined in the same supernatants of repopulated normal and tumor matrices. While tumor ECM repopulated with HCT-15 cells displayed only a tendency to decrease TNF-α levels when in comparison with normal ECM (Figure 23.A), tumor ECM repopulated with HT-29 significantly diminished TNF-α levels (from 109,50±1,89 pg/mL in normal ECM versus 68,08±9,49 pg/mL in tumor ECM) (Figure 23.B).

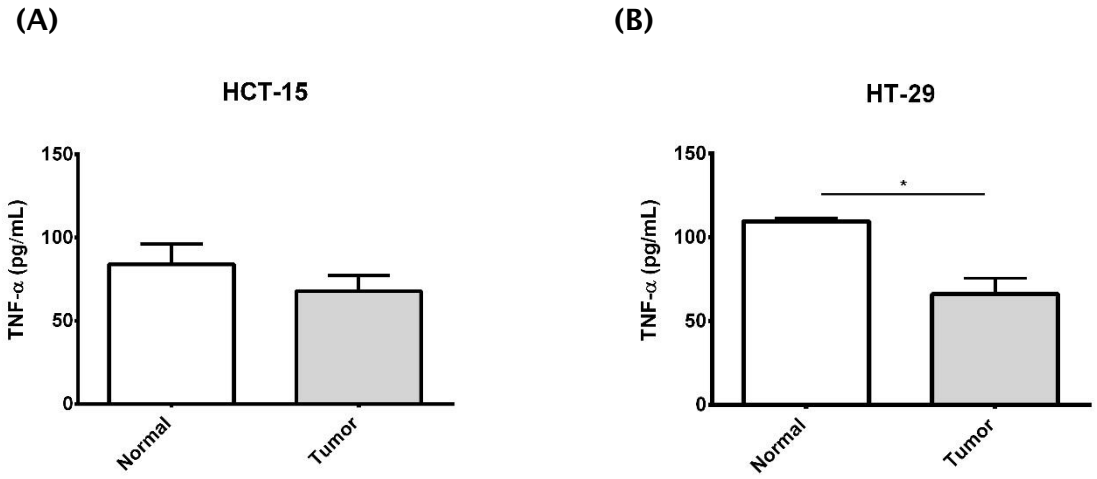


Figure 23. Levels of TNF-α in the supernatants of repopulated matrices. | The levels of TNF-α in conditioned media from HCT-15 and HT-29 cells cultured in normal and tumor matrices were analysed by ELISA. (A) TNF-α levels of normal and tumor ECM repopulated with HCT-15. (B) TNF-α levels of normal and tumor ECM repopulated with HT-29. Data represent experiments performed with matrices from 5 different patients. Bars represent mean values and error bars represent standard error mean (SEM). * significantly different at P<0,05.

4.5 Tumor ECM leads to higher MMP activity

MMPs play crucial roles in ECM remodelling and release of entrapped molecules, such as TGF-β, and also regulation of cell differentiation (142). Therefore, gelatin zymography studies were conducted to evaluate MMP activity on the conditioned media (CM) of normal/tumor ECM repopulated with cancer cells (Figure 24) to determine if there were any differences between the two types of matrices from the same patient (Figure 24.A). Overall, results show that MMP2 and MMP9 activities are increased upon influence of the tumor ECM. In matrices repopulated with HCT-15 cells, proteolytic activity of both gelatinases is increased when cells are within tumor as opposed to paired normal matrices, especially in

the case of MMP2, where this increase is statistically significant (fold increase of 1,36±0,07) (Figure 24.B). The same effect is observed concerning matrices repopulated with HT-29 cells, in which both MMP2 and MMP9 activities significantly increase in tumor relatively to normal matrices (1,35±0,06 and 1,34±0,10, respectively) (Figure 24.B).

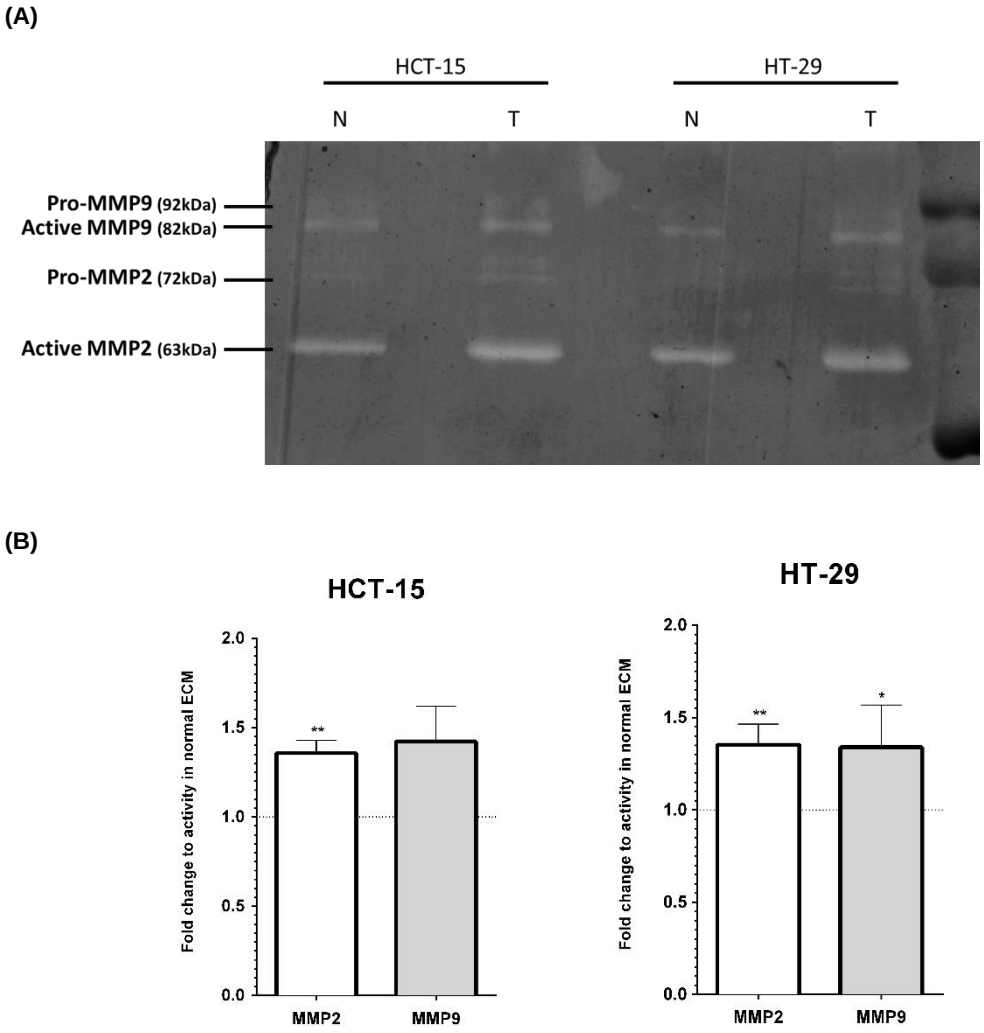


Figure 24. MMP2 and MMP9 activity on supernatants from normal and tumor matrices repopulated with HCT-15 or HT-29 cells. | (A) Conditioned media from normal and tumor matrices repopulated with HCT-15 or HT-29 cells were run on gelatin zymograms. Proteolytic activity was revealed as white bands on a dark background stained with Coomassie. **(B)** Densitometry analysis using ImageJ software allowed quantification of MMP-9 and MMP-2 activities. Proteolytic activity in tumor matrices repopulated with HCT-15 (A) or HT-29 (B) cells was expressed as fold-change of the proteolytic activity in repopulated normal matrices (line at Y=1). Data correspond to mean values of independent experiments performed with paired matrices derived from 5 different patients. Flags indicate standard error mean (SEM); *, significantly different at $P<0.05$

4.6 Tumor ECM influences the ability of HCT-15 cells to resist anoikis

One of the gold standard assays to identify and isolate CSC is the *in vitro* sphere-forming assay, in which cells are grown in serum-free non-adherent conditions in order to enrich the CSC population, the only ones able to survive and proliferate within this environment (differentiated cells die by anoikis - a type of apoptosis occurring when there is no attachment to substrate) (143). Taking this into account, this assay was implemented for the first time in this research group, to assess the effect of normal/tumor ECM on the colonsphere-forming ability of cancer cells. However, the lack of biological material only allowed the execution of this assay using cells that previously repopulated normal and tumor ECM from only one patient. Preliminary results show that HCT-15 cells, after recovery from tumor matrices, were able to form a higher amount of colonspheres, than those cells that underwent the influence of normal ECM, from the same patient (Figure 25). In the case of HT-29, no formation of colonspheres were observed in none of the experimental conditions (data not shown).

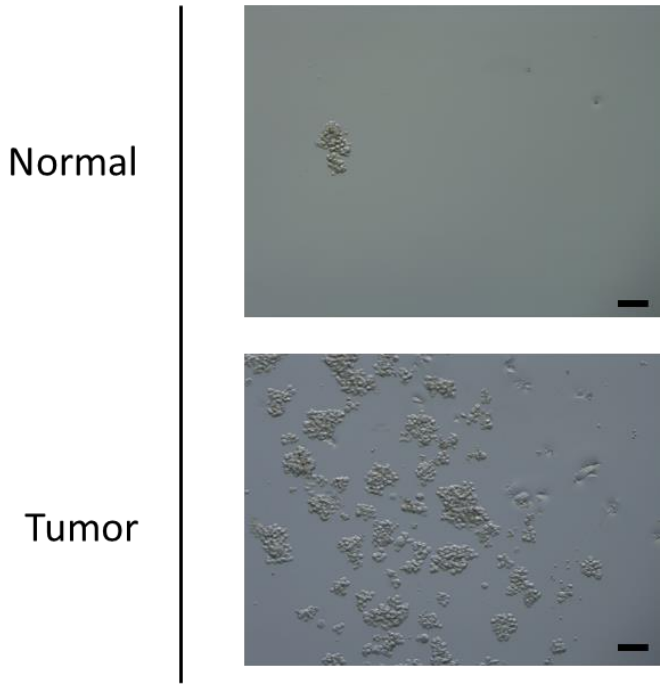


Figure 25. HCT-15 sphere-formation | Formation of colonspheres by HCT-15 cells cultured in normal and tumor matrices. Scale bar = 50 µm.

4.7 Matrigel™ Invasion assays

Recent studies have shown that metastatic cancer cells share stem-like gene expression signatures, raising the hypothesis that CSC from the primary tumor may have the ability to disseminate and contribute to metastasis formation (144, 145). The influence of normal/tumor ECM on the invasive phenotype of HCT-15 and HT-29 cells was evaluated by performing *in vitro* Matrigel™ Invasion assays. Experiments were executed with matrices from only 2 different patients, due to biological material limitations, and unfortunately no consistent results were obtained (Table 3), requiring further studies.

Table 5. Number of invasive HCT-15 and HT-29 cells cultured in normal and tumor ECM. Cells recovered from repopulated normal or tumor ECM were then subjected to Matrigel invasion assays. The experiment was performed using matrices from two different patients.

HCT-15	Patient 1	Patient 2
Normal	59	52
Tumor	113	30

HT-29	Patient 1	Patient 2
Normal	71	235
Tumor	58	68

Chapter V - Discussion

The ECM is a pivotal part of the TME in solid tumours and it is considered a possible driver and promoter of malignancy through its biochemical and biomechanical properties (117). Some studies have also suggested that the ECM could help to constrain cancer progression, depending on the tumor context (146, 147), but more compelling evidence has been gathered concerning the opposite effect. In fact, cues produced by the TME, and the cancer cells themselves, are known to destabilize tissue homeostasis and, consequently, promote malignancy (148-150). In the particular case of CSC, the ECM is thought to regulate and maintain a supportive niche within the TME, and some even hypothesize that the CSC traits are dictated by microenvironmental factors, matrix components included (43).

CSCs are key players on cancer progression since they can be responsible for metastasis, recurrence and resistance to certain therapies (43, 151). Despite much research interest in unravelling the mechanisms associated with the origin, regulation and maintenance of this rare population of cancer cells, it remains to be elucidated the role of the TME, and particularly the ECM, in these processes.

In this work, we aimed to understand if colorectal cancer matrices, as opposed to normal colonic mucosa matrices, had an impact on the stemness of CRC cells. To accomplish this goal, we took advantage of an organotypic model of human decellularized matrices, obtained directly from surgical resections of CRC patients. This model maintains tissue architecture and biochemical composition of both the tumor and the adjacent normal mucosa, sustaining ECM bioactivity and providing a unique tool to disclose ECM/cell interactions and, moreover, to compare between normal and tumor tissues. Although of incalculable valuable, the amount of tissue obtained from surgical resections was extremely restricted, which limited the type of assays conducted and the number of biological replicas for each assay. As each experimental point was obtained with matrices from different patients, as well as samples that could vary in terms of location within the tumor, a high degree of variability was observed. Nevertheless, when results strongly pointed to a trend or were statistically significant, they were considered solid and robust due to the nature of the biological samples and inherent variability.

The decellularization protocol of the same type of samples was previously established by our group (28). Histological, immunohistochemical and ultrastructural evaluations had already demonstrated that tissue structure and major components remain unaltered (collagen type I and IV, laminin, fibronectin and hyaluronic acid) (28). Notwithstanding, we

performed several histological analyses which confirmed the efficiency of this procedure in removing cellular remnants with simultaneous maintenance of ECM architecture.

The great majority of studies on the interaction between cancer cells and the ECM have been performed in 2D systems, with only one ECM component and lacking the spatial dimension of the *in vivo* microenvironment. Currently, three-dimensional culture systems that attempt to re-create the tumor microenvironment are essentially spheroids and organoids, in which the ECM components are produced *de novo*, and natural or synthetic polymer-based matrices, which do not reproduce the dynamic complexity of the ECM (34). Decellularized human tissue is an interesting approach to study the interplay between ECM and cancer, since it enables the maintenance of structural and molecular features of native human tumor matrices, while at the same time, the ability to study its interactions with individual or multiple cell types, through re-cellularization procedures (54, 152, 153). In fact, in this study we repopulated the decellularized tissues with two different colorectal adenocarcinoma cell lines: HCT-15 and HT-29. HCT-15 cells are representative of poorly differentiated tumours, while HT-29 is a colon-like cell line (154). Additionally, they exhibit opposite genomic instability phenotypes: HCT-15 is microsatellite instable and HT-29 is microsatellite stable (155). HT-29 cells are described as pluripotent and with a differentiation potential that varies according to culture conditions (156) as, for example, the substitution of glucose for galactose that induces the reversible enterocytic differentiation of these cells (157). Both cell lines recellularized normal and tumor matrices, although with distinct profiles of colonization. HCT-15 cells exhibited an individualized behaviour while HT-29 cells clustered within the matrices and established structures resembling an epithelium, especially in the normal colonic mucosa in which they appeared to line the crypts. The matrices were recellularized using a passive cell seeding method, nevertheless they were able to penetrate from the surface to the bottom of the structure, even in tumor matrices which have lower porosity and higher density. The cells were in contact with the decellularized matrices for 7 days to allow for an appropriate establishment of cell-matrix and cell-cell interactions, proliferation, any differentiation/dedifferentiation processes and possible selection.

To evaluate the stem-like features of the cells within normal and tumor matrices, several approaches were selected. Unlike breast cancer, in which CSC are widely characterized by a $CD44^{+}CD24^{-}$ and ALDH1 expression phenotype (158), in CRC there is still no consensus on the most accurate markers to identify colonic CSC. Therefore, relying on data from recent studies, both concerning CSC identification and correlation with poor prognosis (104, 133, 159-161), CD44, CD133, CD166 and CD49f were selected. Taking into account that

CSC represent a rare sub-population of cells, high fluctuations in the expression of these cell surface markers were not expected. However, tumor ECM induced a significant increase in $CD44^+CD133^+$, $CD44^+CD166^+$ and $CD133^+CD166^+$ HT-29 populations, when compared to the paired normal ECM. This trend was also observed in HCT-15 cells but to a less extent. Overall, the increase in CD133 expression, both concerning the number of positive cells and number of molecules per cell, is the most consistent and points to a shift in cancer cell stem-like profile under the influence of tumor ECM. Recently, CD133 expression was associated with poor prognosis in stage II colorectal carcinoma, in a cohort of more than 300 patients who had curative surgical resection (162) and, in another study, knockout of CD133 in colon cancer cells by CRISPR/Cas9 gene editing resulted in significant reduction of EMT and colony formation (163). Nevertheless, the combination of at least two cell surface markers usually provides a more reliable CSC identification and, in fact, our results show a significant increase in all double positive populations, upon tumor ECM instruction. CD44 is a particularly important molecule in what concerns cell-matrix interactions since its main ligand is hyaluronic acid (HA), an abundant component of the ECM (164), that upon CD44 activation results in the activation of signaling pathways involved in proliferation, cell survival, cytoskeletal alterations and motility. Additionally, colorectal tumours have increased HA levels when compared to normal tissues (165), and we know that the decellularization protocol used in this study was previously shown not to affect, qualitatively, HA is certainly present in the tissue samples, as we and others observed (28).

In addition to cell surface markers, we also performed gene expression analysis concerning the well described pluripotency-associated stem cell marker complex OCT4/SOX2/NANOG (166). NANOG is a transcription factor that can maintain the pluripotency of human embryonic stem cells since it blocks differentiation (167). It has also been reported as overexpressed in CSCs of CRC (105). Overall, our results show no differences regarding *NANOG* mRNA expression when the cancer cells were cultured in tumor ECM. Contrarily, OCT4 showed a slight trend to increase in both cell lines, as did *SOX2* in HCT-15 cells. SOX2 and OCT4 are two additional stem-related transcription factors that play a critical role on the maintenance of embryonic stem cells (168). A recent study has shown that, when cultured in breast tumor decellularized matrices, breast cancer cells can increase their protein levels of OCT4 and SOX2 (169). These results could also be correlated with the enhanced CD133 expression observed, since deletion of this molecule is known to suppress NANOG, OCT4 and SOX2 expression (170).

We further analysed the expression of EMT-related genes, since this is a process involved in the acquisition and maintenance of stem-cell characteristics (171), and recent studies have shown that CSC often display EMT properties, exhibiting higher migration and metastatic potential, as well as resistance to therapeutic agents (172-174). Moreover, the EMT process has already been linked to the appearance of CSC characteristics on non-stem neoplastic cells (66) and a recent study has shown that decellularized breast tumor ECM can induce the expression of EMT-related genes in a breast cancer cell line (169). During EMT, loss of epithelial markers is mediated by transcriptional repressors such as *SNAIL* (175), *SLUG* (176) and *ZEB1* (172), which are commonly used markers to evaluate this process. Vimentin is a cytoskeletal protein which is upregulated during EMT and furthermore, positively regulates this process by upregulating several EMT-associated genes such as *SLUG* (177). In HT-29 cells, no major changes could be detected in terms of EMT-related genes, upon influence of the tumor ECM, except a slight decrease in *SLUG* expression. Conversely, HCT-15 cells generally increased the expression of all the analysed genes, particularly *SLUG*, suggesting a more mesenchymal phenotype in the presence of tumor ECM and a more epithelial one in normal ECM.

Integrin alpha-6 (also known as CD49f) is a matrix adhesion molecule and also a conserved biomarker in over 30 different stem cell populations (178) and could also have a role in the acquisition of functional characteristics of stem cells. In this study, both cell lines expressed high levels of CD49f, independently of the influence from normal or pathological ECM. However, we further explored this molecule since alternative splicing originates two cytoplasmic variants: integrin alpha-6 isoform A (α6A) and integrin alpha-6 isoform B (α6B), which expression is spatially and temporally regulated and contribute to stem cell differentiation and maintenance of the niche cells(179). In colon, these two isoforms have differential expression along the crypt-villus axis: α6A is more expressed at the base of the crypts, which is characterized by the presence of undifferentiated cells, intestinal stem cells and highly proliferating cells, while α6B is predominantly expressed in the upper crypt/ lower villus, in more differentiated cells(180). The ratio of the two isoforms can determine cellular behaviour and, in colon, a high α6B/α6A ratio favours enterocytic differentiation(181). Due to the lack of reliable antibodies to detect these two cytoplasmic variants, gene expression analysis was performed, and it revealed an unaltered α6B/ α6A ratio whether the cells were within normal or tumor ECM. Interestingly, however, HCT-15 cells had relatively the same expression levels of α6B and α6A while HT-29 exhibited a different profile, with a clear predominance of the α6A relatively to the α6B gene, which could indicate a less differentiated phenotype.

TGF-β is a cytokine with pivotal roles on tissue homeostasis and cancer, which, under certain circumstances, is activated from the ECM where it is entrapped in high amounts (110). Upon release and activation, TGF-β regulates several physiological and pathological processes, such as cell cycle, apoptosis, EMT and even ECM remodelling (110). Dysregulation in TGF-β signaling is associated with malignancy, namely in colon cancer (182, 183), and has been associated with stem-like features of CRC cells in several studies (107, 183, 184). In fact, levels of TGF-β released from normal and tumor decellularized matrices were very similar and demonstrated that this cytokine is maintained entrapped within the matrices despite the decellularization process. In repopulated matrices, however, TGF-β levels significantly increased in HT-29-repopulated tumor matrices than in its normal counterpart, showing a clear effect from the two types of ECM. In HCT-15-repopulated matrices, however, TGF-β levels, whether in normal or tumor ECM, were higher than those observed in non-repopulated matrices.

It is important to notice that, in repopulated matrices, TGF-β detected at the protein level could originate directly from cells but could also derive from the ECM, upon proteolytic cleavage. Regarding HT-29 cells, our results suggest that TGF-β is predominantly, being released from the tumor rather than from the normal ECM, since cells exhibited a lower *TGFB* expression when cultured in tumor than when in normal ECM. The source of TGF-β, in this case, was by cell action on the ECM, indirectly causing its release, and not by direct cellular production. This is supported by the fact that MMP2 and MMP9 activity were also significantly higher in HT-29-repoputaled tumor matrices, and these are crucial matrix metalloproteases that not only remodel the ECM but also activate latent TGF-β (185). In the case of HCT-15 cells, increased TGF-β levels in the presence of tumor matrices could be explained by increased gene expression but also higher release and activation of latent TGF-β, as increased MMP activity is also observed. However, high TGF-β levels in HCT-15-repopulated normal ECM could not be explained through the same mechanisms, since, in these conditions, we detected lower gene expression and MMP activity levels.

Another important characteristic of TGF-β, is that its availability can be regulated by the composition and mechanical properties of the ECM (186, 187). A stiffer ECM, such as that of tumours, facilitates the release and activation of this cytokine (187). Previous work from our group using the same type of biological samples and decellularization protocol, previously demonstrated that not only tumor tissues are stiffer than their normal counterparts but also that the decellularization process does not alter these characteristics and differences (28).

In hepatocellular carcinoma, TGF-β was shown to regulate CD133 expression and increase tumorgenicity (188) and, in another study, TGF-β induced EMT by the transactivation of EGF signaling through HA/CD44 in lung and breast cancer cells, during cancer progression (189). In fact, the increased TGF-β levels in repopulated tumor matrices could be, at least partially, responsible for the increase in CD133 and EMT markers observed in this work. Additionally, in gliomas, TGF-β maintains cancer cell self-renewal by acting directly on Sox4, which then binds to Oct4, and then induce expression of Sox2 (190, 191).

Recently, TGF-β and TNF-α were shown to, synergistically, regulate the stem cell properties in pancreatic (140), but also on breast (192) cancer cells. In HCT-15 repopulated matrices we saw similar levels of TNF-α when the cells were cultured in normal or tumor ECM, reflecting the pattern obtained for TGF-β. In HT-29-repopulated matrices, however, an opposite profile between the two cytokines was observed: TNF-α levels were significantly lower in tumor ECM than in normal ECM. TNF-α has been shown to reduce ECM deposition by the induction of collagenases and by the inhibition of the synthesis of collagen type 1 (193, 194). When cultured in the tumor ECM, cancer cells were under the influence of high levels of collagen type 1. Therefore, we hypothesize that HT-29 cells may be reducing the levels of TNF-α in order to maintain the high levels of collagen type 1 and remain under the influence of a stiff matrix. Also, TGF- β is known to stimulate collagen type 1 expression and its levels were significantly higher in the tumor ECM. The lower secretion of TNF-α can be happening in order to counteract the TGF- β stimulation of collagen type 1 production.

In breast cancer, it has been reported that CSCs are more efficient in remodelling the ECM than their non-cancer stem cell counterparts since they are able to upregulate the expression of different MMP (195) and, actually, repopulated tumor ECM had higher MMP activity relatively to the paired normal ECM. MMP are not only key players in ECM remodelling, but are also responsible for releasing molecules and growth factors entrapped within the matrix and regulating cell signaling and behaviour. Dense matrices, such as those in CRC, enhance the activity of MMP (185), which in turn is required for cell invasion (196).

Since cells cultured in tumor ECM presented higher MMP2 and MMP9 activity, previously associated with cancer cell invasion through ECM degradation, and being invasion a crucial step for cancer progression and metastasis establishment, a hypothesis was raised concerning the invasion-ability of cells from normal and tumor-repopulated matrices. However, preliminary data did not produce consistent results and therefore, the hypothesis will be further investigated in the future, using matrices from at least more 4 different patients.

The sphere forming assay is one of the gold standard methods to assess cell self-renewal potential in vitro. Single cells are plated at low densities in non-adherent conditions, and only the ones with self-renewal ability and anoikis-resistant are able to grow in three-dimensional spheres. The number of spheres that originate from single cells determine the tumorigenic efficiency of a subset of cancer cells. This assay was implemented for the first time during the course of this work and preliminary data obtained with matrices from one patient indicated that the HCT-15 population from tumor ECM *vs* normal ECM was enriched in cells with stem cell properties since a higher amount of spheres was obtained. However, further studies will have to be conducted, not only to optimize the procedure, but also to perform experiments with paired matrices from other patients.

The programmed death ligand-1/programmed death-1 (PD-L1/PD-1) signaling pathway is an important player in the immune tolerance and escape of cancer cells (197) and recently, PD-L1 was described to contribute to the maintenance of CSC self-renewal in CRC (197). Likewise, this study showed that $CD44^{+}CD133^{+}$ cells had a higher expression of PD-L1 than $CD44^{-}CD133^{-}$ cells (197). Due to the unavailability of biological material, PD-L1 expression was analysed in repopulated matrices from only two different patients. Tumor ECM from one of them induced a remarkable increase in PD-L1 expression, both in HCT-15 and HT-29, as opposed to normal ECM. Nevertheless, the same effect was not observed using biological material from another patient, thus requiring further experimental analysis with matrices from other patients. As previously stated, while the normal adjacent mucosa is systematically removed from the same region (at least 10 cm distant from the tumor) and exhibits the same architectural structure between patients, the tumor tissue sample presents higher variability in terms of localization within the tumor, as well as structure. Considering this and adding to the inter-individual variability, our model could be subject of some discrepancies, although closely reflecting the extracellular matrix of normal and tumor colorectal tissues.

With the present study, we were able to show that biochemical, biophysical or biomechanical characteristics of colorectal tumours influence stem-like features of CRC cells, underlying the importance of this tumor microenvironmental component in the maintenance of the CSC niche. The identification of the molecular cues or structural elements responsible for these interactions will be paramount to disclose the modulation mechanisms of cellular differentiation and provide potential targets for therapeutic intervention.

Chapter VI – Conclusion and Future Perspectives

In this work, decellularized CRC matrices were used as an *in vitro* bioactive microenvironment to investigate the influence of ECM on cancer cell stemness. This 3D organotypic model recapitulates the structural and biochemical environment of native tissue from human CRC patients, retains biological activity of small molecules entrapped within the matrix and offers a unique platform to study cell-matrix interactions. With a simple cancer cell recellularization method, we concluded that tumor ECM differently modulated cancer stem cell-associated markers in comparison with normal ECM from the same patient. These observations suggest a role in the promotion/maintenance of stem-like features of cancer cells, or in the selection of CSC existent in the general cell population. Several questions remain to be answered, namely, which are the element(s) in the tumor ECM responsible for this effect; does the area of the tumor from which the ECM is removed affect the results obtained; why is one cell line more susceptible to the ECM than other, etc.

As future work, the *in vitro* sphere forming assay will be optimized and implemented to assess the tumorigenic potential of cancer cells originating from normal or tumor patients derived colorectal matrices. Signaling pathways involved in the stem cell-phenotype will also be analyzed (Hedgehog, Wnt, Notch, PI3K/PTEN and NF-kB pathways) and the effect of main ECM components will be individually studied. It would also be interesting to increase the complexity of the model and to include other cell types, also known to be an important part of the tumor microenvironment and to impact cancer stem cells as, for example, macrophages and fibroblasts. Additionally, the influence of tumor ECM on immune checkpoint molecules and invasion-related activities will also be the subject of extra research studies.

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