Modulation of Colorectal Cancer Stem Cell Pool and its Crosstalk with Tumour Microenvironment by KRas, BRaf, and PIK3CA mutations

André Emanuel Serra Roma

Tese de Candidatura ao grau de Mestre em Investigação, especialização em Oncobiologia, submetida à Faculdade de Medicina da Universidade de Coimbra. O presente trabalho foi executado no IPATIMUP/i3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto sob orientação da Doutora Sérgia Maria Aguiar Velho e coorientação do Professor Henrique Manuel Paixão dos Santos Girão.

This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), project NORTE-01-0145-FEDER-000029, supported by Norte Portugal Regional Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/ Ministério da Ciência, Tecnologia e Inovação in the framework of the projects "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274) and IF/00136/2013/CP1184/CT0003.

Viaje segundo um seu projecto próprio, dê mínimos ouvidos à facilidade dos itinerários cómodos e de rasto pisado, aceite enganar-se na estrada e voltar atrás, ou, pelo contrário, persevere até inventar saídas desacostumadas para o mundo.

José Saramago in Viagem a Portugal

AGRADECIMENTOS

Quero aproveitar esta secção para agradecer a todos os que se cruzaram comigo ao longo deste ano e que deixaram a sua marca no percurso que tracei.

Como não poderia deixar de ser, quero agradecer à Sérgia, pela orientação e pela amizade. Pela confiança no meu trabalho, apesar de por vezes as falhas naturais da imaturidade laboratorial se fazerem sentir. Certamente aprendi a ser mais organizado doravante! Obrigado pelas discussões científicas com pés e cabeça, pelo fomento do espírito crítico e pelo apoio na perseguição das ideias que apareciam pela frente.

Não poderia deixar de agradecer à Raquel, por me ter aberto as portas do seu laboratório e me tenha apresentado a Sérgia com quem tive o prazer de trabalhar. Mas tal não seria possível sem o apoio da Professora Carmen, ao qual agradeço também o apoio que me deu na perseguição dos meus objectivos.

Quero agradecer ao EpIC por me ter acolhido durante este ano e que me continue a acolher daqui para a frente. Em especial à Patrícia, por me ter apoiado em tanto do meu trabalho laboratorial. À Anabela pelas discussões políticas e científicas que acompanhavam o trabalho do laboratório, nunca esquecendo os momentos de música pimba portuguesa! À Joana Figueiredo pelas conversas de rir do princípio ao fim e pelo apoio nos *western blots* que teimavam em não funcionar. Aos óptimos companheiros de almoço do IPATIMUP, onde nunca falta o que conversar, onde não haverá chuva que nos tire o almoço ao ar livre e mesmo agora separados fisicamente, o chamamento para o almoço continua a existir.

Quero deixar especial agradecimento àqueles que não são apenas colegas e amigos de trabalho, mas sim amigos para a vida. À Joana Marques, que mesmo sendo de Marrocos abaixo do Mondego, não é por isso que não continuo a gostar de ti. À Inês, que apesar de a prenda ter acabado por não ser o que se esperava, espero que tenha sempre sido algo de melhor. Aos desabafos dos nossos dramas e aos pensamentos lógicos induzidos por eles. Mas quero deixar o mais especial agradecimento à Babi, por ter sido a minha integradora, e que não podendo ser o meu clone do sexo feminino, certamente é perto disso. Obrigado pelo apoio que me deste ao longo deste ano, pelos ralhetes, pelos momentos de descontração, pelas comédias até altas horas no laboratório "a trabalhar". Que continues assim!

À Maria Inês, Carla, Fábio, Miguel, Café, Tomás e a todos os meus amigos que a faculdade se incumbiu de trazer e que, apesar de longe, nunca me viraram as costas. À Helena, mais uma

grande amiga que certamente será para a vida, à nossa troca de cidades, à nossa cumplicidade e compatibilidade, aos nossos cafés sempre que possível, mas acima de tudo, ao nosso bom gosto musical!! Aos amigos desta cidade e de outras, Eva, Filipa, Joana sabem que fizeram parte deste percurso e que ficarão sempre na minha memória! Que os nossos passeios fotográficos se prolonguem por muito tempo.

Às melhores colegas de casa que poderia ter arranjado, Juliana e Joana, um grande obrigado por tudo, pelos jantares que não cozinhei, pelas toneladas de louça que não lavei. Espero ter compensado com a amizade e com as refeições que também vos proporcionei. Viva a lasanha de soja e o 111!!!

E como não poderia deixar de ser, à minha família, pai, mãe, tia, e especialmente à minha irmã, que sempre fizeram tudo para alcançar os meus objectivos, dando-me o apoio que eu precisava, nunca arredando o pé, por muito difícil que os tempos se avizinhassem. Um grande obrigado por nunca me terem virado as costas.

A todos aqueles que não menciono, possivelmente pela eventualidade do esquecimento, mas que se cruzaram comigo durante a vida e com isso me fizeram crescer como pessoa, obrigado pela marca, boa ou má, que deixaram.

The work presented in this thesis was presented, by myself and others, in the following national and international meetings:

Roma, A.; Carvalho, P.; Seruca, R.; Velho, S. **Modulation of colorectal cancer cell differentiation markers by oncogenic alterations.** *2nd ASPIC International Congress.* Poster and oral presentation. 28-29 April, 2016

Roma, A.; Carvalho, P.; Seruca, R.; Velho, S. **Modulation of colorectal cancer cell stem cell pool by oncogenic alterations.** *9º Encontro de Jovens Investigadores da Universidade do Porto, O*ral presentation. 17-19 February, 2016

Carvalho, P.; Roma, A.; Seruca, R.; Velho, S. **Mutant K-Ras mediates fibroblast-induced colorectal cancer cell invasion.** *2nd ASPIC International Congress.* Poster and oral presentation. 28-29 April, 2016, EACR Best Poster Prize

TABLE OF CONTENTS

LIST OF ABREVIATIONS

ALCAM – Activated Leukocyte Cell Adhesion Molecule

APC – Adenomatous polyposis coli

BMP – Bone Morphogenetic Protein

CAF – Cancer-associated Fibroblasts

CAM – Cell Adhesion Molecules

CCND1 – Cyclin D1

CD44v – CD44 variants

CFE – Colosphere-forming assay

CRC – Colorectal Cancer

CSC – Cancer Stem Cell

EGF – Epithelial Growth Factor

EMT – Epithelial-Mesenchymal Transition

FAK – Focal Adhesion Kinase

FAP – Familial Adenomatous Polyposis

FAP-α – Fibroblasts Activation Protein α

FBS – Fetal Bovine Serum

FSP-1 – Fibroblasts Specific Protein 1

GAP – GTPase-activating protein

GEF – Guanine Nucleotide Exchange Factor

HGF – Hepatocyte Growth Factor

ICD – Intracellular Domain

IGF1/2 – Insulin-like Growth Factor 1/2

IHH – Indian Hedgehog

MET – Mesenchymal-Epithelial Transition

OPN - Osteopontin

PCF – Perycryptal Fibroblasts

PDGF – Platelet-derived Growth Factor

PDGFR-β – PDGF receptor β

PGF-2 – Prostaglandin F2

PIK3CA - Phosphatidylinositol-4,5-bisphosphate 3-kinase Catalytic Subunit Alpha Isoform

Rb – Retinoblastoma Protein

SC – Stem Cell

TME – Tumour Microenvironment

VEGF – Vascular Endothelial Growth Factor

α-SMA – α-Smooth Muscle Actin

LIST OF FIGURES AND TABLES

Supplementary Figures

ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers worldwide and its evolution is associated with multiple and progressive mutations. Although *KRas* mutations have been associated with modulation of cancer stem cell (CSC) properties within the tumour, and therefore affecting tumour progression, other frequent mutations in *BRaf* and *PIK3CA* are still to be associated to stemness modulation in CRC. Along the years, different CSC markers have been proposed, with CD44 and CD133 being extensively studied. The work described in this thesis aims to evaluate the role of oncogenic *KRas*, *BRaf* and *PIK3CA* in the modulation of different CSC and intestinal stem cell markers, as well as E-cadherin as an epithelial-mesenchymal transition (EMT) marker.

We found that KRas is able to modulate CSC markers expression, but BRaf and PIK3CA are not able to fully reproduce the same observations. CD44 is strongly modulated by KRas in HCT-116 cells but not in any other *KRas*-mutated cell line studied. However, when colosphere-forming assay is performed, alteration in stemness is not only common in both *KRas*-inhibited HCT-116 and SW480 cell lines, but is also found in *PIK3CA*-inhibited HCT-116 cells. Resulting from such observations, we explored the involvement of EMT or other possible stem cell markers to explain such alteration of stem-like phenotype. No significant changes were observed in any of the intestinal stem cell markers studied, and neither in the expression of E-cadherin. In order to find a molecular mediator of CSC properties in our cell lines we investigated the expression of integrin α6 as this protein has been described as a possible CSC marker. In fact, our findings support integrin α6 as a possible CSC marker. A reduction in integrin α6 expression is observed in HCT-116, SW480 and RKO cell lines upon *KRas*, *BRaf* and *PIK3CA* inhibitions. Moreover, alterations in the maturation of integrin α6 were found in HCT-116 upon *PIK3CA* inhibition. Also, inhibition of such oncogenic alterations also lead to the modulation of c-Met expression, the hepatocyte growth factor (HGF) receptor important for the communication with the tumour microenvironment. Together with the observations of others that tumour microenvironment is able to modulate CSC pool by secreted factors, like HGF, our findings hint at an eventual role of *KRas*, *BRaf* and *PIK3CA* mutation in the modulation of the communication of CRC cells with the tumour microenvironment. Contrary to what was expected, no alterations were observed in CD44, CD44v6 and E-Cadherin expression upon treatment with fibroblasts' conditioned media.

Overall, this study gives support to the idea that integrin α6 functions as a mediator of KRas-, BRaf- and PIK3CA-induced CRC cell stemness.

RESUMO

O cancro colorectal é um dos tumores mais comuns a nível mundial e a sua evolução está associada à acumulação progressiva de múltiplas alterações genéticas. Apesar de mutações no *KRas* estarem associadas à modulação das propriedades estaminais no tumor, e assim afetar a progressão tumoral, outras mutações frequentes no *BRaf* e *PIK3CA* não estão ainda associadas à modulação da estaminalidade no cancro colorectal. Ao longo do tempo, diferentes marcadores de células estaminais cancerígenas (CSC) têm sido propostos, sendo o CD44 e o CD133 extensivamente estudados. O trabalho apresentado nesta tese tem como objetivo avaliar o papel das mutações do *KRas*, *BRaf* e *PIK3CA* na modulação de diferentes marcadores de CSCs e de células estaminais intestinais, bem como da E-caderina como marcador da transição epitélio-mesenquimal (EMT).

Descobrimos que o KRas é capaz de modular a expressão de marcadores de CSC, no entanto as mesmas observações não são totalmente reproduzidas pelo BRaf e PIK3CA. A expressão de CD44 é fortemente afetada após inibição de *KRas* na linha celular HCT-116 mas não em nenhuma outra linha celular com mutações para *KRas.* No entanto, quando é realizado o ensaio de formação de esferas, alterações da estaminalidade são observadas não apenas em células HCT-116 e SW480 em que o *KRas* está inibido mas também em células HCT-116 com inibição de *PIK3CA*, não se relacionando com as alterações obtidas na expressão dos marcadores de CSC. Como resultado destas observações, explorámos o envolvimento da EMT e de outros possíveis marcadores de células estaminais para explicar a alteração do fenótipo estaminal. Não foram observadas alterações significativas em nenhum marcador de células estaminais intestinais, nem na expressão de E-caderina. De forma a encontrar um mediador molecular de CSC nas nossas linhas celulares, investigamos a expressão de integrina α6, uma vez que se encontra descrito como possível marcador de CSC. De facto os resultados dão suporte ao uso da integrina α6 como marcador de CSC. Uma redução de expressão de integrina α6 é observada nas linhas HCT-116, SW480 e RKO após inibição de *KRas*, *BRaf* e *PIK3CA.* Além disso, alterações na maturação da integrina α6 foram encontradas após inibição de *PIK3CA* em HCT-116. As mesmas inibições levaram também a modulação da expressão de c-Met, o recetor do fator de crescimento de hepatócitos (HGF), importante para a comunicação com o microambiente tumoral. Em conjunto com as observações de outros autores em que é descrito que o microambiente tumoral é capaz de modular as CSC pela secreção de fatores como o HGF, os nossos resultados apontam para um possível papel do *KRas, BRaf* e *PIK3CA* na modulação da comunicação com o microambiente. Contrariamente ao esperado, não foram observadas alterações na expressão de CD44, CD44v6 e E-caderina após tratamento com meio condicionado de fibroblastos.

Em suma, este estudo apoia a ideia de que a integrina α6 funciona como um mediador da estaminalidade celular no cancro colorectal induzida por KRas, BRaf e PIK3CA.

INTRODUCTION

Normal Colon

The colon, or large intestine, is part of the digestive system and extends from the ileocecal junction to the anus (Figure 1). In combination with the small intestine, the colon is responsible for the absorption of nutrients, electrolytes and water, while blocking the entrance of potentially harmful elements such as microorganisms or toxins. During the journey through the colon, nutrients and water from watery chyme (partly digested food) are absorbed, converting it into feces.[1] The ileocecal junction is where the large and small intestines meet, in a 6 cm of size region called cecum. Following the cecum is the colon, a 2-meter-long region that can be divided in four parts: ascending colon, transverse colon, descending colon and sigmoid colon, which ends in the rectum. The large intestine ends in the anal canal that follows the rectum.[1,2]

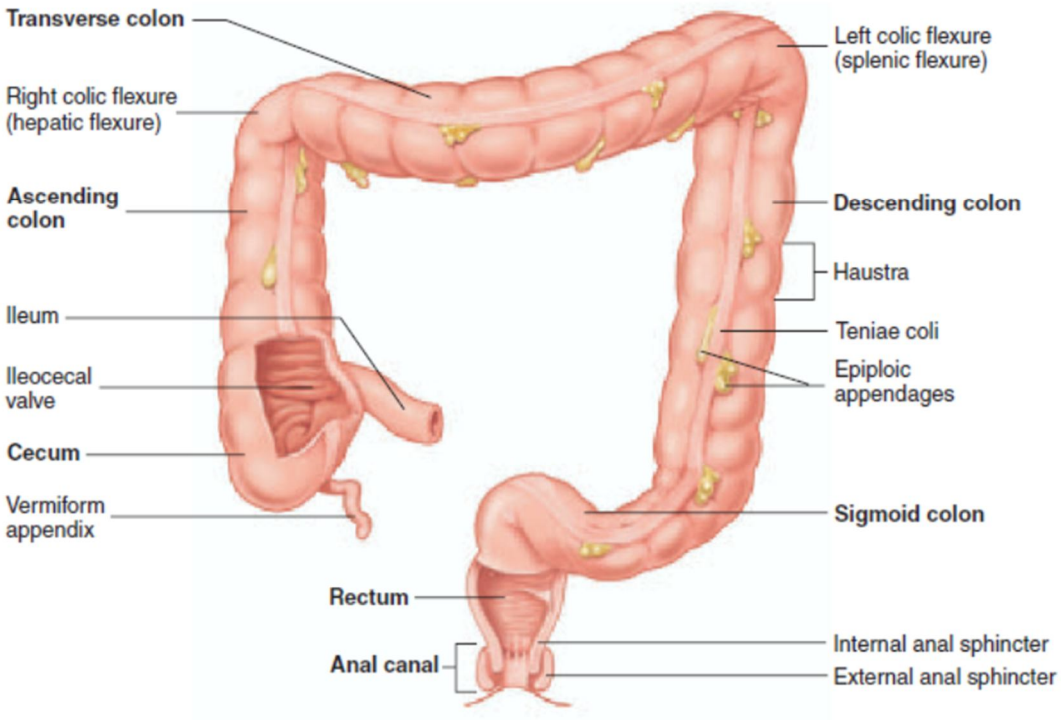

Figure 1. Large intestine. Representation and of large intestinal (colon) and anal canal. Adapted from Seeley et al.[2]

The intestinal tube is composed of four sheets: mucosa, submucosa, muscularis and serosa.[2] The mucosa is the inner sheet and is composed by three layers: an inner simple columnar epithelium, followed by a layer of connective tissue, the lamina propria, and an outer layer of smooth muscle, the muscularis mucosae.[2] In the colon, these layers fold into structures

called crypts of Lieberkühn. The submucosa is a thick layer of connective tissue which contains nerves, blood vessels and glands.[2] Muscularis follows the submucosa layer and is composed of two layers of smooth muscle: one inner layer of circular muscle and an outer longitudinal smooth muscle layer. The last outer layer is the serosa, another layer of connective tissue that is a continuation of the *peritoneum.* [1,2]

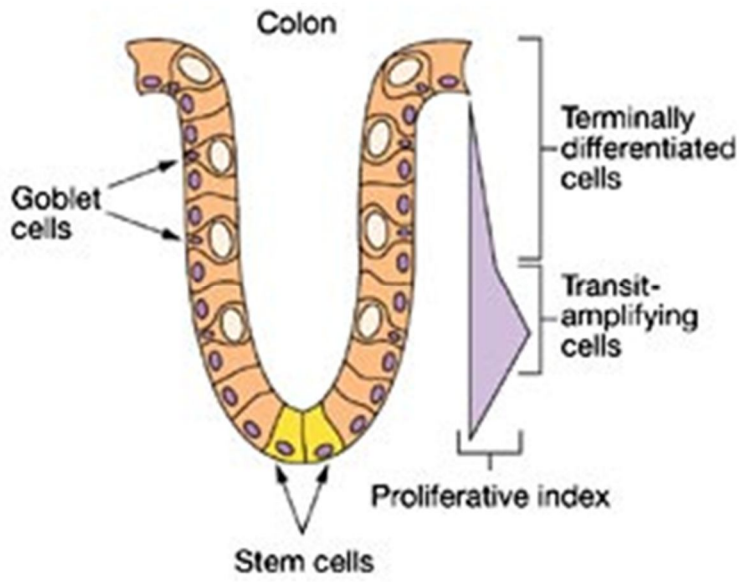

Figure 2. Schematic of the colonic crypt and localization of the stem cell pool. Stem cells are located at the bottom of the crypt. Cell proliferation occurs mainly at the transit-amplifying region. Adapted from McDonald et al.[3]

The colonic crypts are the functional unit of the colon. Normal human colon is composed of millions of such units, each with about 2000 cells. Such cells are constantly renovated, with such turnover normally occurring every two to seven days, increasing under damaging conditions.[4] This renovation of the colonic epithelium is regulated by adult stem cells (SC) and although many discussed about its true localization, it was found that Lgr5-expressing cells at the bottom of the crypt constitute the stem cell niche. These cells were capable of generating all epithelial lineages of the crypt, and were able to re-create a crypt *in vitro.*[5,6] Curiously, recent studies found that Lrig1 identified a different SC population at the bottom of the crypt, at position +4, with a quiescent phenotype.[7] Moreover, Lrig1-expressing cells significantly differ from those expressing Lgr5.[7]

Due to the constant renewing, more than 100 billion of intestinal cells are produced daily, initiating its differentiation along a vertical axis, from the bottom of the crypt, where SC lie,

towards its luminal surface, where they undergo apoptosis (Figure 2). During this process, four types of differentiated epithelial cells are generated: goblet, enteroendocrine, Tuft cells (all secretory cells) and colonocytes (absorptive cells).[8] Furthermore, cells that are in transit from the SC pool and are not yet differentiated in the types mentioned above, called transit-amplifying cells, have the potency to function as SCs when submitted to the right stimulus.[9] Nevertheless, it is still uncertain the importance of transit-amplifying cells for the homeostasis of the colon crypt, with hypothesis that cellular stage is responsible for the renewal of the crypt with rare divisions by SC.[8]

Differentiation of colonic epithelial cells along the vertical axis of the crypt is highly dependent on the epithelial cells themselves and the microenvironment, with particular focus on fibroblasts.[10] Hedgehog, platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP), Wnt/β-catenin, Notch and Eph/Ephrin pathways work cooperatively to construct and renew the crypt, and to define the patterning along the intestine.[11] Communication between pathways is precisely and tightly regulated, often leading to dysplasia and cancer when such crosstalk is disrupted.

Colorectal Cancer

Colorectal cancer (CRC) is the third most common type of cancer worldwide, with almost one and a half million new cases diagnosed every year. Its prevalence and death rate is strongly affected by social and geographic components. While incidence rate is higher in developed countries, the number of deaths is higher in less developed countries, reflecting the inaccessibility to diagnostic tools.[12] Almost 20% of all CRC patients have a familial risk, while 5 to 10% inherit the risk in an autosomal-dominant manner.[13]

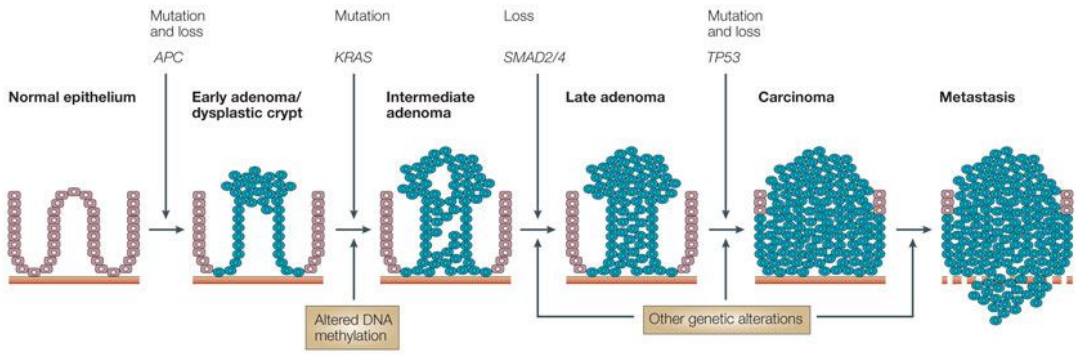

Figure 3. Colorectal cancer progression model. Progressiom from normal epithelium to colorectal carcinoma is characterized by the accumulation of different mutations. From Coleman et al.[14]

The initiation and progression of CRC is still matter of debate.[15] However, the model proposed by Vogelstein *et al.* back in 1988 not only helped the development of CRC research but is also still fairly accepted with minor alterations.[16] Vogelstein model suggests a linear progression, where mutations affecting at least one oncogene and several tumour-suppressor genes are accumulated along the years of cancer development (Figure 3).[17] The first of this multi-stage process is characterized by a hyperproliferation of the crypt and the development of an adenoma, which commonly bear mutations in the *Adenomatous Polyposis Coli* (*APC*) gene or in other Wnt/β-catenin signalling pathway components.[18] Such adenoma develop into an intermediate adenoma, by the accumulation of activating mutations in *KRas* or *BRaf* genes.[17,19] Further development towards carcinoma *in situ* occurs with the accumulation of mutations in, for example, TGF-β signalling pathway (*SMAD-4* and *TGFBR2*), Hedgehog pathway (*GLI1* and *FOXM1*), BMP pathway, *p53*, *DCC*.[20–24]

Adenomatous polyposis coli

Early studies in familial adenomatous polyposis (FAP) identified loss within the long arm of chromosome 5 as the reason behind this disease.[25,26] Such loss lead to the appearance of

numerous colonic adenomas, the precursors of CRC carcinomas, resulting in 100% cases of CRC in these individuals by the age of 40 years.[27] Later it was found that the gene affected in the long arm of chromosome 5 was in fact the *APC* gene, also involved in approximately 85% of the CRC cases.[28,29] Inactivation of APC appears to be an early event in the tumorigenesis process, as loss-of-function somatic mutations occur in 5 % of hyperplastic crypts, 30 to 70% of sporadic adenomas and 72% of sporadic tumours.[16]

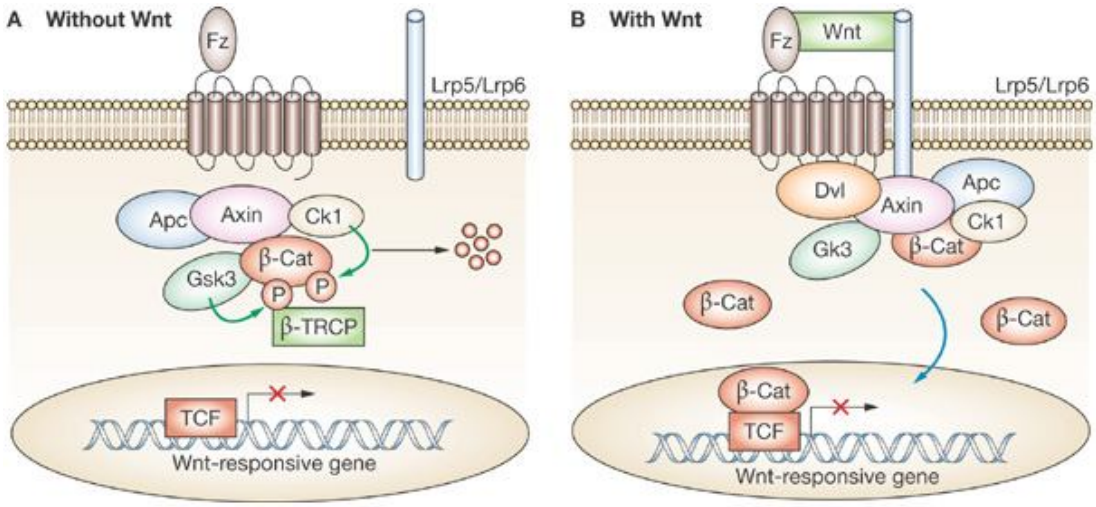

Figure 4. Wnt/β-catenin signalling in normal conditions. Without Wnt signals, APC leads to the degradation of β-catenin. When Wnt signals activate the pathway, β-catenin is released from APC and used as a transcription regulator. When APC is mutated it is unable to bind to β-catenin, and activates the transcription similar to when Wnt signals are present. From Deal, C.[30]

APC is important for the regulation of the Wnt/β-catenin signalling (Figure 4). In normal conditions, APC is complexed with β-catenin, leads to its phosphorylation and posterior degradation by the ubiquitin-proteasome system. When a Wnt signal is present, APC dissociates from β-catenin, which enters the nucleus and activates transcription by association with transcription factors. However, when *APC* is mutated, like in colon cancer, phosphorylation of β-catenin does not occur, therefore it is not degraded and enters the nucleus, leading to the aberrant activation of Wnt/ β-catenin pathway.[18,31,32]

KRas and BRaf

KRas is a small GTPase protein, which function is controlled by the presence of GTP or GDP. In the presence of an extracellular stimulus, guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, activating the protein.[33] This activation leads to the subsequent activation of different downstream pathways, such as RAF-MEK-MAPK,

PI3K/AKT/mTOR or RalGEF/Ral pathways.[33] KRas has a low intrinsic GTPase activity, therefore, after activating the downstream pathways, GTPase activating proteins (GAPs) stimulate the conversion of GTP to GDP, leading to the inactivation of KRas.[33]

KRas mutations are found in different human cancers, like pancreatic, lung cancer and CRC.[34,35] In the specific case of CRCs, it accounts for 40 to 50% of all cases.[36,37] Activating mutations in KRas occur more frequently at codons 12 and 13, but mutations in codon 61 and 146 are also known.[37] The more frequent missense mutations substitute glycine for aspartate (G12D and G13D) or glycine for valine (G12V), which locks KRas in a GTP-bound active state. This lead to the continuous activation of the downstream pathways, responsible for regulating actin organization, cell proliferation and differentiation, cell cycle arrest, prevention of apoptosis and drug resistance (Figure 5).[38–41]

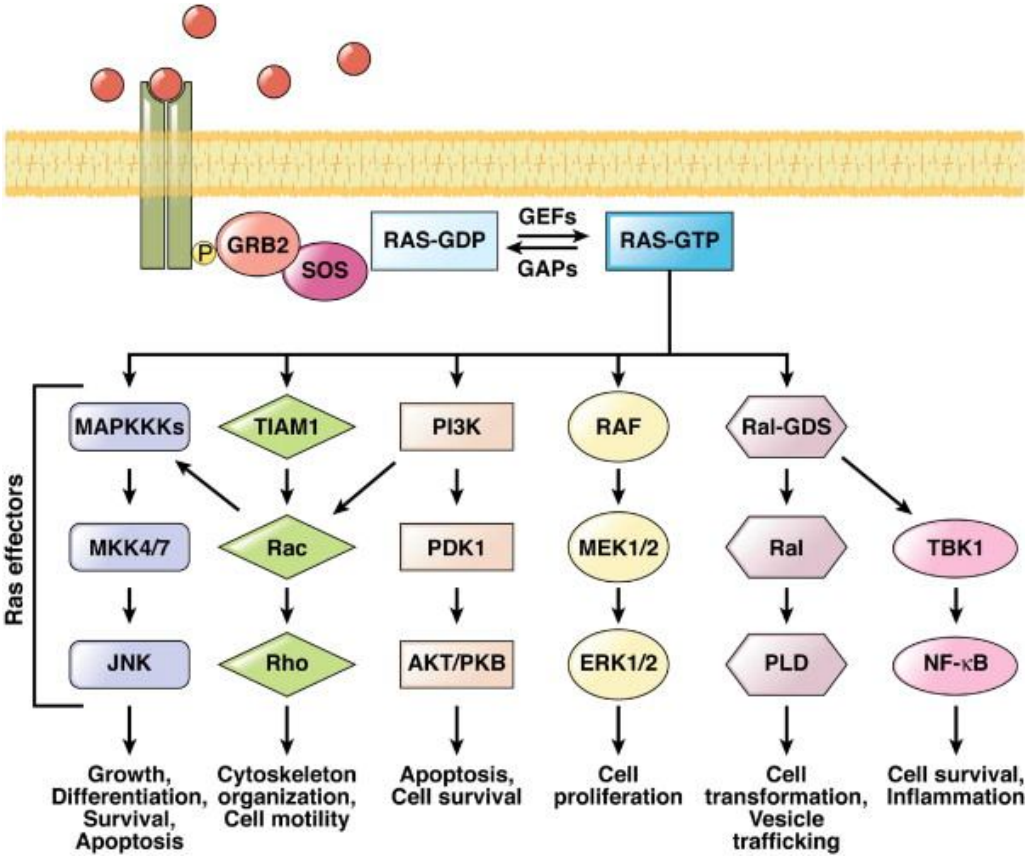

Figure 5. KRas downstream signalling pathways. In normal conditions, growth factors from activate KRas, leading to the activation of the downstream pathways represented. However, when *KRas* is mutated, KRas is constitutively active. From Pino et al.[16]

BRaf is a serine/threonine protein kinase which is a downstream effector of KRas. About 10% of sporadic CRC cases have oncogenic mutations in *BRaf*, being the most common mutation the substitution of valine for glutamate in the codon 600 (V600E).[42,43] Mutations in *KRas* and *BRaf*, and the fact that *KRas* and *BRaf* mutations rarely occur simultaneously, indicate the importance of the RAF/MEK/ERK pathway in CRC.[36,42,44,45]

PIK3CA

Catalytic subunit alpha of type I phosphatidylinositol 3 kinase (PIK3CA) is responsible for the phosphorylation of the phosphatidylinositol and consequently the activation of downstream signalling pathways, like PI3K/Akt/mTOR, which is responsible for regulating cell growth, proliferation and survival.[46] About 20% of CRCs have mutations in *PIK3CA*, with E542K (glutamate to lysine), E545K and H1047R (histidine to arginine) being oncogenic in CRC models and increasing the lipid kinase activity.[21,47] Evidence suggests an association of *PIK3CA* mutations with CRC development and progression. However, concomitant mutation of *KRas* or *BRaf* mutations and *PIK3CA* mutations is found more frequently than PIK3CA mutations alone, challenging the understanding of the true role of PIK3CA in the tumour progression.[48,49]

Cancer stem cells

The elucidation of the morphogenesis of CRC is pivotal for the better understanding of tumour development. However, the origin of the colon cancer is still matter of debate.[50] Shih et al. proposed a top-down model, where the dysplastic cells proliferate at the top of the crypt and displace the normal epithelium.[51] In contrast, Preston et al. defend a bottom-up approach for the CRC initiation, where transformation occurs in the SC population present in the bottom of the crypt, which expands and leads to crypt fission. The bottom-up theory does not neglect the occurrence of top-down phenomena, attributing it the role for tumour expansion by overgrowing adjacent crypts.[52]

Although the controversy is still present, both theories identify the cellular origin of the cancer within SCs. Colon cancer stem cells (CSC) were first identified back in 2007. Dick and De Maria groups identified CSC using a cancer neural SC marker, CD133, and CRC samples.[53,54] Sorting of $CD133^+$ cells and subsequent implantation in immunocompromised mice allowed for the development of tumours using less cells than when $CD133^-$ or even non-separated cells were injected. Moreover, the tumours formed by $CD133^+$ cells reproduced the original tumour, a characteristic attributed only to CSC.[53] These observations support the SC theory for carcinogenesis, which suggests that tumours are generated and maintained by

a small pool of SCs able to self-renew and differentiate into all the tumour cells.[55] Besides being able to generate a whole primary tumour, CSC also have the capacity to circulate in the blood and initiate a tumour elsewhere in the body, giving rise to distant metastasis.[56] Moreover, the presence of CSC within a tumour may also help to explain why cancer therapy often fails to cure patients. In fact, chemotherapy targets rapidly dividing cells, however CSC are often quiescent.[55] Furthermore, CSCs have higher multidrug resistance, increasing their defence against chemotherapy.[55] Also, many pathways commonly mutated in CRC (such as Wnt or p53 pathways) are related with proliferation and self-renewal, which due to its deregulation often leads to the expansion of the cell population, including CSCs.[55] In fact, Wnt/β-catenin signalling is of major importance in the establishment of the SC niche in the bottom of the crypt, as well as to define colon CSC.[18,57]

Cancer stem cell markers

CD133 reliability as a SC marker for all CRCs has been questioned, leading to the proposal of new SC markers, such as CD24, CD44, CD166 and different isoforms of α6 integrin, and the use of multiple SC markers in combination.[58–60]

CD24

CD24 antigen is a sialoglycoprotein commonly expressed at the surface of B cells and granulocytes, but its expression was also observed in the normal epithelium and in some solid tumours.[61]

CD24 has been used, in combination with CD44, to identify $CD44^{+}/CD24^{-}$ cells with tumour-initiating properties in breast cancer cells. Although, such characterization fails to be ubiquitous to all breast cancer cells, indicating other possible combination of markers to identify cells with stem-like properties.[62] Nonetheless, CD24 was also associated with the carcinogenesis of other organs, such as the ovary, prostate and urinary bladder.[63] Moreover, overexpression of CD24 was found to be correlated with poor prognosis in different tumours, which might indicate a role in the tumorigenesis process.[64–66] Such conclusions are in line with the observation that CD24 expression increased with the tumour progression in CRC. Furthermore, CD24 appears to promote cell proliferation through the activation of Raf-ERK and p38 MAPK signalling pathway.[67]

CD44

CD44 belongs to the transmembrane glycoprotein group of cell adhesion molecules (CAM), which mediate cell-cell or cell-matrix interactions. Its involvement in the metastatic process has been proposed as it is associated with an invasive phenotype.[68] CD44 has different

isoforms due to alternative splicing, which differ from each other at the extracellular domain, where a variable region is found. The smallest isoform, which misses the variable domain, is called CD44s. The variable region has 10 different isoforms (v1 to v10 according to the exon transcribed, respectively 6 to 15), and give rise to CD44 variant isoforms (CD44v).[68]

In colon, overexpression of CD44 appears to occur early in the colorectal adenoma-carcinoma sequence, which might indicate an association with early mutational events, such as APC or other deregulation of the Wnt/β-catenin signalling pathway.[69–71] Nonetheless, CD44v6 is normally expressed in the lower crypt epithelium, where normal colonic SCs are found, and has the tumour progress, its expression is observed in the neoplastic tissue.[70] This observation indicates that CD44v6 might be responsible for tumour progression as well as a marker for CSC. Moreover, its interaction with osteopontin (OPN), HGF and its receptor Met, among other cytokines, might reveal its importance in the communication with the tumour microenvironment, as well as in the cell migration and invasion.[68,72]

CD133

CD133, also called Prominin-1, is a transmembrane glycoprotein. Although its mRNA is found in many cell lines, the expression of AC133 (the epitope recognized by CD133 antibodies) is more restricted to undifferentiated cells, possibly indicating a isoform specific for SCs.[73]

CD133 has been used to identify CSC do to its correspondence with normal SC phenotype. In CRC, it has been reported that CD133 identifies cells with increase tumour initiation phenotype, alongside with higher cell motility.[74] Others have noted that, although a AC133 reduction upon CSC differentiation was observed, overall expression of CD133 at the membrane was not altered. One possible explanation for this observation is that different glycosylation patterns might be indicative of SC phenotype rather than the whole CD133 protein expression.[75] However, other authors question the role of CD133 as a valid SC marker, by presenting results that $CD133^+$ do not present tumour-initiating capacity nor radiotherapy resistance.[76] Moreover, others report that $CD133^-$ are also able to develop tumours in immunocompromised mice and that such tumours are more aggressive than those formed by $CD133^+$ cells.[77] Therefore, although the use of CD133/AC133 as a SC marker already lead to the identification of CSC in different types of tumours, its reliability as a good marker is not yet validated.[4,74,76,77]

CD166

CD166, also known ALCAM (Activated Leukocyte Cell Adhesion Molecule), is a transmembrane glycoprotein involved in cell-cell adhesion, thought to have a role in processes such as development, maintenance of the tissue integrity and tumour progression. Its expression during development occurs mostly in proliferating cells, with an important function during neurogenesis. [78]

Due to its importance in cell adhesion, its role in cell migration in tumours has been addressed. The function of CD166 seems to be conserved in tumours of different progression stages, suggesting that it is not involved in the invasive process.[78] However, others have found that antibody inhibition of CD166 lead to reduction of cell invasion and tumour growth in CRC, supporting its role as a pro-invasive molecule.[79]

Furthermore, its use as a SC marker is also questionable has its use has been based on its presence during normal development and the prognosis association in different cancers. Haraguchi et al. observed that CD166 had a small variation after differentiation induction of CRC cell lines, compared with other SC markers.[60] Moreover, downregulation of CD166 was observed in blood circulating tumour cells, although such cells also expressed higher levels of other SCs markers.[80] However, other have suggested the role of the downregulation of CD166 in the establishment of a more invasive and aggressive phenotype, once again questioning the value of CD166 has a CSC marker.[78]

Integrin α6

Integrin α6, also known as CD49f or ITGA6, is a transmembrane protein that regulates cell-matrix interactions in epithelial cells when in a complex with other integrins such as integrin β4, also known as CD104 or ITGB4, or integrin β1.[81] It is known to be involved in tumour cell invasion and migration in CRC.[81,82] Due to its involvement in the metastatic process, the possibility to work as a CSC marker was evaluated in different cancers. In breast cancer, CD49f was associated with increase SC activity and invasion. [83] In CRC, it was observed that CD49f, along with CD44, revealed a high variation in the expression profile when the differentiation was induced, showing a decrease of 74,4% and 98,2%, respectively. [60] Moreover, observations made in $CD44^{high/}CD24^{low}$ breast cancer cells indicated that the isoform α6A was associated with an epithelial phenotype and α6B with mesenchymal properties. Furthermore, integrin α6B was responsible for CSC phenotype and tumour initiation, which integrin α6A was unable to perform.[84] Interestingly, in CRC integrin α6A appears to be responsible for the regulation of proliferation, through the involvement with Wnt/β-catenin signalling pathway, while integrin α6B was found in quiescent and differentiated epithelial cells.[85,86]

Intestinal stem cell markers

Normal intestinal SC markers have been extensively studied to identify the stem population within the colon crypt. But the overexpression of some of these markers has also been associated with poor prognosis and chemotherapy resistance in CRC, which opens new doors for the study of CSCs.[87]

BMI1

BMI1, known as B cell-specific Moloney murine leukemia virus insertion site 1, is a polycomb repressive complex 1 component, involved in the regulation of the gene silencing by modifications in the chromatin.[87] It targets a locus encoding important proteins involved in retinoblastoma (Rb) and p53 signalling pathways, both deregulated in many types of cancer including CRC.[88] It is localized in the bottom of the colon crypt, at cells called +4 putative SCs, characterized for having quiescent phenotype and regenerative potential.[89,90]

Although some were able to correlated BMI expression with CRC progression, BMI expression at the protein and mRNA levels have been evaluated as a prognostic tool, with conflicting results.[91] While some identified BMI1-positive tumours with a higher tendency for metastasis and tumour recurrence, others reveal that high BMI1 expression correlates with a better prognosis.[92,93]

LGR5

LGR5, or Leucine-rich repeat-containing G-protein coupled receptor 5, is expressed at the bottom of the colon crypt, possibly identifying columnar cells with stem properties.[6] Although LGR5 has been identified as a Wnt/β-catenin target gene, which supports its use as a SC marker, the regulation of such signalling through LGR5 is still under debate.[87,94]

Nonetheless, LGR5 silencing has been reported to reduce colony formation, alongside decreased proliferation, migration and increased apoptosis, revealing its role as a CRC SC marker.[95] Moreover, increased levels of LGR5 mRNA in blood from CRC patients strongly correlates with poor outcome, possibly reflecting the stem-like phenotype from circulating tumour cells.[96] However, others found that $LGR5^-$ cells are also able to initiate tumour, as well as reveal resistance to radiotherapy.[97] Furthermore, Walker et al. also found that LGR5 negatively regulates tumorigenesis and cell adhesion, questioning the function of LGR5 in the maintenance/establishment of SC phenotype.[98] These results support the idea that different SC markers might identify cells present in different positions of the colon crypt, all with stem properties.[89,97]

LRIG

LRIG, also known as leucine-rich repeats and immunoglobulin-like domains, is a transmembrane family composed of three members. LGR1 is known for being a negative regulator of tyrosine kinase receptors, such as ErbB, Met and Ret receptors, by enhancing its lysosomal degradation.[99] Although in different cancers LRIG1 expression appears to be decrease, in CRC its expression is heterogeneous.[100] Nonetheless, LRIG1 knockdown led to the upregulation of Met receptor in intestinal epithelia and to expansion of intestinal SCs, leading to crypt dysplasia.[7,101] Powell et al. also described Lrig1 as an intestinal SC marker that identifies cells with a different transcriptome that $Lgr5^+$ cells. Moreover, APC loss in $LRIG^+$ cells lead to the development of histologically advanced adenomas.[7]

OLFM4

OLFM4, known as olfactomedin-4, is a glycoprotein that has been proposed as an intestinal SC marker for LGR5 SCs by histochemical studies.[102] Although its function is still to be elucidated, studies with members of the same family in *Xenopus* proposes role as a BMP antagonist.[102,103]

In CRC, OLFM4 is highly expressed in colon carcinomas, comparably to the adjacent normal tissue.[102] Some have associated high expression of OLFM4 with early stage CRC, with a decrease or complete loss in its expression in later stages.[104,105] Moreover, recent studies demonstrate that Olfm4 deletion in $Apc^{min/+}$ mice leads to the development of adenocarcinoma and upregulation of Wnt/β-catenin signalling genes.[103]

NANOG

Nanog is a transcription factor crucial for the maintenance of the embryonic SC phenotype and used to induce pluripotency in differentiated cells.[106] Studies in colorectal cell lines found that Nanog is expressed in six cell lines evaluated, with two presenting cytoplasmic expression and the remaining four presenting nuclear localization. Moreover, inhibition of Nanog lead to growth inhibition both *in vitro* and *in vivo*.[107] Studies evaluating co-localization of LGR5 and Nanog found that Nanog was not expressed in SC clusters of poorly differentiated colon cancer nor in normal colonic tissue, while being expressed mainly in SC clusters of highly differentiated tumour. Furthermore, LGR5 did not co-localized with Nanog in such highly differentiated tumours.[108]

It is also proposed that Nanog expression is a later event during tumour development, with a role as a driving force for the development of malignant metastatic colon cancer, opening the possibility of being used as a prognosis biomarker.[108] Thus, Nanog has been related with

the prognosis of many types of cancer, and in the specific case of CRC, its presence has been associated with tumour progression and a poor outcome.[109]

Curiously, the induction of p53 was described has responsible for the reduction of Nanog expression, in normal SCs.[110] Also, p53, GLI1 and NANOG form a network responsible for the regulation of apoptosis and CSC maintenance.[111,112] Together with p53, Nanog is also know to interact with focal adhesion kinase (FAK), playing a role in cell survival.[113]

EPHB2

EPHB2, or ephrin B2 receptor, is a tyrosine kinase receptor involved in cell signalling pathways during development.[114] In the intestine, EphB receptors are responsible for the positioning of cells within the SC niche. Moreover, EphB signalling coordinate cell migration and proliferation in the intestinal SC niche, through the regulation of the cell cycle re-entry.[115]

In CRC evidences suggest that EphB2 expression marks colon progenitor cells, but its expression declines over tumour progression. Interestingly, different EphB receptors appear to have different gene regulation mechanisms, with EphB2 being regulated by TCF/β-catenin signalling.[116] Reduced expression of EhpB2 expression is observed in later stages of CRC and associated with invasion and metastasis.[117] Moreover, high levels of expression of EphB2 in CRC patients correlates with better prognosis.[118] Some have proposed a model where some Eph genes, including EphB2, became upregulated in the transition from normal colon into an early stage CRC, and eventually a downregulation as consequence of the progression towards late stages CRC.[114]

Tumour Microenvironment

The tumour microenvironment (TME) has gain special attention from researchers as its importance during the course of tumorigenesis increased. The idea that a tumour is a homogeneous bulk of cancer cells is reductionist, as not only many different types of cancer cells have been identified within the same tumour, but also "normal" cells are present and can contribute for either tumour regression or progression.

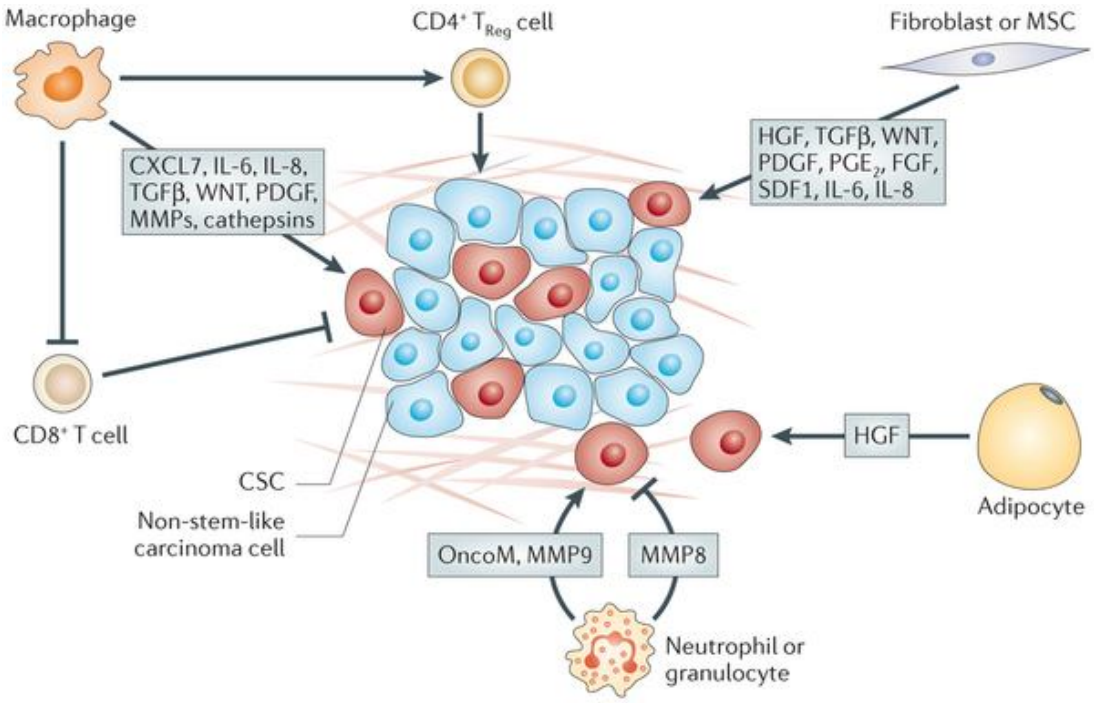

Figure 6. Tumour microenvironment interplay with cancer cells and cancer stem cells. Stromal cells secrete factors that influence the formation of CSC and help maintain stemness. From Pattabiraman et al.[119]

The TME is composed not only by the cancer cells and CSC, but also by endothelial cells and pericytes that form and sustain the vasculature, immune cells that react to the presence of the foreign body, extracellular matrix which physically supports for the cells to rest, and importantly cancer-associated fibroblasts which give support to the tumour and constitute its main stromal population.[120]

Many characteristics of tumour progression, and even therapeutic responses, can be modulated by TME. For instance, its known that immune cells are able to selectively kill cancer cells, but they can also potentially select cancer cells with high malignancy, which are

able to evade immune detection.[121] Different activation patterns of macrophages have different effects on tumour growth and progression.[122] Furthermore, it was found that stable vasculature induced dormancy in cancer cells, while neovasculature, characteristic of tumours, allowed cells to grow.[123] Fibroblasts have also an important role in tumour progression and patient outcome. Its known that cancer-associated fibroblasts (CAF) are able to modulate the therapeutic response of tumour cells, regulate stemness, promote tumour progression and metastasis.[57,124–127]

Normal Fibroblasts and Cancer-associated Fibroblasts

Fibroblasts are present in the lamina propria and are α-smooth muscle actin (α-SMA) negative.[128] Pericryptal fibroblasts (PCF), surround the crypts and are adjacent to the colonic epithelium. PCFs are classified as myofibroblasts due to the expression of α-SMA, vimentin and are desmin-negative.[129] The position of the PCFs relative to the colonic epithelium allows the intercommunication between the SCs and the cells in differentiation therefore being important for the SC niche maintenance, regulation of replication, differentiation and migration of the normal cells.[130]

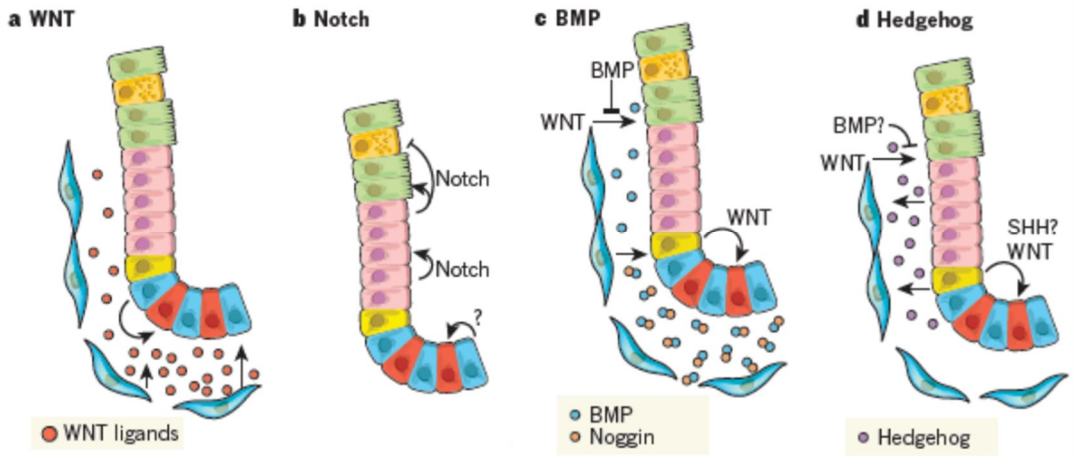

Figure 7. Wnt/b-catenin, Notch, BMP and Hedgehog pathways. Fibroblasts create gradients of different proteins, in order to induce differentiation along the vertical axis of the crypt, while maintaining the stem cell pool at the bottom of the crypt. Adapted from Medema et al.[10]

The architecture of the colon crypt reveals the importance of fibroblasts in intestinal homeostasis, where they regulate different signalling pathways to induce the differentiation of cells along the vertical axis of the crypt. Fibroblasts at the bottom of the crypt secrete Wnt

ligands, leading to the activation of Wnt/β-catenin signalling and inducing SC properties. Such secretion of Wnt ligands at the bottom of the crypt creates a decreasing gradient towards the top of the crypt that, in combination with Notch pathway activation in the epithelia, drives proliferation at the bottom of the crypt, where Wnt signals are high, and enterocyte differentiation at the top of the crypt, where Wnt/β-catenin signalling is reduced.[10] BMPs are also secreted by fibroblasts at the bottom of the crypt, but its function is blocked by the simultaneous secretion of BMP inhibitors like noggin and Gremlin proteins. [10] At the top of the crypts, fibroblasts secrete BMPs, but not BMP inhibitors, leading to the inactivation of Wnt/β-catenin signalling, halting proliferating, and inducing cell differentiation.[131] The Hedgehog signalling also has a role in the crypt differentiation, where Indian Hedgehog (IHH) is secreted by the epithelia at the top of the crypt, inducing the release of BMPs by surrounding fibroblasts, possibly to inhibit the Wnt/β-catenin signalling.[132,133] The involvement of different and important signalling pathways prompt to conclude that disruption of such precise regulation frequently leads to neoplasia.

CAFs, also known as activated fibroblasts or myofibroblasts, are identified using α-SMA, fibroblasts activation protein (FAP-α), fibroblast-specific protein-1 (FSP-1) and platelet-derived growth factor receptor β (PDGFR- β).[134] In the context of cancer, the number CAFs are increased and α-SMA^{-} fibroblasts are differentiated into α-SMA^{+} phenotype.[135] Moreover, CAFs can also be originated from mesenchymal SCs differentiation, due to recruitment of such cells by the tumour microenvironment.[136]

CAFs play a role in supporting different aspects of the tumour. They are able to sustain proliferation, to induce resistance to cell death and evade growth suppression. Colonic CAFs are known to secrete HGF, epidermal growth factor (EGF), insulin-like growth factor 1/2 (IGF1/2), prostaglandin-E (PGE-2), PDGF and vascular endothelial growth factor (VEGF). Such ligands are able to activate the RAF-MEK-MAPK and PI3K/AKT/mTOR pathway, leading to regulation of cell proliferation, survival and invasion, among others.[137–139]

TGF-β secreted from CAFs promotes metastasis formation.[140] Interestingly, incubation of CAFs with CRC cells conditioned media increased the TGF-β secretion.[141] The treatment of CAFs with TGF-β also increases the expression of type I collagen, fibronectin, metalloproteinases, tenascin-C and laminin-B1, ECM proteins involved in invasion and metastasis.[134,141] On the other hand, the treatment of CRC cells with conditioned media from CAFs induced their invasion and migration.[129,142,143] Such communications are an example of the crosstalk between tumour cells and CAFs in order to promote tumour progression.

RATIONAL AND AIMS

CSC are known to be capable to initiate tumour growth, being therefore associated with recurrence and metastasis. Some recent studies suggest that *KRas* mutation on APC loss background enhanced the CSC pool activation, and therefore tumour growth. Moreover, it was also found that this activation of CSC was attained through the activation of Wnt/β-catenin and Ras/ERK/MEK pathways.[144] Therefore, in the first part of this work we questioned if *BRaf* and *PIK3CA* activating mutations, which are KRas downstream targets important for CRC progression, are also able to modulate the CSC pool like *KRas* mutations.

In the normal homeostasis of the colon crypt, fibroblasts and SCs crosstalk in a tight and precise manner to control differentiation, in order to renew the colonic epithelium and to maintain the SC pool. However, such fibroblasts are also known to alter their phenotype in a cancer context, and become supportive of the disease.[10] In fact, Vermeulen et al. found that CAFs were able to modulate the Wnt/β-catenin in CSC, being important to confer stemness to cancer cells.[57] Due to the fact that CAFs can modulate stemness, we questioned if that modulation of stemness can be revealed by alterations in different CSC markers. Moreover, due to the involvement of KRas in the modulation of stemness, we ought to know if it can alter the response of KRas-mutated cells to CAF-released factors.

To address our questions, we aimed to:

1. Clarify the role of KRas activation in the modulation of different SC markers
2. Understand the role of BRaf and PIK3CA as alternative or complementary pathway for the modulation of SC markers
3. Study the response of *KRas* mutated cells to activated fibroblasts in terms of modulation of different SC markers

MATERIALS AND METHODS

Cell culture

Human colorectal cancer cell lines HCT-116, HCT-15, SW480 and RKO, which present different mutations (Table 1), were available from the IPATIMUP cell bank. Cells were routinely maintained at 37°C and 5% CO2 in the following media (Invitrogen Ltd, UK): RPMI-1640 for HCT-116, HCT-15 and SW480 and DMEM for RKO. All media contained 10% heat-inactivated fetal bovine serum (FBS) (Greiner bio-one, Belgium) and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, USA).

Table 1. Genetic characteristics of four colorectal cancer cell lines. Abbreviations: wt – wild-type, MSI – microsatellite instability, MSS – microsatellite stability, CIN – chromosomal instable, CSS – chromosomal stable. Adapted from Ahmed et al.[145]

Cell Line	Mutations		
	KRas	BRaf	PIK3CA
HCT-15	G13D	wt	E545K;D549N
HCT-116	G13D	wt	H1047R
SW480	G12V	wt	wt
RKO	wt	V600E	H1047R

The normal human intestinal fibroblasts-CCD18-Co cell line was purchased from ATCC. Cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Gibco) in a humidified incubator at 37ºC with 5% carbon dioxide. The medium was renewed twice a week and when the confluence reached values close to 80% the cells were subcultured and the same number of cells were divided to two T75 flasks. When the confluence was reached, cells were washed twice with PBS1x and the following conditions were used:

- 1 flask with DMEM supplemented only with 1% penicillin/streptomycin
- 1 flask with DMEM supplemented only with 1% penicillin/streptomycin plus
- 10ng/mL rhTGF-β1 (Immunotools)

In parallel the same conditions were employed using culture flasks with no cells. After four days the conditioned media from all the conditions were harvested, centrifuged at 1200rpm during 5 minutes, filtered through a 0,2µm filter and stored at -20ºC. The cells were tripsinized and counted, and total protein extraction was performed. The confirmation of

fibroblasts' activation was assessed through the evaluation of alpha smooth muscle actin (α-SMA) expression by western blot.

Primary Antibodies

The primary anti-human antibodies shown in Table 2 were used for western blot and flow cytometry.

Table 2. Antibodies used for flow cytometry and western blot studies. FC – flow cytometry, WB – western blot

Antibody	Clone	Cat. No	Manufacturer	Dilution	Application	Blocking agent
CD24 PE-conjugated	-	555428	BD Pharmingen, USA	1:100	FC	PBS + 0,1% FBS
CD44 FITC-conjugated	-	130-098-210	Miltenyi Biotec, Germany	1:100		
CD44v6	2F10	BBA13	R&D Systems, USA	0,25µg/10^6 cells		
CD133/1 APC-conjugated	AC133	130-098-847	Miltenyi Biotec, Germany	1:100		
CD166 APC-conjugated	-	130-106-619	Miltenyi Biotec, Germany	1:100		
Mouse IgG1-PE	-	130-098-845	Miltenyi Biotec, Germany	1:100		
Mouse IgG1-FITC	-	130-098-847	Miltenyi Biotec, Germany	1:100		
Mouse IgG1-APC	-	130-098-846	Miltenyi Biotec, Germany	1:100		
REA Control (S)-APC	-	130-104-614	Miltenyi Biotec, Germany	1:100		
E-cadherin	HECD1	13-1700	Invitrogen Ltd, UK	1:100		
c-Met	C-12	SC-10	Santa Cruz Biotechnologies, USA	1:1000	WB	Milk 5%
CD49f (integrin α6)	-	HPA012696	Sigma Life Science, Sweden	1:1000		BSA 4%
CD104 (integrin β4)	H-101	SC-9090	Santa Cruz Biotechnologies, USA	1:5000		BSA 4%
p-Src (Tyr416)	-	2101	Cell Signaling Technology, USA	1:1000		BSA 4%

Src	-	2108	Cell Signaling Technology, USA	1:1000		BSA 4%
p-ERK (Thr202/Tyr204)	D13.14.4E	4370	Cell Signaling Technology, USA	1:1000		BSA 4%
ERK	-	9102	Cell Signaling Technology, USA	1:2000		Milk 5%
p-Akt (Ser473)	D9E	4060	Cell Signaling Technology, USA	1:2000		BSA 4%
Akt	-	9272	Cell Signaling Technology, USA	1:1000		Milk 5%
B-Raf	F-7	SC-5284	Santa Cruz Biotechnologies, USA	1:1000		BSA 4%
PIK3CA	C73F8	4249	Cell Signaling Technology, USA	1:1000		BSA 4%
GAPDH	0411	SC-47724	Santa Cruz Biotechnologies, USA	1:10000		Milk 5%
α-Tubulin	DM1A	T6199	Sigma Life Science, Sweden	1:10000		Milk 5%

siRNA transfection

Gene silencing was performed using a pool of 4 small interfering RNAs (siRNA), specific for *KRAS* (L-005069-00-0010), *BRAF* (L-003460-00-0010) and *PIK3CA* (L-003018-00-0010). All siRNAs are ON-TARGETplus SMARTpool, from Dharmacon, GE Healthcare, USA. Transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, UK), according to manufacturer's recommended procedures. Briefly, 250µL of optiMEM and 3µL of Lipofectamine RNA iMAX were mixed and incubated for 5 min. To that solution was added a mixture of 1µL of siRNA and 250µL of optiMEM and incubated for additional 20 minutes. 500µL were added per well in a 6-well plate. siRNA final concentration was 10nM. A scrambled siRNA sequence, with no homology to any gene, was used as a negative control (Qiagen, USA) at the same concentration as the siRNA targeting the genes of interest. Subsequent studies were performed after 72 hours of cell transfection, except when referred.

Protein Extraction and Western Blotting

Protein lysates were prepared from cells, using RIPA lysis buffer [50mM TrisHCl pH 7.5, 1% (v/v) NP-40 (Sigma-Aldrich, USA), 150mM NaCl and 2mM EDTA] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany) and 1:100 phosphatases inhibitors cocktail (Sigma Aldrich, Israel). Cells were lysed with the help of a scraper and centrifuged at 14000 rpm at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (BioRad Protein Assay kit, USA). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 min at 95°C. Samples were separated by SDS–PAGE and proteins were transferred into nitrocellulose membranes [Amersham Protran Premium 0.45µm nitrocellulose blotting membranes]. For immunostaining, membranes were blocked with 5% (w/v) non-fat dry milk or 4% (w/v) bovine serum albumin in PBS containing 0.5% (v/v) Tween20 and incubated overnight with primary antibodies. After washing with PBS-Tween20, membranes were incubated with HRP-conjugated anti-mouse or rabbit secondary antibodies (Santa Cruz Biotechnologies, USA). Proteins were then detected using ECL reagent (Clarity Western ECL Substrate, Bio-Rad, USA) as a substrate. Quantity One software (Bio-Rad, USA) was used for quantification of the differences in protein expression comparing with GAPDH or tubulin expression.

RNA extraction, PCR and quantitative Real-Time-PCR

TripleXtractor (Grisp, Portugal) was added to the cells and cell lysate was collected. RNA was isolated according to the manufacturer's recommended procedure. cDNA was synthesized using qScript XLT cDNA SuperMix (Quanta BioSciences, USA) using 1µg of total RNA. Quantitative-Real-Time-PCR (qRT-PCR) reaction was performed with TaqMan Gene Expression Assays (Applied Biosystems, USA), using gene-specific probes shown in Table 3. Briefly, 0,5µL of cDNA, 4,5µL of DNAse/RNAse free water, 5µL of TaqMan Universal PCR Master Mix No AmpErase (Applied Biosystems, UK) and 0,5µL of probe were added to each well, in triplicate for each sample.

Table 3. Genes and probe reference used for quantitative Real Time PCR.

Gene	Reference	Manufacturer
KRAS	Hs00270666_m1	Applied Biosystems, USA
LGR5	Hs00969422_m1	
LRIG1	Hs00394267_m1	
CCND1	Hs00277039_m1	
OLFM4	Hs.PT.58.19581735	Integrated DNA Technologies, Inc., USA
EPHB2	Hs.PT.58.19695111	
NANOG	Hs.PT.58.21480849	
BMI1	Hs.PT.56a.18691455	
GAPDH	Hs.PT.39a.22214836	

Analysis was performed with the ABI PRISM 7700 Sequence Detection System Instrument and software (Applied Biosystems, USA), following the manufacturer's recommendations. The internal standard human *GAPDH* was used to normalize cDNA quantity. Data was analysed by the comparative $2^{(-\Delta CT)}$ method.

Colosphere forming efficiency (CFE) assay

After 48h of the siRNA transfection, cells were enzymatically harvested using Trypsin, manually dissociated with a 25-gauge needle to form a single-cell suspension, and resuspended in cold PBS. Cells were plated at a density of 500 cells/cm^2 in non-adherent culture conditions, in 6-well plates coated with 1.2% poly(2-hydroxyethylmethacrylate)/95%ethanol (Sigma-Aldrich, USA) and allowed to grow for 5 days, in phenol red-free RPMI-1640 containing 1x B27 supplement (Life Technologies, UK),

1x N2 supplement (Life Technologies, UK), 20 ng/ml EGF (Sigma-Aldrich, USA), 10 ng/mL bFGF (Life Technologies, UK) and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, UK) in a humidified incubator at 37°C and 5% (v/v) CO_2. Colosphere forming efficiency was calculated as the number of spheres (≥50 µm) formed divided by the number of cells plated, being expressed as a percentage.

Flow Cytometry

For flow cytometry analysis, cells were harvested with trypsin/EDTA or, for E-cadherin analysis, Versene (Life Technologies, UK), washed with PBS supplemented with 0.5% FBS and re-suspended in the wash buffer. Single cell suspension was labelled with PE-conjugated CD24, FITC-conjugated CD44, APC-conjugated CD133, REA-(S)-APC-conjugated CD166, E-cadherin (HECD1) and CD44v6 antibodies. Fluorochrome-conjugated antibodies were incubated at room temperature, in the dark, for 15 minutes. For HECD1 and CD44v6 staining, primary antibodies were incubated at 4°C, in the dark, for 1 hour and washed twice. Secondary Alexafluor488-conjugated goat anti-mouse IgG (Invitrogen, UK) was incubated for 30 min, in the dark, at 4°C in a 1:250 dilution. Labelled cells were then washed in wash buffer and analysed on a FACS Canto-II or BD Accuri C6 (BD Biosciences, USA). Data was analysed used FlowJo cytometry analysis program.

Statistical Analysis

Results are representative of three or more independent experiments. Quantifications are expressed as mean ± SD of the biological replicates considered. Statistical analyses were performed using GraphPad Prism. For all data comparisons, the two-sided Student's t-Test was used and considered as significant when *P* value was less than 0.05.

RESULTS

PART 1 – Role of oncogenic alterations in the stem cell pool modulation

In the first part of this work, we aimed to understand if *BRaf* and *PIK3CA* mutations, which are downstream components of the KRas signalling, affect the cancer stem cell population similarly to what was described for mutant *KRas*.[144] To do so, we started by analysing, using flow cytometry, alterations in cancer stem cell markers upon inhibition of *KRas*, *BRaf* and *PIK3CA* expression, whether mutated or not. Alterations in the stem cell markers were observed, leading us to understand if the capacity of developing colospheres was in fact affected by such alteration. Moreover, an exploratory approach targeting other cancer stem cell and intestinal stem cell markers was performed, through western blot and real time quantitative PCR, in order to identify viable stem cell markers.

I – *KRas* is the only oncogene capable of modulating cell surface expression of cancer stem cell markers

The expression of different stem cell markers was evaluated in a set of colorectal cancer cell lines with distinct mutational profiles (Table 1).

CD44 expression was studied upon *KRas* and *BRaf* in HCT-116 cell line (Figure 8 and Figure *9*, and supplementary Figure S 1 and Figure S 2). In addition to the previous markers, CD24, CD133 and CD166 were also evaluated in *PIK3CA*-inhibited HCT-116 cell line (Figure 10, and supplementary Figure S 3). Alterations in the expression of cancer stem cell markers were only observed after *KRas* inhibition. CD44 expression presented a drastic decrease clearly observed by the enrichment in the $CD44^-$ ($P<0,0001$) population (Figure 8B). No alterations are observed in any cancer stem cell marker in either *BRaf* or *PIK3CA*-inhibited HCT-116 cells (Figure 9 and Figure *10*).

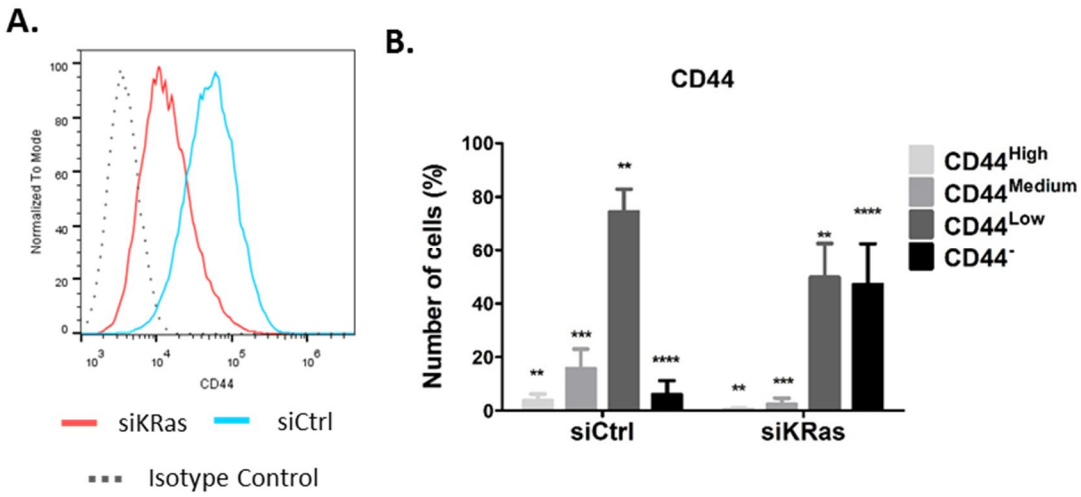

Figure 8. Expression of CD44 in *KRas*-inhibited HCT-116 cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, CD44 n=7

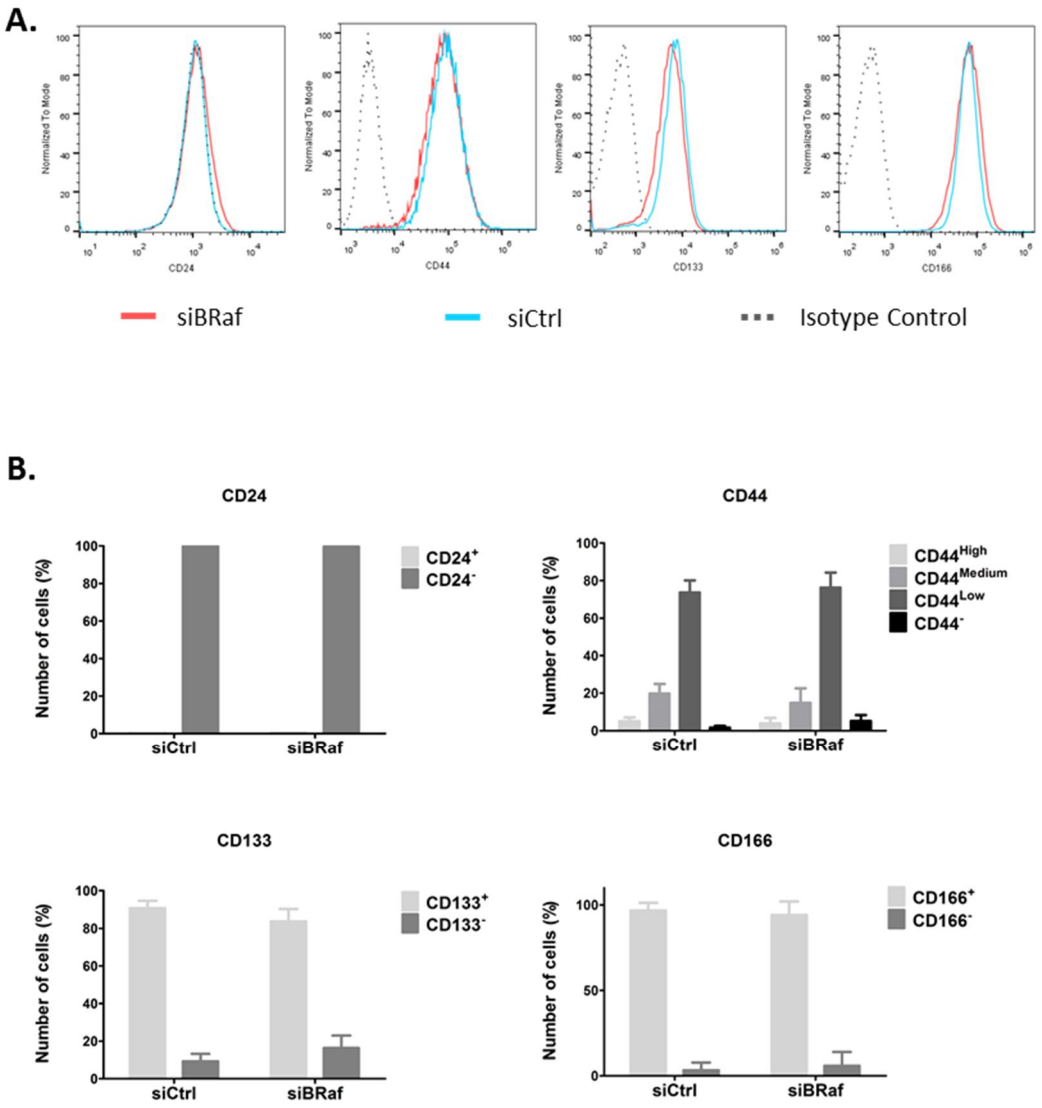

Figure 9. Expression of CD44, CD133 and CD166 in *BRaf*-inhibited HCT-116 cell line. A. Representative fluorescence histograms. **B** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=2, CD24 n=1.

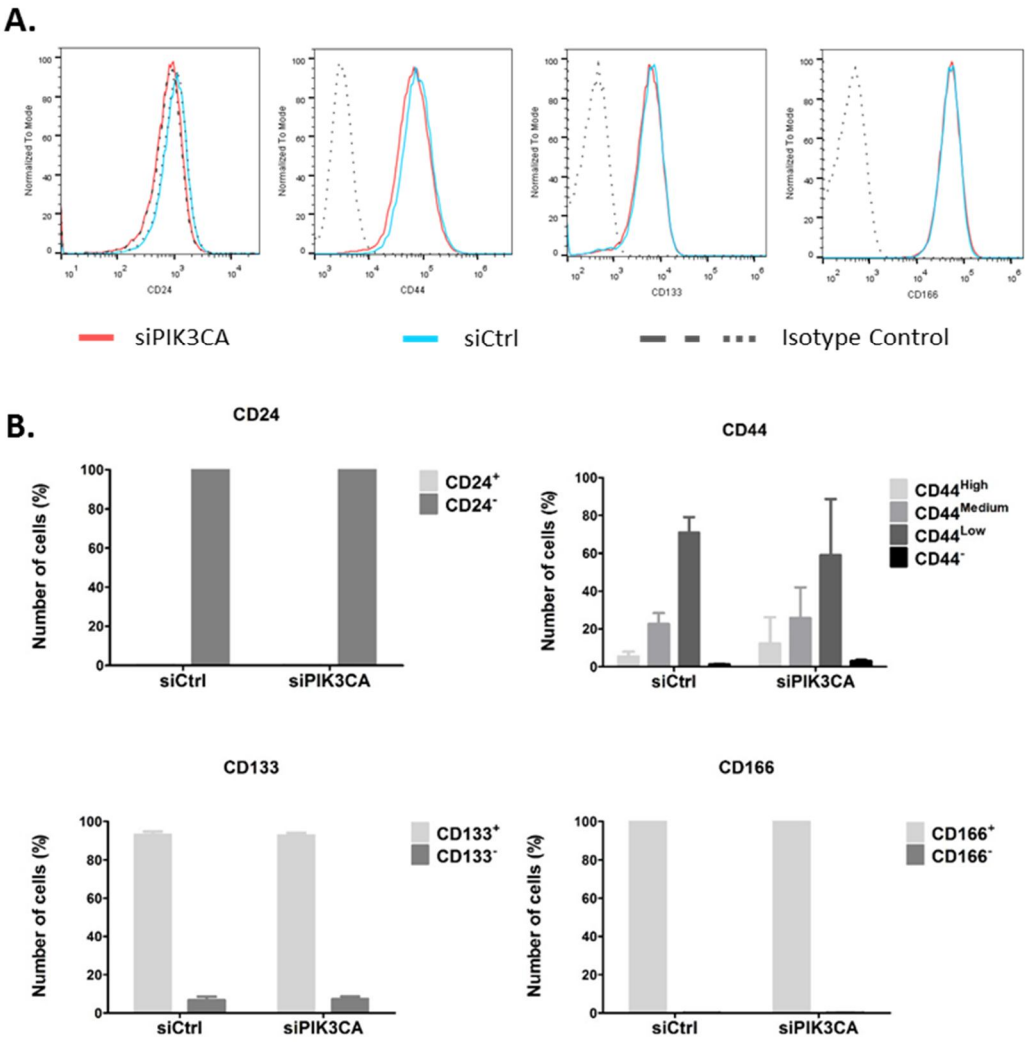

Figure 10. Expression of CD24, CD44, CD133 and CD166 in *PIK3CA*-inhibited HCT-116 cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=2.

The same markers were evaluated in another *KRas* mutated cell line, SW480 cell line. Curiously, no alterations in all the markers evaluated were observed, either after *KRas* (Figure 11 and supplementary Figure S 4) or *PIK3CA* inhibition (Figure 12 and supplementary Figure S 5).

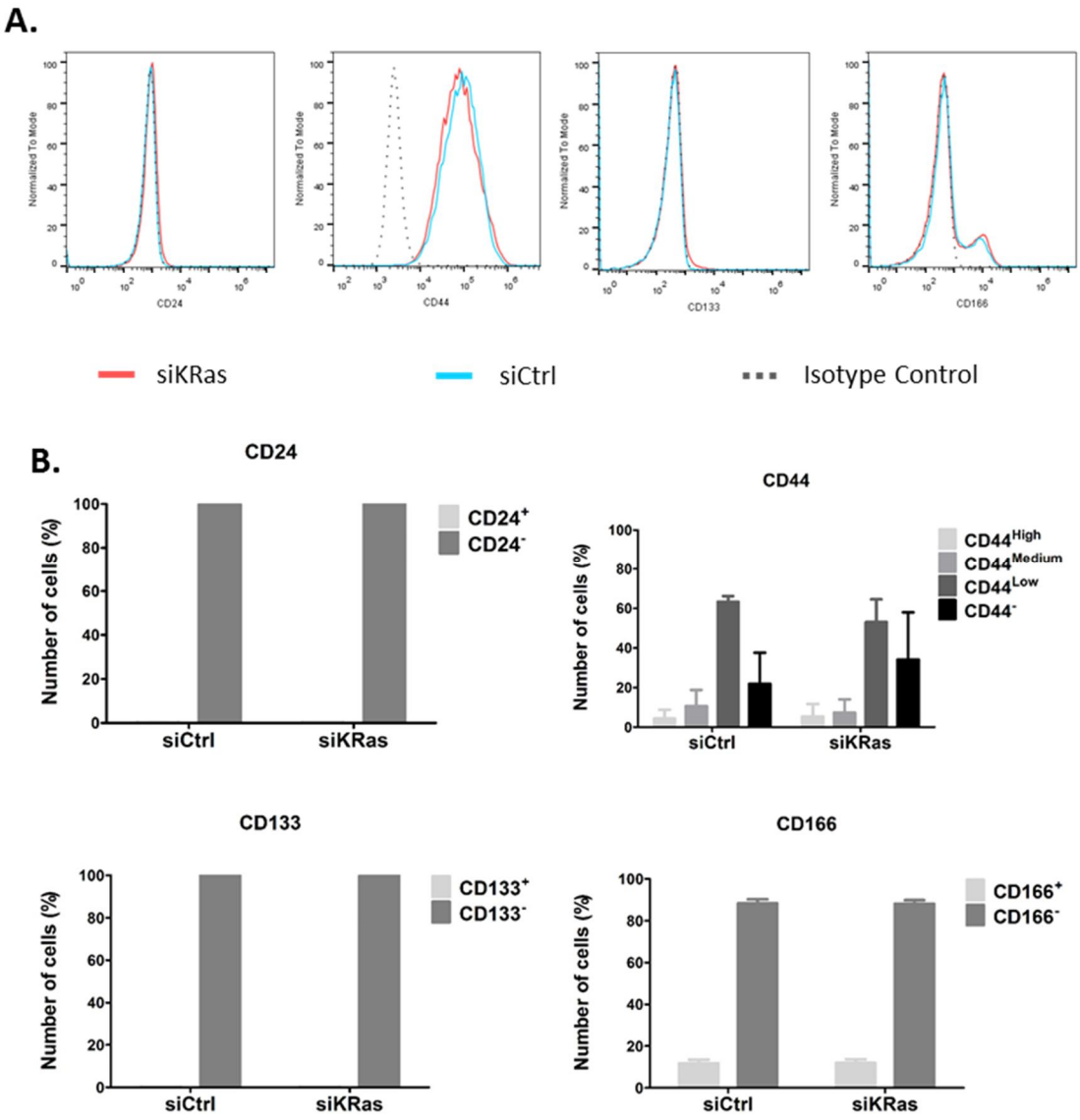

Figure 11. Expression of CD24, CD44, CD133 and CD166 in *KRas*-inhibited SW480 cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=3, and CD24 n=2.

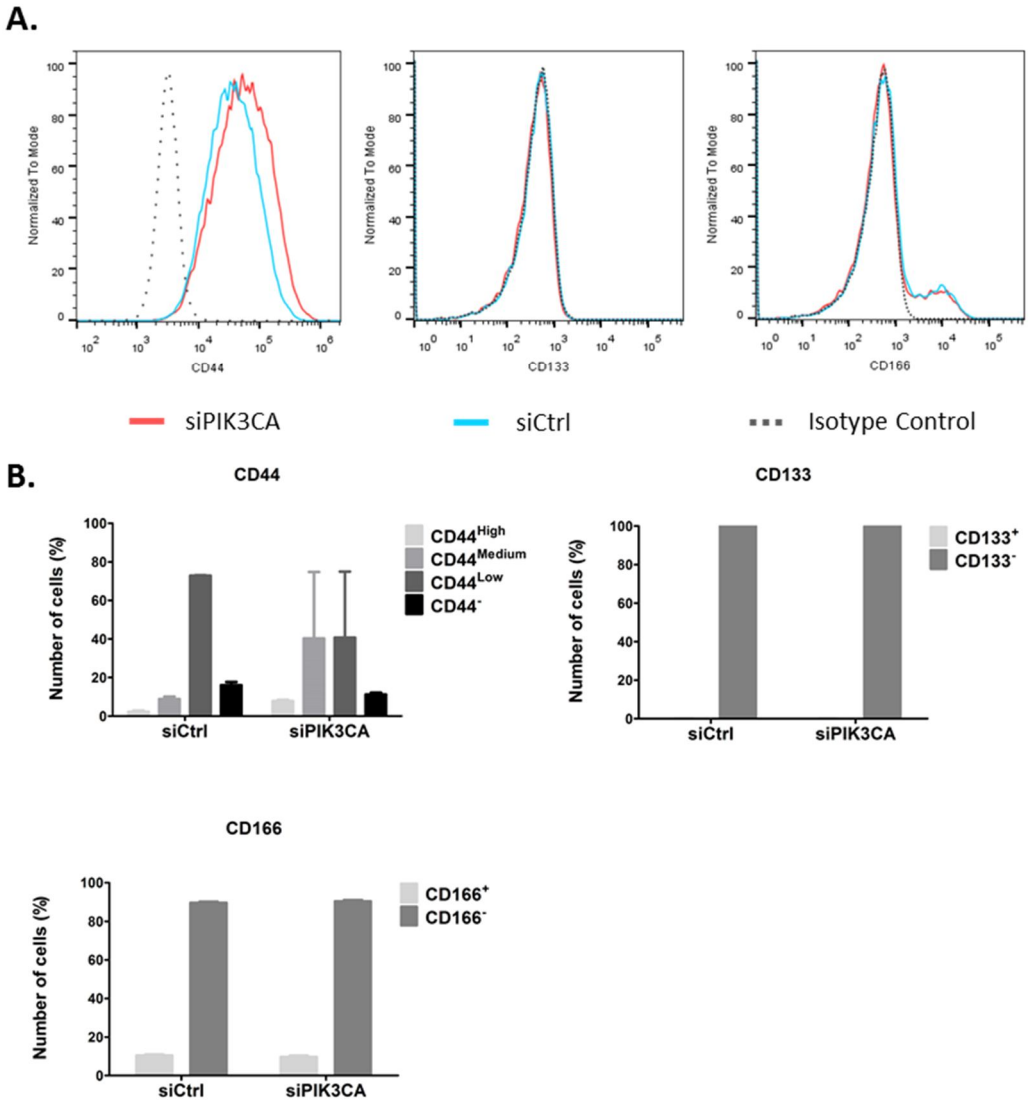

Figure 12. Expression of CD44, CD133 and CD166 in *PIK3CA*-inhibited SW480 cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=2.

The same cancer stem cell markers were evaluated in RKO cell line after *BRaf* (Figure 13 and supplementary Figure S 6) and *PIK3CA* inhibitions (Figure 14 and supplementary Figure S 7) with no alterations found.

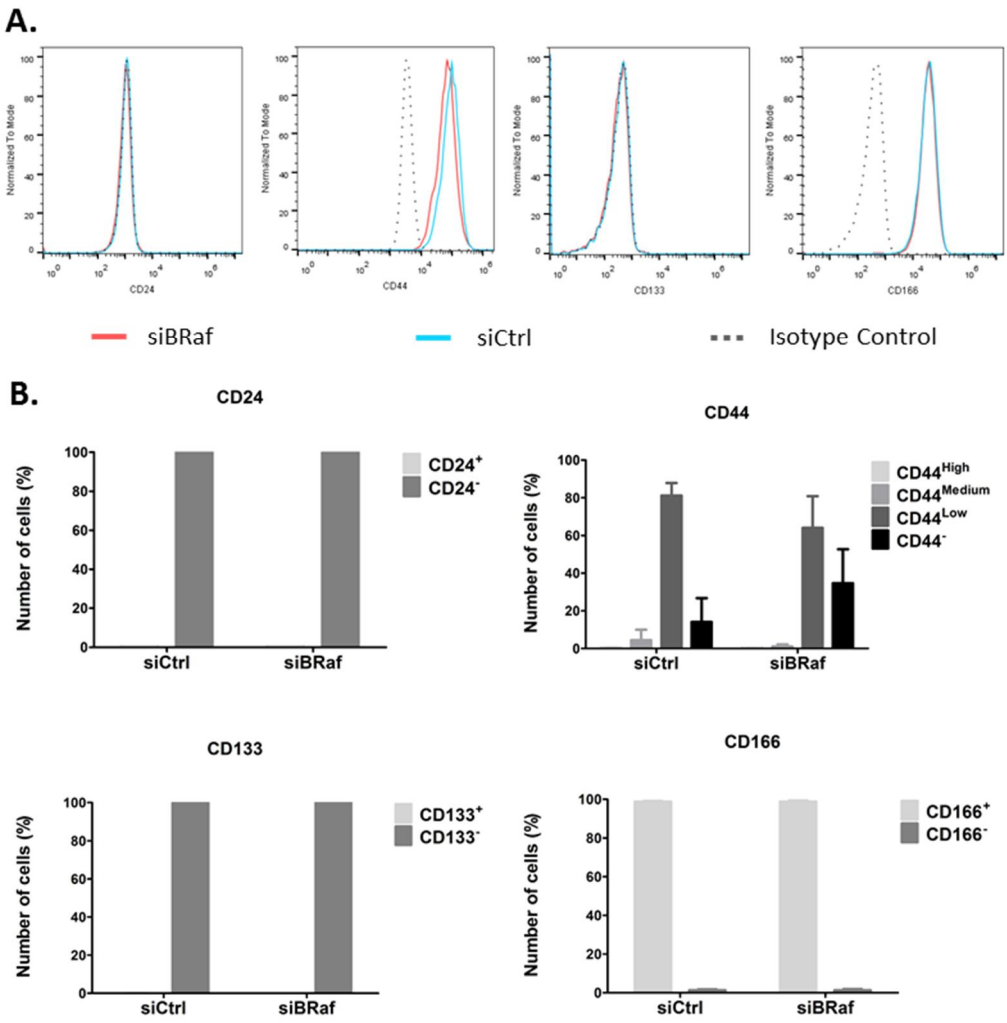

Figure 13. Expression of CD24, CD44, CD133 and CD166 in *BRaf*-inhibited RKO cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=2, and CD24 n=1.

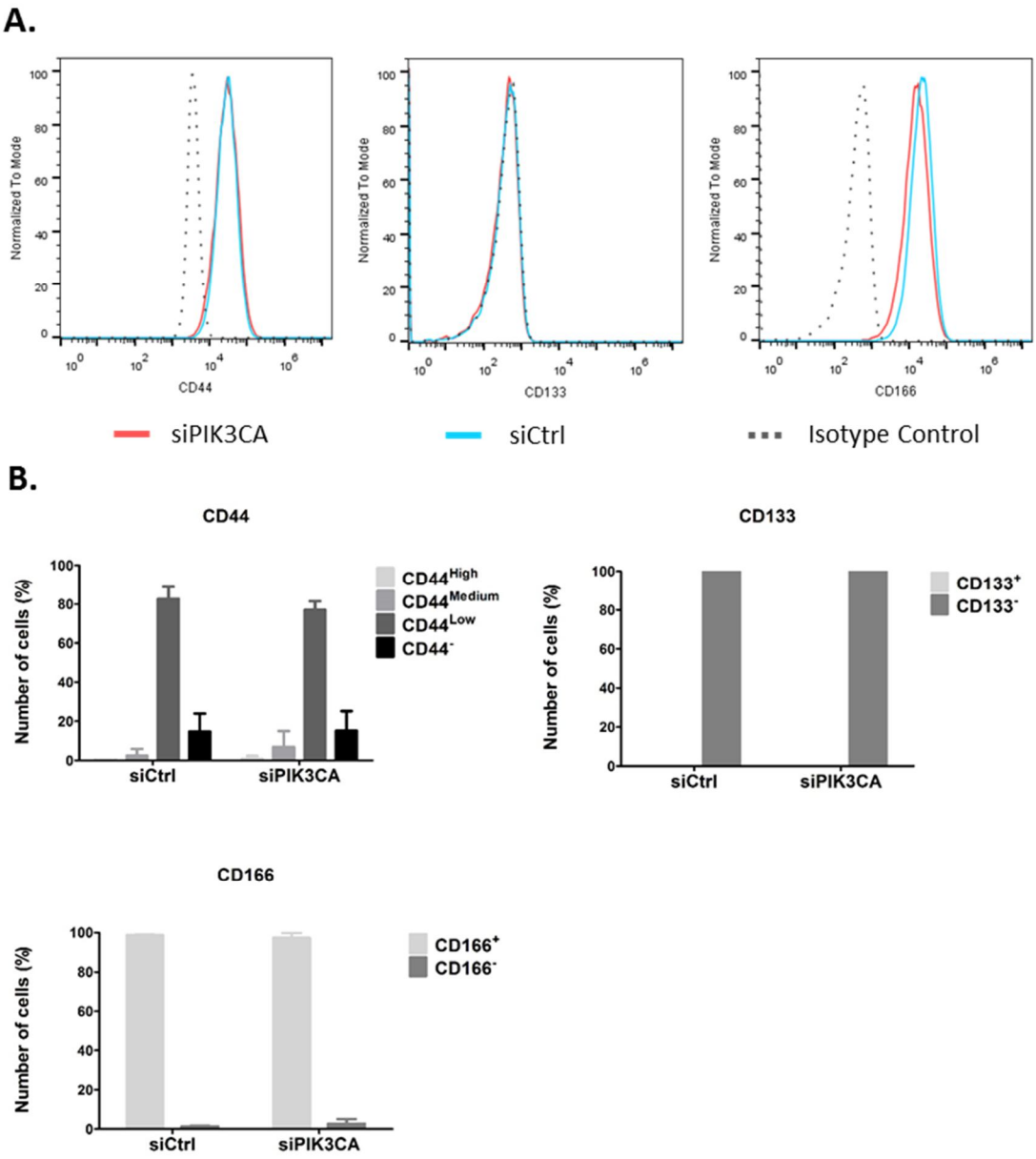

Figure 14. Expression of CD44, CD133 and CD166 in *PIK3CA*-inhibited RKO cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=3.

The analysis of the effects on CD24, CD44, CD133 and CD166 cancer stem cell markers were evaluated upon *KRas* inhibition in HCT-15 colorectal cancer cell line (Figure 15 and supplementary Figure S 8). HCT-15 cell line is CD24-, CD44- and CD133-negative. Although no alterations in CD44 or CD133 are observed when cells are treated with siRNA for *KRas*, CD24 expression slightly increases (P= 0,0316). Moreover, CD166 expression is reduced when *KRas* is inhibited (P= 0,0316).

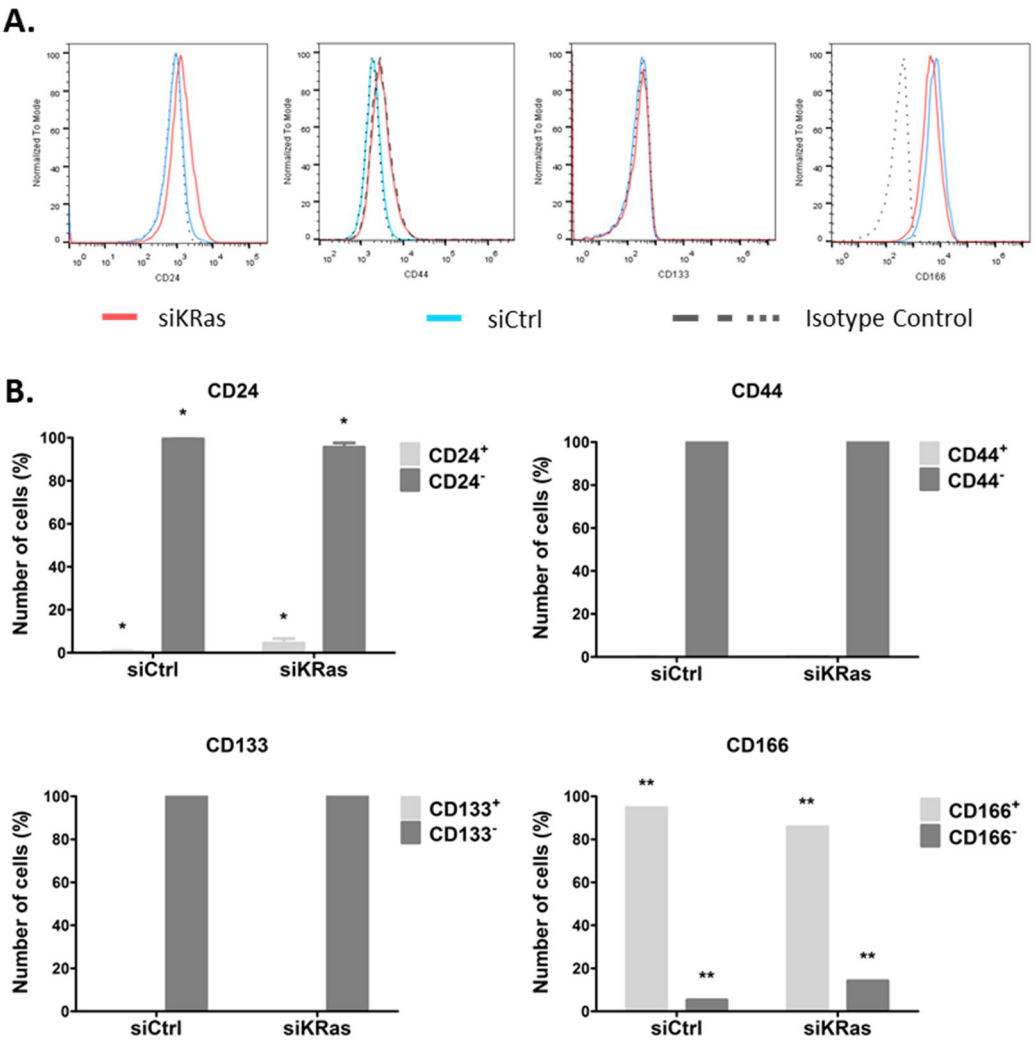

Figure 15. Expression of CD24, CD44, CD133 and CD166 in *KRas*-inhibited HCT-15 cell line. A. Representative fluorescence histograms. **B.** Percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=3.

Taken together, our results suggest that only KRas is able to modulate the expression level of cancer stem cell markers. Nevertheless, the effect is not common to all mutant KRas cell lines analysed and the markers affect differed between cell lines.

Table 4. Variations in the expression of the cancer stem cell markers evaluated through flow cytometry in HCT-116, SW480, RKO and HCT-15 upon *KRas*, *BRaf* and *PIK3CA* inhibitions. + - increased expression, - - decreased expression, — - larger decrease of expression, = - no variation of expression. Strikethrough represent markers not evaluated.

Cell Line	Cancer Stem Cell Marker			
	CD24	CD44	CD133	CD166
HCT-116 siKRas		—		
HCT-116 siBRaf	+	=	-	=
HCT-116 siPIK3CA	=	=	=	=
SW480 siKRas	=	=	=	=
SW480 siPIK3CA		=	=	=
RKO siBRaf	=	=	=	=
RKO siPIK3CA		+	=	=
HCT-15 siKRas	+	-	=	-

II – *KRas* and *PIK3CA*-inhibited HCT116 and SW480 present reduced capacity to establish colospheres

In order to evaluate whether the alterations in the expression level of cancer stem cell markers triggered by *KRas* inhibition had a real impact on the stem properties of the cell lines, we performed the colosphere-forming assay, which selects cells with a stem-like phenotype due to its ability to grow individually in suspension. For this assay, we selected the HCT116 cell line in which *KRas* inhibition lead to a drastic decrease of CD44, and SW480 in which *KRas* inhibition did not affect the expression of any of the markers. Additionally, the assay was also performed upon *PIK3CA* inhibition in order to further support the unique role of KRas in the induction/maintenance of a cancer stem cell phenotype.

Contrary to our assumptions, alterations in the morphology and number of the spheres formed upon *KRas* (in both cell lines) and *PIK3CA* (in HCT-116) inhibitions were clearly observed (Figure 16). After *KRas* inhibition, spheres formed in both cell lines are smaller and have an irregular morphology (Figure 16). Moreover, *KRas* inhibition shows more individualized and apparently quiescent cells than in the control or even in the si*PIK3CA* conditions. In terms of sphere forming efficiency, *KRas* inhibition lead to a reduction of about 50% in the capacity of formation in both HCT-116 and SW480 cell lines. Inhibition of *PIK3CA* also lead to a severe decrease in the capacity of sphere formation, shown in Figure 16 and Figure 17. Inhibition of cells were confirmed and are shown in supplementary Figure S 9.

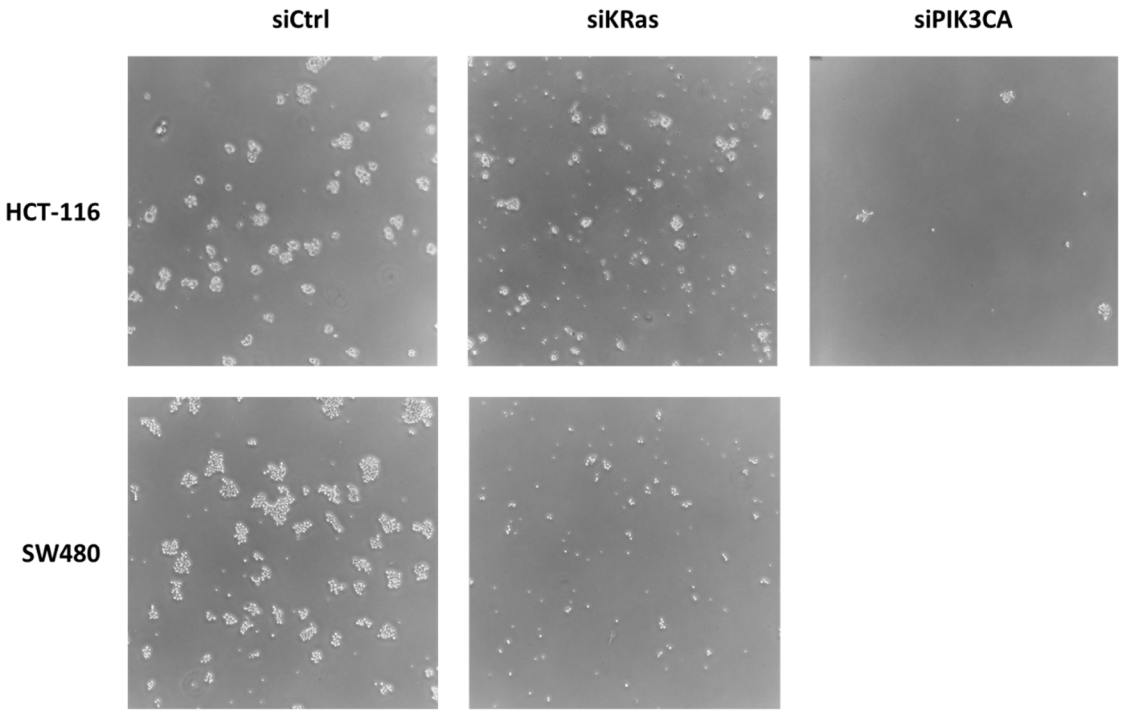

Figure 16. Colosphere-forming assay in *KRas* and *PIK3CA* inhibited HCT-116 and SW480 cell lines. Images were acquired after 5 days in culture using a 20x objective. 5.000 cells were plated for both siCtrl and siKRas, while 60.000 cells were plated for siPIK3CA conditions.

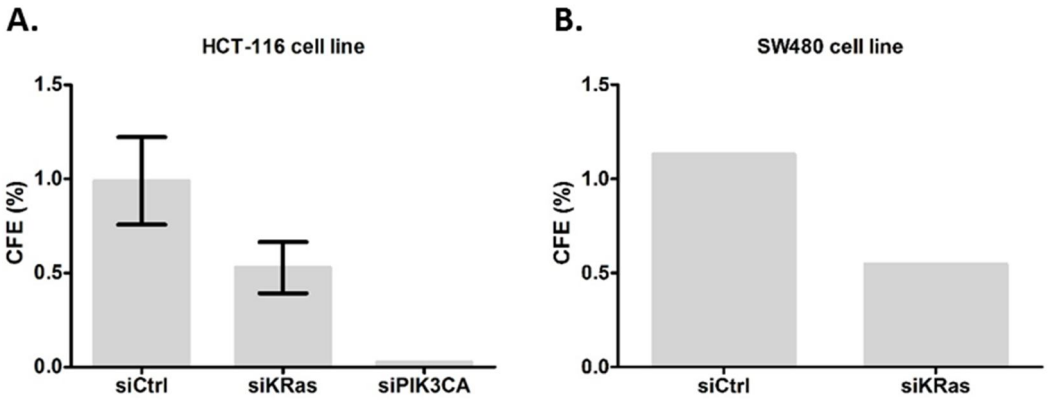

Figure 17. Colosphere-forming efficiency in *KRas* and *PIK3CA*-inhibited HCT-116 (A) and in *KRas*-inhibited SW480 cell line (B). Spheres with a diameter bigger than 50µm were considered and counted after 5 days in culture. HCT-116 siCtrl and si*KRas* n=4, si*PIK3CA* n=1; SW480 n=1.

Overall, the results indicate that, independently of affecting or not the expression of cancer stem cell markers, *KRas* inhibition does affect the stem properties of the cell lines studied.

Moreover, *PIK3CA* inhibition also induced alterations in the stem phenotype without affecting the expression of any of the cancer stem cell markers previously analysed. These observations suggest that KRas and PIK3CA modulate cancer cell stemness through alternative molecules.

III – Intestinal stem cell markers show no alteration upon *KRas* inhibition in HCT-116 or SW480 cell lines

In order to identify the molecular mediators of KRas- and PIK3CA-induced, we explored alterations in normal intestinal stem cell markers and cyclin D1 (*CCND1*), a Wnt pathway target and a cell cycle regulator, through real time quantitative PCR in two KRas-mutated cell lines, HCT-116 and SW480.

In HCT-116 cell line, results shown in Figure 18 confirm that *KRAS* gene expression was drastically reduced (P<0,0001). However, such efficient inhibition only lead to a small reduction of *BMI1* and *CCND1* gene expression (P=0,0360 and P=0,0105, respectively).

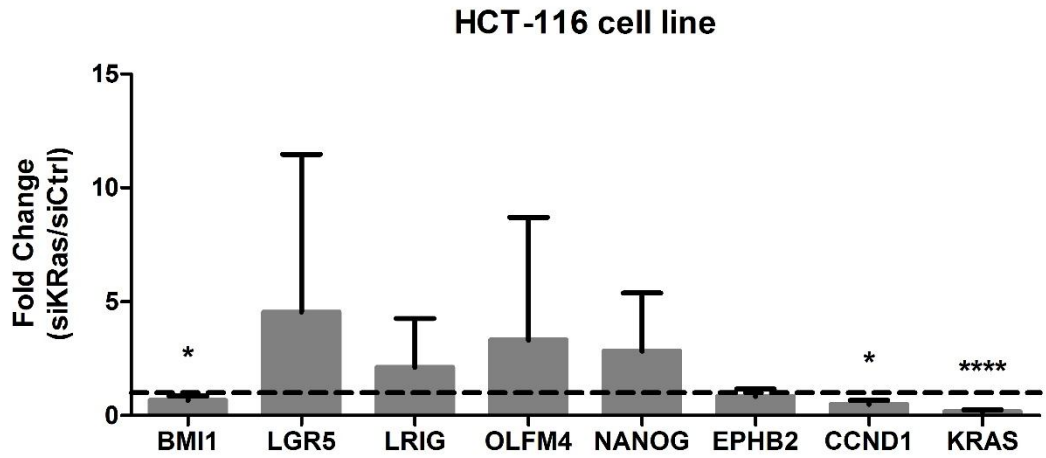

Figure 18. mRNA expression of intestinal stem cell markers in *KRas*-inhibited HCT-116 cell line. Significant decreases were observed in *BMI1*, and Cyclin D1 (*CCND1*). Results are shown as average ± SD, all n=4 except KRAS n=6 and LRIG n=3.

In SW480 cell line, *KRas* expression was also efficiently inhibited, however no statistically significant alterations were found in any of the genes analysed (Figure 19). Actually, *LGR5* gene shows a tendency towards an increase of expression (P=0,0501).

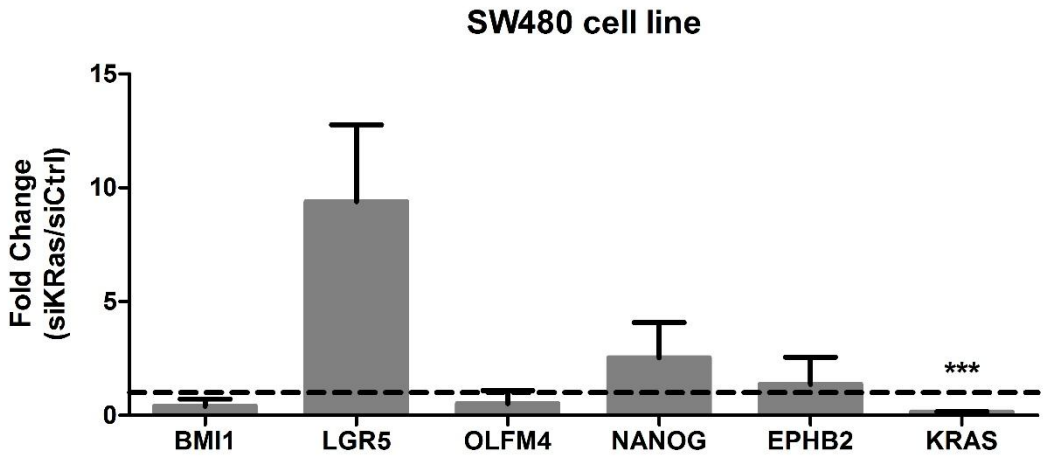

Figure 19. mRNA expression in *KRas*-inhibited SW480 cell line. The expression of several intestinal stem cell markers is not affected upon *KRas* inhibition. *LGR5* reveals a tendency towards an increase upon KRas inhibition. Results are shown as average ± SD, n=3.

As no alterations in the intestinal stem cell markers previously evaluated were observed, we hypothesized that epithelial-mesenchymal transition (EMT/MET) could be involved in the reduction of stemness. E-cadherin expression was explored in HCT-116, SW480 and RKO after *KRas*, *BRaf* and *PIK3CA* inhibitions (Figure 20 and supplementary Figure S 10). No variation in positive versus negative E-cadherin population were observed, however *KRas*-inhibited HCT-116 cells present a different E-cadherin profile, with an increase in the overall expression of E-cadherin (Figure 21). Yet, such results are still incompatible with the observations made in the colosphere-forming assay.

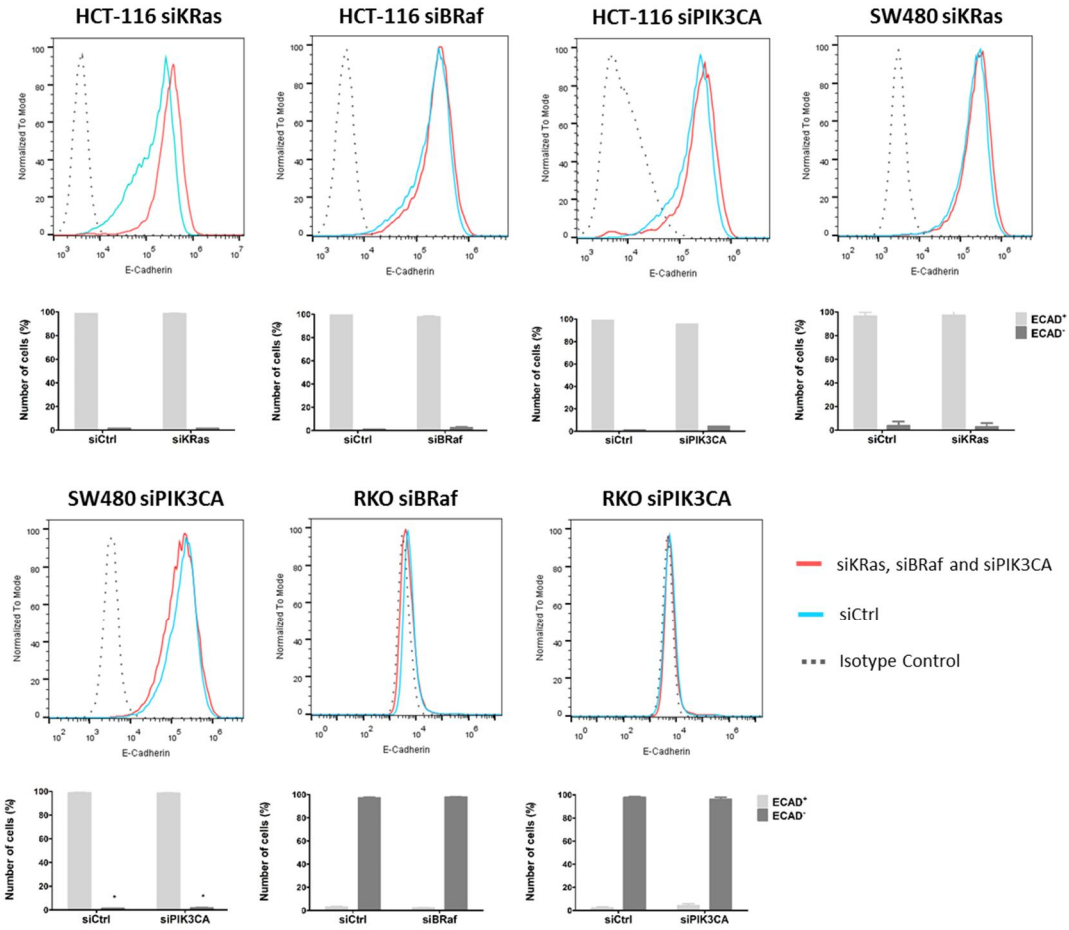

Figure 20. E-cadherin expression in HCT-116, SW480 and RKO upon *KRas*, *BRaf* and *PIK3CA* inhibition. Representative fluorescence histograms and graphs showing percentage of positive and negative cells in the total population. Results are shown as average ± SD, n=2 except HCT-116 siPIK3CA n=1, RKO siPIK3CA n=1, RKO siPIK3CA n=4.

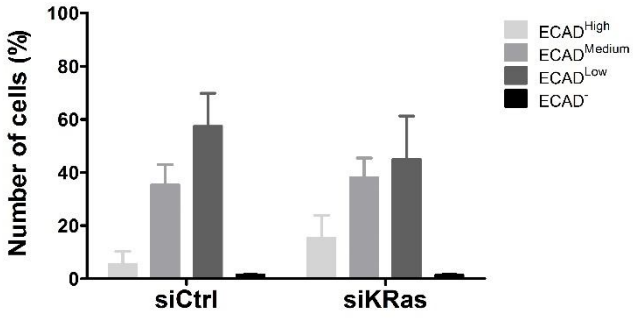

Figure 21. E-cadherin expression in *KRas* inhibited HCT-116 cell line. Expression was evaluated in terms of high, medium, low and negative expression of E-cadherin to analyse differences in terms of expression. n=2

In order get further insight on the KRas and PIK3CA downstream events mediating the capacity to form colospheres, we evaluated the expression of integrin α6, also known as CD49f, and its co-receptor integrin β4 (or CD104) as integrin α6 has been described as a

cancer stem cell marker.[60,85] Moreover, c-Met expression, a HGF receptor, was also evaluated as this receptor dimerizes with CD44, more specifically CD44v6, and integrin α6 when present at the cell membrane.[68] The expression of integrins α6 and β4, and c-Met was evaluated in RKO cells upon BRaf and PIK3CA as well. Integrin α6 expression reveal that its expression is downregulated in all the cell lines upon inhibition of *KRas*, *BRaf* and *PIK3CA* (Figure 22, Figure 23 and supplementary Figure S 11), with different degrees of decrease except in *PIK3CA*-inhibited HCT-116 cell line. Interestingly, inhibition of *PIK3CA* in HCT-116 cells lead to alterations in the maturation of integrin α6, with an increase of pro-integrin α6 and a decrease of the mature form of the protein (Figure 24).

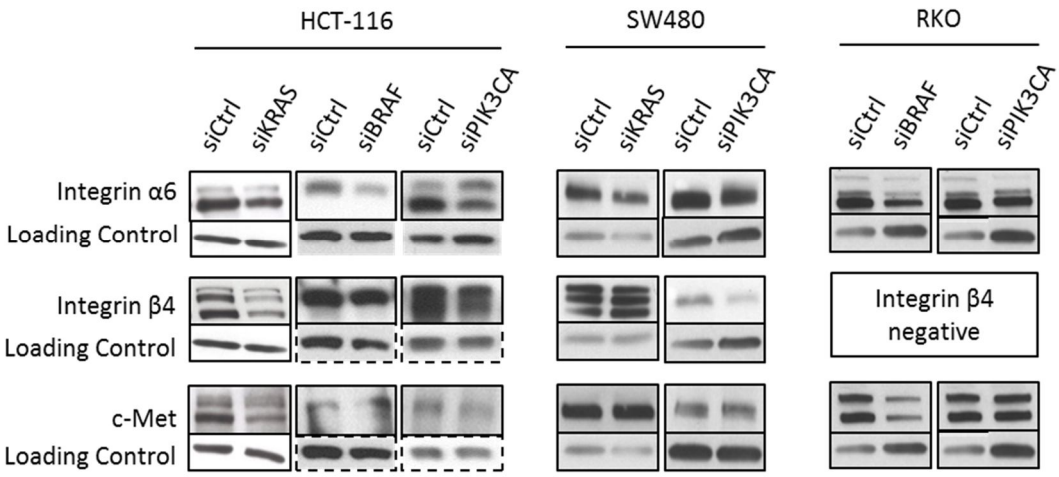

Figure 22. Expression of c-Met, integrins α6 and β4 (CD49f and CD104, respectively) in HCT-116, SW480 and RKO cell lines. Images are representative of one experiment. GAPDH was used as loading control, unless in the images with dashed borders, where β-tubulin was used. n=3 except in HCT-116 siBRaf, RKO siBRaf and RKO siPIK3CA: c-Met and CD49f n=2. n=3 except in HCT-116 si*BRaf*, RKO si*BRaf* and RKO si*PIK3CA*: c-Met and CD49f n=2; HCT-116 and SW480 si*KRas* n=2.

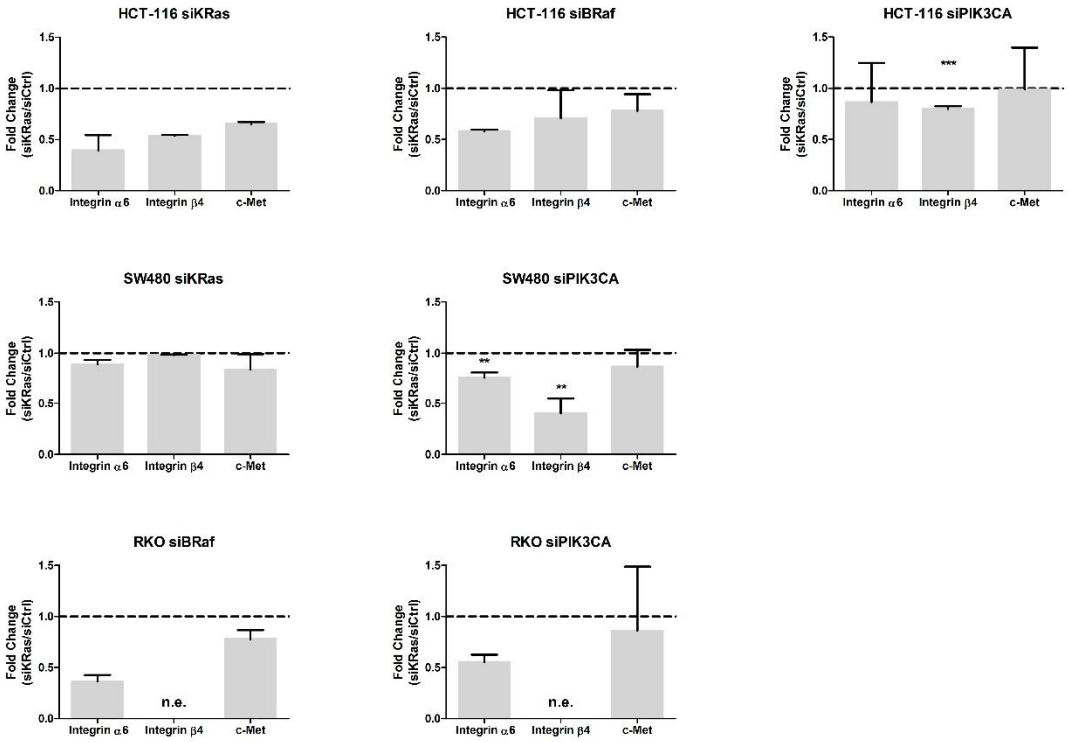

Figure 23. Quantification of c-Met, integrins α6 and β4 expression in HCT-116, SW480 and RKO cell lines. RKO cell line do not express integrin β4 (n.e.). Results are shown as relative average to siCtrl-treated cells. n=3 except in HCT-116 siBRaf, RKO siBRaf and RKO siPIK3CA: c-Met and CD49f n=2; HCT-116 and SW480 siKRas n=2.

Expression of integrin β4, which dimerizes with integrin α6, revealed that it is downregulated in HCT-116 si*KRas* and si*PIK3CA*, and in si*PIK3CA*-treated SW480 cell line, but curiously not in siKRas-treated SW480 cells. It should be noted that RKO cell line is integrin β4 negative. Furthermore, expression of c-Met was also sharply downregulated in HCT-116 si*KRas* but almost no alterations exist in the other inhibitions or cell lines.

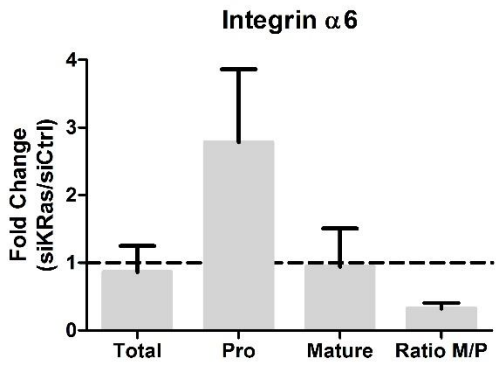

Figure 24. Expression of total, pro and mature integrin α6 in HCT-116 siPIK3CA and ratio mature/pro-integrin α6. Bars represent the average ± SD from three independent experiments.

In summary, among all the markers analyzed integrin α6 is the only molecule which expression is commonly altered in all the cell lines upon inhibition of either *KRas*, *PIK3CA* or *BRaf*. Therefore, it represents the most promising molecule to explain the loss of stem properties of the inhibited cells.

Part 2 – Microenvironmental regulation of stem cell markers

Tumour microenvironment is known to have a role in the regulation of the stem cells, performed through Wnt/β-catenin signalling pathway.[57] Moreover, oncogenic KRas is also known to promote activation of Wnt/β-catenin signalling and a stem cell-like program in colorectal cancer initiation.[146,147] To understand if tumour microenvironment, more specifically activated fibroblasts, is able to modulate the cancer stem cell properties induced by oncogenic alterations we started by evaluating the expression levels of CD44, CD44v6 and E-cadherin by flow cytometry in *KRas*-inhibited HCT-116 cell line.

Results in Figure 25 show that CD44 expression is not altered when siCtrl-treated HCT-116 cells are exposed to either control (medium and TGFβ) or test (from fibroblasts and activated fibroblasts) conditioned media. No alterations were also found when *KRas*-inhibited HCT-116 cells were treated with conditioned media (Figure 25B).

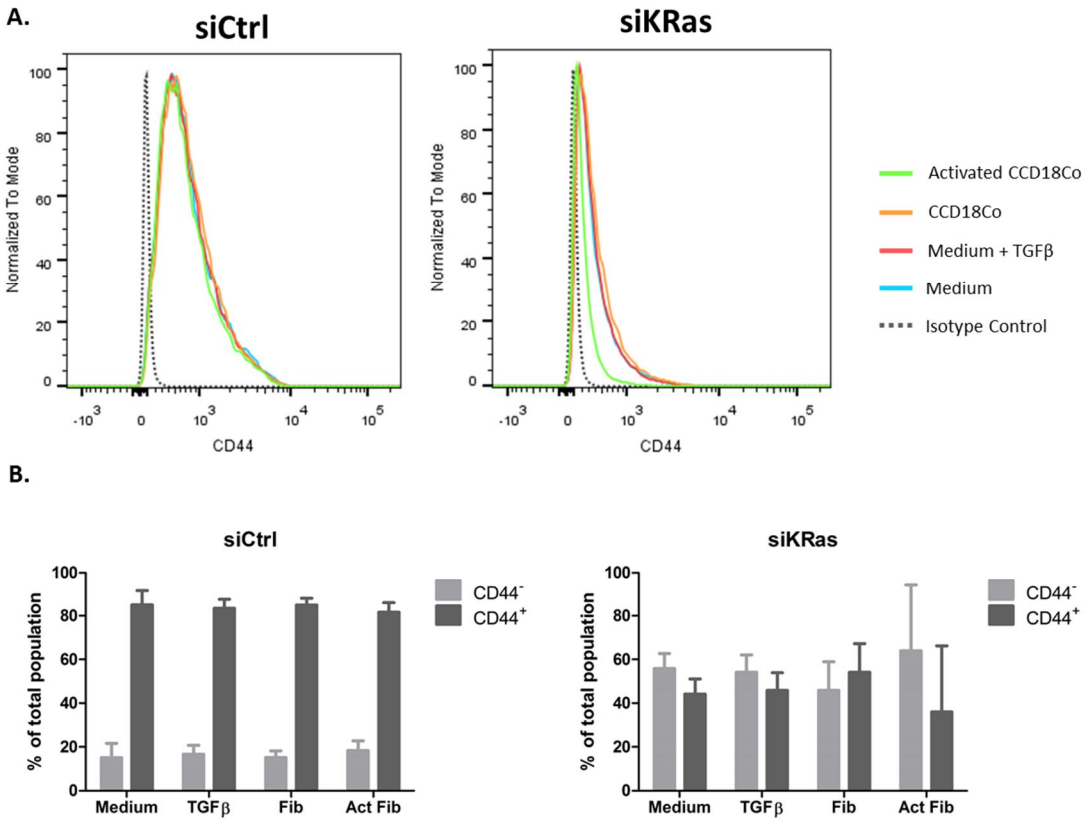

Figure 25. CD44 expression in *KRas* inhibited HCT-116 cell line after treatment with fibroblasts and activated fibroblasts. A. Fluorescence histograms from one biological replica. **B.** Quantification of CD44-positive versus negative population in each of the conditions studied: Medium, TGFβ, CCD18Co fibroblasts (Fib) and activated CCD18Co fibroblasts (Act Fib). Results are shown as average, n=2.

CD44v6 was also evaluated in the same conditions as CD44 due to its role in the dimerization with c-Met receptor. Like CD44 expression in *KRas*-inhibited HCT-116 cells, CD44v6 expression is also reduced by the inhibition of *KRas*, although the decrease is not as sharp (Figure 26 and supplementary Figure S 12). No alterations were found upon treatment with the conditioned media (Figure 26B).

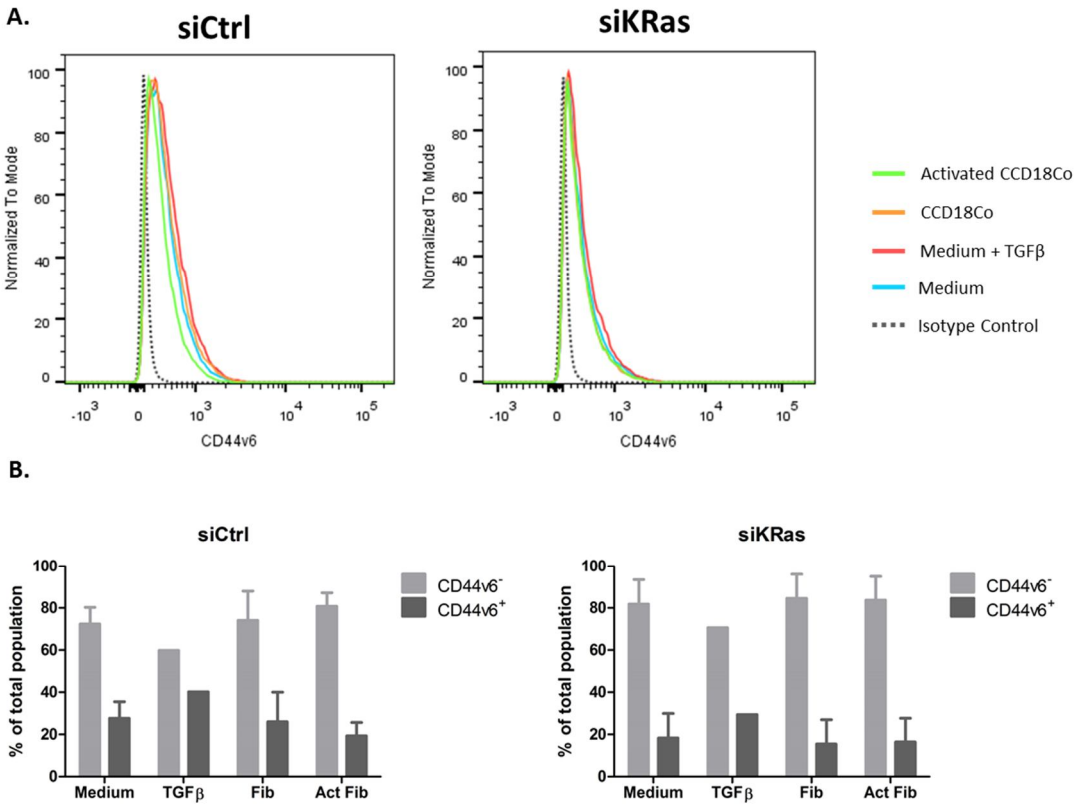

Figure 26. CD44v6 expression in *KRas* inhibited HCT-116 cell line after treatment with fibroblasts and activated fibroblasts. A. Fluorescence histograms from one biological replica. **B.** CD44v6-positive versus negative percentage of the total population in each of the conditions studied: Medium, TGFβ, CCD18Co fibroblasts (Fib) and activated CCD18Co fibroblasts (Act Fib). Results are shown as average, n=2, except TGFβ conditions where n=1.

Alterations in E-cadherin expression were also evaluated after HCT-116 cells were treated with the previously used conditions. No alterations in E-cadherin expression (Figure 27B) and expression profile (Figure 27A and supplementary Figure S 13) were observed in both siCtrl and si*KRas*-treated HCT-116 cells, independently of the conditioned media used.

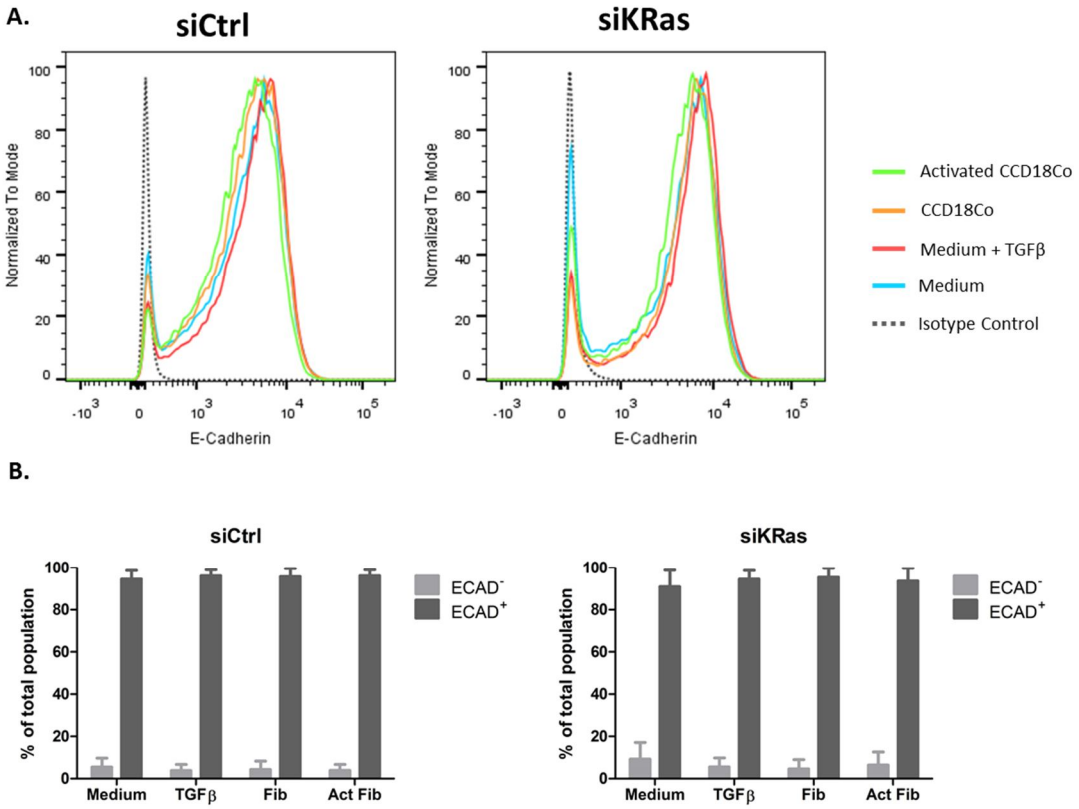

Figure 27. E-cadherin expression in *KRas* inhibited HCT-116 cell line after treatment with fibroblasts and activated fibroblasts. A. Fluorescence histograms from one biological replica. **B.** Quantification of E-Cadherin-positive and negative population in each of the conditions studied: Medium, TGFβ, CCD18Co fibroblasts (Fib) and activated CCD18Co fibroblasts (Act Fib). Results are shown as average, n=2.

The results show that in HCT-116 cell line, independently of KRas activation, basal levels of CD44, CD44v6 and E-Cadherin are not affected by fibroblasts' conditioned media (fibroblasts activation is confirmed in supplementary Figure S 14).

DISCUSSION

The results presented provide evidence on the effect of *KRas*, *BRaf* and *PIK3CA* oncogenic alterations in the modulation of stem cell phenotype in colorectal cancer cell lines.

Our findings indicate that *KRas* inhibition alters the expression of different CSC markers, essentially CD24, CD44 and CD166, but not in all the cell lines evaluated. Moreover, *BRaf* and PIK3CA inhibition did not fully reproduce the alterations observed with *KRas* inhibitions, suggesting that modulation of CSC markers expression by *KRas* is attained through other downstream signalling pathways such as RalGEF/Ral or Tiam/Rac. Furthermore, the fact that alterations in the expression of CSC markers induced by *KRas* is not common to all the *KRas* mutant cell lines suggests that regulation of such expression must the modulated by other factors that differ in the cell lines used. In accordance to our results, it was demonstrated that oncogenic *KRas* is capable of activating the CSC pool through Wnt/β-catenin signalling, and downregulation of this pathway can lead to a decrease in CD44 expression in HCT-116 cell line.[144,148] Moreover, recent findings indicate that both p53 and SMAD can modulate the Wnt/β-catenin pathway through BMP signalling, depending on the mutational status of both proteins.[149] In a p53 wild-type context, BMP pathway can inhibit Wnt/β-catenin signalling but only if SMAD4 is present. Therefore, our data regarding CD44 regulation by mutant KRas in HCT-116 fits the models described in the literature. Furthermore, the association between BMP-p53-SMAD4 signalling and the regulation of Wnt/β-catenin signalling may also help to explain why KRas inhibition has no effect on the expression of CD44 in SW480 as this cell line has high Wnt activity due to mutations in *p53*.[149] Nevertheless, in HCT-15 *KRas* inhibition slightly changed the expression of CD24 and CD166 which suggests that these cancer stem cell markers might be modulated by a Wnt/β-catenin-independent mechanism.

In this part of the study we evaluated a set of cell lines with different genetic backgrounds, showing that the effect of *KRas* is cell line dependent. Thus, inhibition of *KRas*, *PIK3CA* and *BRaf* should be completed in our panel of cell lines and further extended to other cell lines as their effect on the modulation of CSC expression might also be cell line specific.

With the assumption that KRas might regulate CSC pool in HCT-116 but not in the other cell lines due to the alteration in CD44 expression, colosphere-forming assay was performed. Our results show that both *KRas*-inhibited HCT-116 and SW480 cell lines present a decreased capacity for sphere formation, independently of the alterations observed in CD44. Moreover, *PIK3CA*-inhibited HCT116 cells, which did not show alterations in CD44 expression or in any of the other CSC markers, also show reduced capacity to form spheres, which is in accordance published results showing that PI3K/Akt/mTOR pathway inhibitors also lead to a reduction

in the sphere-forming capacity.[150] However, it is important to mention that PIK3CA-inhibited HCT-116 results should be read carefully, as we have only performed one experiment, and *PIK3CA* inhibition was not confirmed due to lack of sufficient biologic material. However, before plating for the colosphere assay the number of cells was counted and found to be reduced for half of the control, which is in accordance to other results where *PIK3CA* inhibitions were confirmed. Together, these observations support the idea that alterations in stemness do not correlate with CD44 expression. Despite the results we obtained, the reliability of our approach is questionable. Inhibitions using siRNA technique are transient and sphere-forming assays end one week after the inhibitions are performed. Therefore, we cannot exclude that the cells form spheres because expression of the oncogenes is recovered during the assay. Also, since the inhibition by siRNA is not 100% efficient, we cannot exclude that spheres are formed from non-inhibited cells, which limits further analysis. Nonetheless, it is true that the capacity to form spheres is affected. In a near future, stable inhibition approaches should be adopted, such as the use of KRas, PIK3CA and BRaf isogenic cell lines, CRISPR/Cas9 or shRNA in order to ensure that inhibition is maintained throughout the assay, and to allow the execution of longer experiments such as *in vivo* tumorigenic assays. Furthermore, our study also questions the true validity of CD44 as a CSC marker. Therefore we believe further studies must the executed to understand the role of CD44 and its variants in the modulation of stemness. Curiously, in breast cancer, overexpression of CD44 intracellular domain (ICD) was found to increase stemness and tumorigenesis by the activation of stemness factors, like Sox2 and Oct4.[151] Our results only evaluated the extracellular portion of CD44, questioning if the regulation of stemness in CRC cell lines is due to reduction of the cleaved ICD of CD44 instead of the protein present at the membrane. Furthermore, if such role of the CD44 ICD is verified, is natural to question the role of KRas, BRaf and PIK3CA in the modulation of CD44 cleavage.

Analysis of mRNA expression of intestinal stem cell markers and Cyclin D1 did not show relevant alterations in its expression that could explain the decreased capacity to form spheres. In *KRas*-inhibited HCT-116 cells was only observable a slight decrease in *BMI1* expression, possibly indicating a reduction in the stemness, and in *CCND1*, possibly associated with cell cycle arrest and halted proliferation.[89,152] Analysis of gene expression in *KRas*-inhibited SW480 cell line also show no alterations, with *LGR5* having a tendency to increase, either indicating an increase in the stemness, which is refuted by the findings in the colosphere assay, or an increase in cell adhesion, reduction of clonogenicity and tumourigenicity.[95,98] Evaluation of other stem cell markers could be explored, such as

Musashi-1 or Sox9,[87] and, if altered, the analysis could be extended to other *KRas*, *BRaf* and *PIK3CA* inhibited cell lines, to clarify the alterations that might occur upon such inhibitions in the different cell lines.

Alterations in stemness could also be explained by the induction of EMT/MET by *KRas* and *PIK3CA* inhibitions. CSC are known to have a more invasive and motile phenotype, characteristics that are shared with mesenchymal cells.[153] Moreover, not only CSC have been reported to have increased expression of EMT-related genes, but Wnt/β-catenin signalling as also been reported to promote EMT in CRC cells.[153,154] Due to such possibility, E-cadherin expression, which is lost upon EMT induction, was evaluated.[153] The results suggested that EMT/MET do not play a role in the alterations observed in stemness, as only *KRas*-inhibited HCT-116 cell line show alterations in the expression profile. In breast cancer, loss of E-cadherin is not necessary for EMT.[155] Therefore evaluation of EMT-targeted genes, such as microRNA 200 family and ZEB, should be made, to ensure EMT/MET is not involved in the loss of stemness observed.

In order to find putative molecules that could explain the reduction in stemness, we evaluated the expression of integrin α6. Integrin α6, and in particular its splice variants, has been proposed to have a key role in the modulation of cancer stem cells and as a regulator of the Wnt pathway.[60,85] Following this idea, we showed that inhibition of *KRas*, *BRaf* and *PIK3CA* resulted in a reduction of integrin α6 in all the cell lines evaluated as well as alterations in its maturation, observed upon *PIK3CA* inhibition in HCT-116 cell line. These are the first findings suggesting that mutant forms of *KRas*, *BRaf* and *PIK3CA* are able to modulate the expression of integrin α6, and are in accordance with recent data showing that in human chondrosarcoma cells the RAS/RAF/MEK/ERK pathway is important to mediate amphiregulin-induced integrin α6 expression.[156] Moreover, these results do not exclude the possibility of other *KRas* downstream pathways to have a role in such modulation of integrin α6 expression. As integrin α6 is downregulated in both *KRas*-inhibited HCT-116 and SW480 and *PIK3CA*-inhibited HCT-116 cell lines, and the capacity to form colospheres is severely affected by such inhibitions, we hypothesized that integrin α6 might be a viable cancer stem cell marker. It is also important to refer that the downregulation of integrin α6 could be linked with the decrease of integrin β4 due to the association of both as a complex at the membrane. However, RKO cell line is integrin β4-negative and still has downregulation of integrin α6, revealing the downregulation is performed through an integrin β4-independent mechanism. Moreover, our work also shows that although *KRas*-inhibited SW480 cells do not

show alterations in integrin β4, alterations are observed in si*PIK3CA*-treated SW480 cell line, suggesting a role of wildtype PIK3CA in the modulation of integrin β4 expression.

With the results presented, integrin α6 appears to be the best candidate as a feasible and reliable colorectal cancer stem cell marker. However further studies must the accomplished to better understand the role of KRas, BRaf and PIK3CA, or other KRas downstream pathways, in the modulation of integrin α6 expression, splicing and functionality. Moreover, it is important to clarify if integrin α6 marks CSC as a consequence of the activation of stem signalling pathways, such as Wnt/β-catenin, or if it is in fact responsible for the stemness of such cells. Also, by inhibiting integrin α6 expression, functional studies such as sphere-forming assay or *in vivo* tumour formation must be performed to evaluate if the capacity to form new tumours is affected. Furthermore, differences in the integrin α6 splice variants mRNA expression upon *KRas*, *BRaf* and *PIK3CA* inhibitions should be determined by real time quantitative PCR, in order to understand the ratio between both variants. The necessity for such understanding is based on the observation that the ratio of integrin α6A to integrin α6B is altered in later stages of colon tumours, and that integrin α6A knockout is associated with reduced capacity to form new tumours *in vivo*.[85]

Finally, downregulation of c-Met was observed after the inhibition of *KRas* in HCT-116 cell line, but only slightly in the other inhibitions and cell lines. This might also reveal an important role of *KRas* and its downstream pathways in the modulation of crosstalk with the tumour microenvironment. Altogether, due to the role of integrins and c-Met in the communication with the microenvironment, the decrease of integrins α6 and β4 and c-Met receptor might reveal a pathway to reduce the crosstalk with tumour microenvironment, likely impacting on CSC properties. In fact, cancer-associated fibroblasts have been known to play a role in the modulation of stem cell phenotype, through Wnt/β-catenin signalling activation and upregulation of stem cell markers, like LGR5, by the secretion of soluble factors, mainly HGF.[57] Here we tried to understand if stemness modulation attained by cancer-associated fibroblasts was reflected in the CD44 and CD44v6 expression and whether mutant *KRas* mediates such effect. Moreover, due to the knowledge that HGF, TGFβ and EGF are able to induce EMT, resulting in reduction of E-cadherin, alterations in E-cadherin expression were evaluated.[153] No alterations in CD44 expression were observed in siCtrl-treated HCT-116 cells, possibly due to the already high expression of this protein. When *KRas* is inhibited we observed a decrease in CD44 expression, as previously demonstrated, but no alterations were registered upon treatment with conditioned media. This is expected due to the observation that c-Met, the HGF receptor, is downregulated in *KRas*-inhibited HCT-116

cells. When CD44v6 was analysed, also no variation in its expression was observed upon treatment with the conditioned media. This support the idea that CD44 is not a reliable cancer stem cell marker, as cells that were expected to have a more stem-like phenotype do not alter its expression of CD44 nor CD44v6. Furthermore, no alterations in E-cadherin were observed, suggesting that cells did not underwent MET/EMT. Due to the high expression of CD44 in HCT-116 cell lines, other KRas-inhibited cell lines, with a medium expression of CD44, should be studied upon treatment with fibroblasts' conditioned media. Also, *BRaf* and *PIK3CA* inhibitions should be studied in these conditions in order to evaluate the role of such pathways in the microenvironmental regulation of stemness. Importantly, the effect of fibroblasts' conditioned media on the expression of integrin α6 should be performed in the presence or absence of KRas, BRaf and PIK3CA oncogenes.

CONCLUSION

CSC have for long been associated with metastasis and chemotherapy resistance, eventually resulting in tumour relapse. Our work is in agreement with the observations of others supporting the putative role of integrin α6 as CSC marker. Although further studies must be performed to consolidate the observations made, the present work awards integrin α6 a key role as a colorectal CSC marker that could be used in research, to improve cancer stem cell studies, and in the clinic, as a possible prognostic marker. Moreover, this study gives preliminary results and establishes a path to be pursued in order to identify and better understand signalling pathways that can alter the stem-like phenotype and communication with the tumour microenvironment, hopefully leading to the development of new therapeutic targets to improve the approach to battle colorectal cancer in the clinics.

REFERENCES

1. Silverthorn, D. U. *Human Physiology: An Integrated Approach*. **5,** (Pearson Higher Education, 2010).

2. Seeley, R., Stephens, T. & Tate, P. *Anatomy and Physiology*. (Mc Graw Hill Higher Education, 2013).

3. McDonald, S. a C., Preston, S. L., Lovell, M. J., Wright, N. a & Jankowski, J. a Z. Mechanisms of disease: from stem cells to colorectal cancer. *Nat. Clin. Pract. Gastroenterol. Hepatol.* **3,** 267–274 (2006).

4. Todaro, M., Francipane, M. G., Medema, J. P. & Stassi, G. Colon Cancer Stem Cells: Promise of Targeted Therapy. *Gastroenterology* **138,** 2151–2162 (2010).

5. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459,** 262–5 (2009).

6. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449,** 1003–1007 (2007).

7. Powell, A. E. *et al.* The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* **149,** 146–158 (2012).

8. Crosnier, C., Stamataki, D. & Lewis, J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat. Rev. Genet.* **7,** 349–359 (2006).

9. Potten, C. S. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos. Trans. R. Soc. London B Biol. Sci.* **353,** 821–830 (1998).

10. Medema, J. P. & Vermeulen, L. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. *Nature* **474,** 318–326 (2011).

11. Biswas, S. *et al.* Microenvironmental control of stem cell fate in intestinal homeostasis and disease. *J. Pathol.* **237,** 135–145 (2015).

12. Ferlay, J. *et al.* Cancer incidence and mortality worldwide : sources , methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **136,** (2014).

13. Lynch, H. T. & de la Chapelle, A. Hereditary colorectal cancer. *N Engl J Med* **348,** 919–932 (2003).

14. Davies, R. J., Miller, R. & Coleman, N. Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* **5,** 199–209 (2005).

15. Smith, G. *et al.* Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9433–8 (2002).

16. Pino, M. S. & Chung, D. C. The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology* **138,** 2059–2072 (2010).

17. Vogelstein, B. *et al.* Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.* **319,** (1988).

18. Logan, C. Y. & Nusse, R. The Wnt Signaling Pathway in Development and Disease. *Annu. Rev. Cell Dev. Biol.* **20,** 781–810 (2004).

19. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417,** 949–954 (2002).

20. Iacopetta, B. TP53 mutation in colorectal cancer. *Hum. Mutat.* **21,** 271–276 (2003).

21. Samuels, Y. *et al.* High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304,** 554 (2004).

22. Hedrick, L. *et al.* The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev.* **8,** 1174–1183 (1994).

23. Grady, W. M. *et al.* Mutation of the type II transforming growth factor-beta receptor is coincident with the transformation of human colon adenomas to malignant carcinomas. *Cancer Res.* **58,** 3101–3104 (1998).

24. Grady, W. M. & Markowitz, S. D. Genetic and epigenetic alterations in colon cancer. *Annu. Rev. Genomics Hum. Genet.* **3,** 101–128 (2002).

25. Leppert, M. *et al.* The Gene for Familial Polyposis Coli Maps to the Long Arm of Chromosome 5. *Science (80-.).* **40,** 40–42 (1987).

26. Bodmer, W. F. *et al.* Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* **328,** 614–6 (1987).

27. Half, E., Bercovich, D. & Rozen, P. Familial adenomatous polyposis. *Orphanet J. Rare Dis.* **22,** 105–112 (2009).

28. Groden, J. *et al.* Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66,** 589–600 (1991).

29. Solomon, E. *et al.* Chromosome 5 allele loss in human colorectal carcinomas. *Nature*

328, (1987).

30. Deal, C. Potential new drug targets for osteoporosis. *Nat Clin Pr. Rheum* **5,** 20–27 (2009).

31. Kinzler, K. *et al.* Identification of FAP locus genes from chromosome 5q21. *Science (80-.).* **253,** 661–665 (1991).

32. Morin, P. J. *et al.* Activation of beta -Catenin-Tcf Signaling in Colon Cancer by Mutations in beta-Catenin or APC. *Science (80-.).* **275,** 1787–1790 (1997).

33. Scheffzek, K. & Ahmadian, M. R. GTPase activating proteins: Structural and functional insights 18 years after discovery. *Cell. Mol. Life Sci.* **62,** 3014–3038 (2005).

34. Schubbert, S., Shannon, K. & Bollag, G. Hyperactive Ras in developmental disorders and cancer. *Nat. Rev. Cancer* **7,** 295–308 (2007).

35. Lau, K. & Haihis, K. Non-Redundancy within the RAS Oncogene Family Family: Insights into Mutational Disparities in Cancer. *Mol. Cell Biol.* **28,** 1–6 (2009).

36. Arvelo, F., Sojo, F. & Cotte, C. Biology of colorectal cancer. *Ecancermedicalscience* **9,** 1–20 (2015).

37. Velho, S. & Haigis, K. M. Regulation of homeostasis and oncogenesis in the intestinal epithelium by Ras. *Exp. Cell Res.* **317,** 2732–2739 (2011).

38. Trahey, M. & McCormick, F. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238,** 542–545 (1987).

39. Neel, N. F. *et al.* The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. *Genes Cancer* **2,** 275–287 (2011).

40. Danielsen, S. A. *et al.* Portrait of the PI3K/AKT pathway in colorectal cancer. *Biochim. Biophys. Acta - Rev. Cancer* **1855,** 104–121 (2015).

41. Mccubrey, J. A. *et al.* Roles Of The RAS/MEK/ERK pathway in Cell Growth, Malignant Transformation and Drug Resistance. *Biochim. Biophys. Acta - Mol. Cell Res.* **1773,** 1263–1284 (2007).

42. Oliveira, C. *et al.* KRAS and BRAF oncogenic mutations in MSS colorectal carcinoma progression. *Oncogene* **26,** 158–163 (2007).

43. Tan, Y. H. *et al.* Detection of BRAF V600E mutation by pyrosequencing. *Pathology*

40, 295–298 (2008).

44. Sancho, E., Batlle, E. & Clevers, H. Signaling Pathways in Intestinal Development and Cancer. *Annu. Rev. Cell Dev. Biol.* **20,** 695–723 (2004).

45. Sahin IH, Kazmi SM, Yorio JT, Bhadkamkar NA, Kee BK, G. C. Rare Though Not Mutually Exclusive: A Report of Three Cases of Concomitant KRAS and BRAF Mutation and a Review of the Literature. *J Cancer* **4,** 320–322 (2013).

46. Samuels, Y. & Velculescu, V. E. Oncogenic Mutations of PIK3CA in Human Cancers. *Cell Cycle* **3,** 941–945 (2004).

47. Ikenoue, T. *et al.* Functional analysis of PIK3CA gene mutations in human colorectal cancer. *Cancer Res.* **65,** 4562–4567 (2005).

48. Lipsyc, M. & Yaeger, R. Impact of somatic mutations on patterns of metastasis in colorectal cancer. *J. Gastrointest. Oncol.* **6,** 645–9 (2015).

49. Velho, S. *et al.* The prevalence of PIK3CA mutations in gastric and colon cancer. *Eur. J. Cancer* **41,** 1649–1654 (2005).

50. Wright, N. A. & Poulsom, R. Top down or bottom up? Competing management structures in the morphogenesis of colorectal neoplasms. *Gut* **51,** 306–308 (2002).

51. Shih, I. M. *et al.* Top-down morphogenesis of colorectal tumors. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2640–2645 (2001).

52. Preston, S. L. *et al.* Bottom-up histogenesis of colorectal adenomas: Origin in the monocryptal adenoma and initial expansion by crypt fission. *Cancer Res.* **63,** 3819–3825 (2003).

53. Ricci-Vitiani, L. *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* **445,** 111–115 (2007).

54. Brien, C. A. O., Pollett, A., Gallinger, S., Dick, J. E. & O'Brien, C. a. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445,** 106–110 (2007).

55. Boman, B. M. & Huang, E. Human colon cancer stem cells: A new paradigm in gastrointestinal oncology. *J. Clin. Oncol.* **26,** 2828–2838 (2008).

56. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and

cancer stem cells. *Nature* **414,** 105–111 (2001).

57. Vermeulen, L. *et al.* Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat. Cell Biol.* **12,** 468–476 (2010).

58. Kai, K. *et al.* Maintenance of HCT116 colon cancer cell line conforms to a stochastic model but not a cancer stem cell model. *Cancer Sci.* **100,** 2275–2282 (2009).

59. Human, E., Cancer, C. & Lines, C. CD133+, CD166+CD44+, and CD24+CD44+ Phenotypes Fail to Reliably Identify Cell Populations with Cancer Stem Cell Functional Features in Established Human Colorectal Cancer Cell Lines. *Stem Cells Transl. Med.* **60,** 592–603 (2012).

60. Haraguchi, N. *et al.* CD49f-positive cell population efficiently enriches colon cancer-initiating cells. *Int. J. Oncol.* **43,** 425–430 (2013).

61. Hough, M. R., Rosten, P. M., Sexton, T. L., Kay, R. & Humphries, R. K. Mapping of CD24 and Homologous Sequences to Multiple Chromosomal Loci. *Genomics* 154–161 (1994).

62. Honeth, G. *et al.* The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast cancer Res.* **10,** R53 (2008).

63. Lee, J.-H., Kim, S.-H., Lee, E. & Kim, Y.-S. CD24 overepression in cancer development and progression: A meta-analysis. *Oncol. Rep.* **22,** 1149–1156 (2009).

64. Kristiansen, G. *et al.* CD24 Expression Is a New Prognostic Marker in Breast Cancer. *Clin. Cancer Res.* **9,** 4906–4913 (2003).

65. Agrawal, S. *et al.* CD24 expression is an independent prognostic marker in cholangiocarcinoma. *J. Gastrointest. Surg.* **11,** 445–451 (2007).

66. Chou, Y.-Y. *et al.* Cytoplasmic CD24 expression is a novel prognostic factor in diffuse-type gastric adenocarcinoma. *Ann. Surg. Oncol.* **14,** 2748–58 (2007).

67. Wang, W. *et al.* CD24-dependent MAPK pathway activation is required for colorectal cancer cell proliferation. *Cancer Sci.* **101,** 112–119 (2010).

68. Orian-Rousseau, V. CD44, a therapeutic target for metastasising tumours. *Eur. J. Cancer* **46,** 1271–1277 (2010).

69. Wielenga, V. J. M. *et al.* Expression of CD44 Variant Proteins in Human Colorectal

Cancer Is Related to Tumor Progression. *Cancer Reasearch* **53,** 4754–4756 (1993).

70. Wielenga, V. J. *et al.* Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. *Am. J. Pathol.* **154,** 515–523 (1999).

71. Zeilstra, J. *et al.* Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. *Cancer Res.* **68,** 3655–3661 (2008).

72. Orian-Rousseau, V., Chen, L., Sleeman, J. P., Herrlich, P. & Ponta, H. CD44 is required for two consecutive steps in HGF / c-Met signaling. *Genes Dev.* 3074–3086 (2002).

73. Shmelkov, S. V, St Clair, R., Lyden, D. & Rafii, S. AC133/CD133/Prominin-1. *Int. J. Biochem. Cell Biol.* **37,** 715–719 (2005).

74. Elsaba, T. M. a *et al.* The stem cell marker CD133 associates with enhanced colony formation and cell motility in colorectal cancer. *PLoS One* **5,** (2010).

75. Kemper, K. *et al.* The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. *Cancer Res.* **70,** 719–729 (2010).

76. Dittfeld, C. *et al.* CD133 expression is not selective for tumor-initiating or radioresistant cell populations in the CRC cell line HCT-116. *Radiother. Oncol.* **94,** 375–383 (2010).

77. Sergey V. Shmelkov, Jason M. Butler, Andrea T. Hooper, Adilia Hormigo, Jared Kushner, Till Milde, Ryan St. Clair, Muhamed Baljevic, Ian White, David K. Jin, Amy Chadburn, Andrew J. Murphy, David M. Valenzuela, Nicholas W. Gale, Gavin Thurston, George D. Y, S. R. CD133 expression is not restricted to metastatic colon cancer cells initiate tumors. *J. Clin. Invest.* **118,** 2111–2120 (2008).

78. Swart, G. W. M. Activated leukocyte cell adhesion molecule (CD166/ALCAM): developmental and mechanistic aspects of cell clustering and cell migration. *Eur. J. Cell Biol.* **81,** 313–321 (2002).

79. Wiiger, M. T., Gehrken, H. B., Fodstad, O., Maelandsmo, G. M. & Andersson, Y. A novel human recombinant single-chain antibody targeting CD166/ALCAM inhibits cancer cell invasion in vitro and in vivo tumour growth. *Cancer Immunol. Immunother.* **59,** 1665–1674 (2010).

80. Schölch, S. *et al.* Circulating tumor cells exhibit stem cell characteristics in an

orthotopic mouse model of colorectal cancer. *Oncotarget* **7,** (2016).

81. Robertson, J. H., Yang, S. Y., Winslet, M. C. & Seifalian, A. M. Functional blocking of specific integrins inhibit colonic cancer migration. *Clin. Exp. Metastasis* **26,** 769–780 (2009).

82. Sakthianandeswaren, A. *et al.* PHLDA1 expression marks the putative epithelial stem cells and contributes to intestinal tumorigenesis. *Cancer Res.* **71,** 3709–3719 (2011).

83. Brooks, D. L. P. *et al.* ITGA6 is directly regulated by hypoxia-inducible factors and enriches for cancer stem cell activity and invasion in metastatic breast cancer models. *Mol. Cancer* **15,** 26 (2016).

84. Goel, H. L. *et al.* Regulated Splicing of the α6 Integrin Cytoplasmic Domain Determines the Fate of Breast Cancer Stem Cells. *Cell Rep.* **7,** 747–761 (2014).

85. Groulx, J.-F. *et al.* Integrin α6A splice variant regulates proliferation and the Wnt/β-catenin pathway in human colorectal cancer cells. *Carcinogenesis* **35,** 1217–27 (2014).

86. Dydensborg, A. B. *et al.* Integrin α6Bβ4 inhibits colon cancer cell proliferation and c-Myc activity. *BMC Cancer* **9,** 223 (2009).

87. Marcker Espersen, M. L., Olsen, J., Linnemann, D., Høgdall, E. & Troelsen, J. T. Clinical Implications of Intestinal Stem Cell Markers in Colorectal Cancer. *Clin. Colorectal Cancer* **14,** 63–71 (2014).

88. Roussel, M. F. The INK4 family of cell cycle inhibitors in cancer. *Oncogene* **18,** 5311–5317 (1999).

89. Yan, K., Chia, L. & Li, X. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Pnas* **109,** 466–471 (2012).

90. Tian, H. *et al.* A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* **478,** 255–9 (2011).

91. Du, J., Li, Y., Li, J. & Zheng, J. Polycomb group protein Bmi1 expression in colon cancers predicts the survival. *Med. Oncol.* **27,** 1273–1276 (2010).

92. Benard, A. *et al.* Prognostic value of polycomb proteins EZH2, BMI1 and SUZ12 and histone modification H3K27me3 in colorectal cancer. *PLoS One* **9,** (2014).

93. Li, D. W. *et al.* Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer. *J. Cancer Res. Clin. Oncol.* **136,** 997–1006 (2010).

94. Hsu, H. C. *et al.* LGR5 regulates survival through mitochondria-mediated apoptosis and by targeting the Wnt/β-catenin signaling pathway in colorectal cancer cells. *Cell. Signal.* **26,** 2333–2342 (2014).

95. Hirsch, D. *et al.* LGR5 positivity defines stem-like cells in colorectal cancer. *Carcinogenesis* **35,** 849–858 (2014).

96. Valladares-Ayerbes, M. *et al.* Evaluation of the adenocarcinoma-associated gene AGR2 and the intestinal stem cell marker LGR5 as biomarkers in colorectal cancer. *Int. J. Mol. Sci.* **13,** 4367–4387 (2012).

97. Asfaha, S. *et al.* Krt19+/Lgr5− Cells Are Radioresistant Cancer-Initiating Stem Cells in the Colon and Intestine. *Cell Stem Cell* **16,** 627–638 (2015).

98. Walker, F., Zhang, H. H., Odorizzi, A. & Burgess, A. W. LGR5 is a negative regulator of tumourigenicity, antagonizes wnt signalling and regulates cell adhesion in colorectal cancer cell lines. *PLoS One* **6,** (2011).

99. Gur, G. *et al.* LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *EMBO J.* **23,** 3270–81 (2004).

100. Ljuslinder, I. *et al.* LRIG1 expression in colorectal cancer. *Acta Oncol.* **46,** 1118–22 (2007).

101. Wong, V. W. Y. *et al.* Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat. Cell Biol.* **14,** 401–8 (2012).

102. van der Flier, L. G., Haegebarth, A., Stange, D. E., van de Wetering, M. & Clevers, H. OLFM4 Is a Robust Marker for Stem Cells in Human Intestine and Marks a Subset of Colorectal Cancer Cells. *Gastroenterology* **137,** 15–17 (2009).

103. Liu, W. *et al.* Olfactomedin 4 deletion induces colon adenocarcinoma in Apc(Min/+) mice. *Oncogene* 1–11 (2016).

104. Besson, D. *et al.* A Quantitative Proteomic Approach of the Different Stages of Colorectal Cancer Establishes OLFM4 as a New Nonmetastatic Tumor Marker. *Mol. Cell. Proteomics* **10** (2011).

105. Seko, N. *et al.* Olfactomedin 4 (GW112, hGC-1) is an independent prognostic marker

for survival in patients with colorectal cancer. *Exp. Ther. Med.* **1,** 73–78 (2010).

106. Mitsui, K. *et al.* The Homeoprotein Nanog Is Required for Maintenance of Pluripotency in Mouse Epiblast and ES Cells. *Cell* **113,** 631–642 (2003).

107. Zhang, J. *et al.* NANOGP8 is a retrogene expressed in cancers. *FEBS J.* **273,** 1723–1730 (2006).

108. Amsterdam, A. *et al.* Differential localization of LGR5 and Nanog in clusters of colon cancer stem cells. *Acta Histochem.* **115,** 320–329 (2013).

109. Meng, H. M. *et al.* Overexpression of nanog predicts tumor progression and poor prognosis in colorectal cancer. *Cancer Biol. Ther.* **9,** 295–302 (2010).

110. Lin, T. *et al.* p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* **7,** 165–171 (2005).

111. Zbinden, M. *et al.* NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. *EMBO J.* **29,** 2659–74 (2010).

112. Po, A. *et al.* Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *EMBO J.* **29,** 2646–58 (2010).

113. Ho, B. *et al.* Nanog Increases Focal Adhesion Kinase (FAK) Promoter Activity and Expression and Directly Binds to FAK Protein to Be Phosphorylated. *J. Biol. Chem.* **287 ,** 18656–18673 (2012).

114. Herath, N. I. & Boyd, A. W. The role of Eph receptors and ephrin ligands in colorectal cancer. *Int. J. Cancer* **126,** 2003–2011 (2010).

115. Holmberg, J. *et al.* EphB Receptors Coordinate Migration and Proliferation in the Intestinal Stem Cell Niche. *Cell* **125,** 1151–1163 (2006).

116. Kumar, S. R. *et al.* Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression. *Cancer Res.* **69,** 3736–3745 (2009).

117. Guo, D. L. *et al.* Reduced expression of EphB2 that parallels invasion and metastasis in colorectal tumours. *Carcinogenesis* **27,** 454–464 (2006).

118. Jubb, A. M. *et al.* EphB2 is a Prognostic Factor in Colorectal Cancer. *Clin Cancer Res* **11,** 5181–5187 (2005).

119. Pattabiraman, D. R. & Weinberg, R. A. Tackling the cancer stem cells - what

challenges do they pose? *Nat Rev Drug Discov* **13,** 497–512 (2014).

120. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).

121. Teng, M. W. L., Galon, J., Fridman, W. H. & Smyth, M. J. From mice to humans: Developments in cancer immunoediting. *J. Clin. Invest.* **125,** 3338–3346 (2015).

122. Italiani, P. & Boraschi, D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front. Immunol.* **5,** 1–22 (2014).

123. Ghajar, C. M. *et al.* The perivascular niche regulates breast tumour dormancy. *Nat. Cell Biol.* **15,** 807–817 (2013).

124. Hirata, E. *et al.* Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin β1/FAK Signaling. *Cancer Cell* **27,** 574–588 (2015).

125. Colak, S. & Medema, J. P. Human colonic fibroblasts regulate stemness and chemotherapy resistance of colon cancer stem cells. *Cell Cycle* **4101,** 00–00 (2014).

126. Tommelein, J. *et al.* Cancer-Associated Fibroblasts Connect Metastasis-Promoting Communication in Colorectal Cancer. *Front. Oncol.* **5,** 1–11 (2015).

127. Bai, Y.-P. *et al.* FGF-1/-3/FGFR4 signaling in cancer-associated fibroblasts promotes tumor progression in colon cancer through Erk and MMP-7. *Cancer Sci.* n/a–n/a (2015).

128. Martin, M., Pujuguet, P. & Martin, F. Role of stromal myofibroblasts infiltrating colon cancer in tumor invasion. *Pathol. Res. Pract.* **192,** 712–7 (1996).

129. Herrera, M. *et al.* Functional heterogeneity of cancer-associated fibroblasts from human colon tumors shows specific prognostic gene expression signature. *Clin. Cancer Res.* **19,** 5914–5926 (2013).

130. Mutoh, H. *et al.* Pericryptal fibroblast sheath in intestinal metaplasia and gastric carcinoma. *Gut* **54,** 33–39 (2005).

131. He, X. C. *et al.* BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* **36,** 1117–1121 (2004).

132. van den Brink, G. R. *et al.* Indian Hedgehog is an antagonist of Wnt signaling in

colonic epithelial cell differentiation. *Nat. Genet.* **36,** 277–282 (2004).

133. Van Dop, W. A. *et al.* Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine. *Gastroenterology* **139,** 1665–1676.e10 (2010).

134. Rupp, C. *et al.* IGFBP7, a novel tumor stroma marker, with growth-promoting effects in colon cancer through a paracrine tumor-stroma interaction. *Oncogene* 1–11 (2014).

135. Adegboyega, P. A., Mifflin, R. C., DiMari, J. F., Saada, J. I. & Powell, D. W. Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps, and adenomatous colorectal polyps. *Arch. Pathol. Lab. Med.* **126,** 829–836 (2002).

136. Harper, J. & Sainson, R. C. A. Regulation of the anti-tumour immune response by cancer-associated fibroblasts. *Semin. Cancer Biol.* **25,** 69–77 (2014).

137. Nakagawa, H. *et al.* Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene* **23,** 7366–7377 (2004).

138. Peddareddigari, V. G., Wang, D. & Dubois, R. N. The tumor microenvironment in colorectal carcinogenesis. *Cancer Microenviron.* **3,** 149–166 (2010).

139. De Boeck, A. *et al.* Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression. *Proteomics* **13,** 379–388 (2013).

140. Calon, A. *et al.* Dependency of Colorectal Cancer on a TGF-β-Driven Program in Stromal Cells for Metastasis Initiation. *Cancer Cell* **22,** 571–584 (2012).

141. Hawinkels, L. J. a C. *et al.* Interaction with colon cancer cells hyperactivates TGF-β signaling in cancer-associated fibroblasts. *Oncogene* **33,** 97–107 (2014).

142. De Boeck, A. *et al.* Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. *Gut* **62,** 550–560 (2013).

143. Henriksson, M. L. *et al.* Colorectal cancer cells activate adjacent fibroblasts resulting in FGF1/FGFR3 signaling and increased invasion. *Am. J. Pathol.* **178,** 1387–1394 (2011).

144. Moon, B. S. *et al.* Role of oncogenic K-Ras in cancer stem cell activation by aberrant Wnt/β-catenin signaling. *J. Natl. Cancer Inst.* **106,** 1–10 (2014).

145. Ahmed, D. *et al.* Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* **2,** e71 (2013).

146. Lemieux, E., Cagnol, S., Beaudry, K., Carrier, J. & Rivard, N. Oncogenic KRAS signalling promotes the Wnt/β-catenin pathway through LRP6 in colorectal cancer. *Oncogene* **34,** 4914–27 (2015).

147. Rolle, A. Le *et al.* Oncogenic KRAS activates an embryonic stem cell-like program in human colon cancer initiation. *Oncogene* **7,** (2016).

148. Kanwar, S. S., Yu, Y., Nautiyal, J., Patel, B. B. & Majumdar, A. P. N. The Wnt/beta-catenin pathway regulates growth and maintenance of colonospheres. *Mol. Cancer* **9,** 212 (2010).

149. Voorneveld, P. W. *et al.* The BMP pathway either enhances or inhibits the Wnt pathway depending on the SMAD4 and p53 status in CRC. *Br. J. Cancer* **112,** 122–30 (2015).

150. Chen, J. *et al.* PI3K/Akt/mTOR pathway dual inhibitor BEZ235 suppresses the stemness of colon cancer stem cells. *Clin. Exp. Pharmacol. Physiol.* **42,** 1317–26 (2015).

151. Cho, Y., Lee, H., Kang, H., Kim, H. & Chun, K. Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. *Oncotarget* **6,** 8709–8721 (2015).

152. Shtutman, M. *et al.* The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5522–7 (1999).

153. Findlay, V. J., Wang, C., Watson, D. K. & Camp, E. R. Epithelial-to-mesenchymal transition and the cancer stem cell phenotype: insights from cancer biology with therapeutic implications for colorectal cancer. *Cancer Gene Ther.* **21,** 181–7 (2014).

154. Guo, Y. *et al.* Wnt / β-catenin pathway transactivates microRNA-150 that promotes EMT of colorectal cancer cells by suppressing CREB signaling. *Oncotarget* (2016).

155. Hollestelle, A. *et al.* Loss of E-cadherin is not a necessity for epithelial to mesenchymal transition in human breast cancer. *Breast Cancer Res. Treat.* **138,** 47–

57 (2013).

156. Chen, J.-C. *et al.* Amphiregulin enhances alpha6beta1 integrin expression and cell motility in human chondrosarcoma cells through Ras/Raf/MEK/ERK/AP-1 pathway. *Oncotarget* **6,** 11434–46 (2015).

SUPPLEMENTARY FIGURES

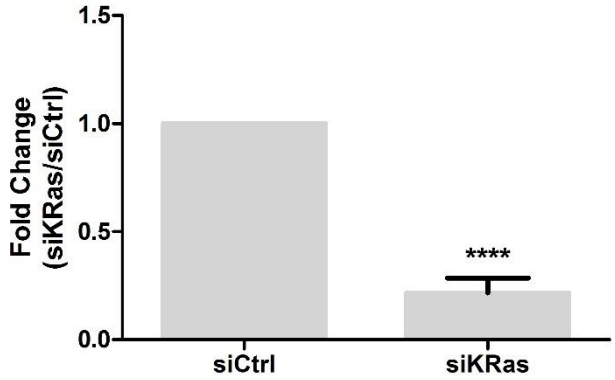

Figure S 1. Confirmation of KRas inhibition in HCT-116 cell line (P<0,0001). Results are shown as average ± SD, n=7.

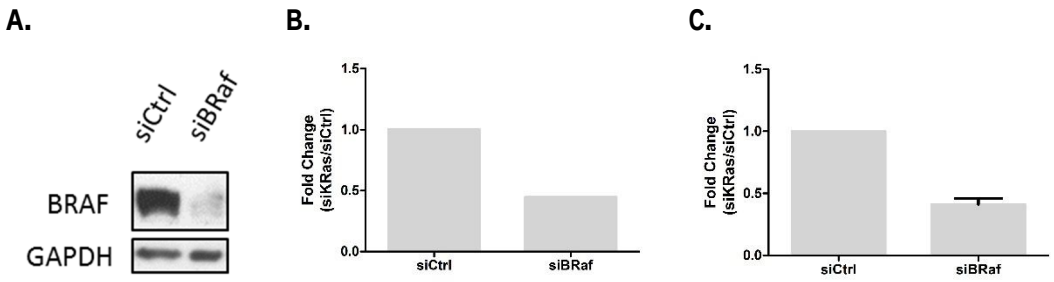

Figure S 2. Confirmation of *BRaf* inhibition in HCT-116 cell line. A. Western blot for BRaf in HCT-116 cell line. **B.** Inhibition confirmation for samples used to evaluate CD24 expression, n=1 **C.** Inhibition confirmation for samples used to evaluate CD44, CD133 and CD166 expression, n=2. Results are shown as average ± SD.

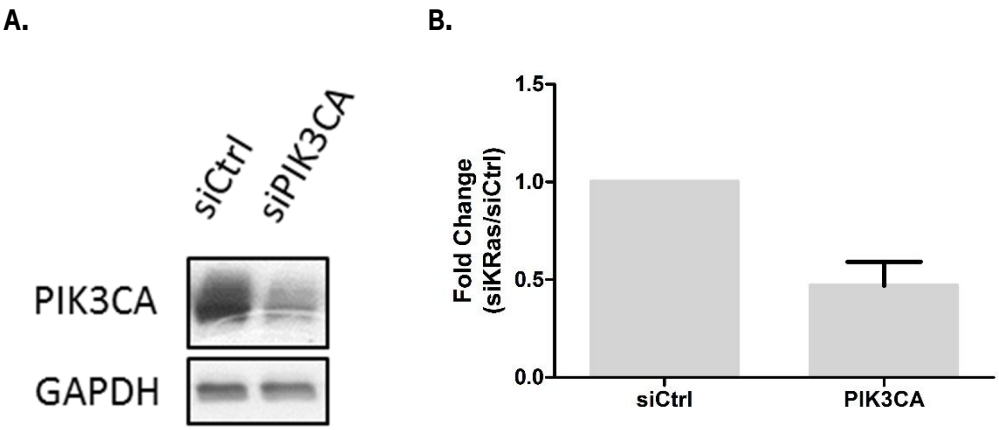

Figure S 3. Confirmation of *PIK3CA* inhibition in HCT-116 cell line. A. Western blot for PIK3CA in HCT-116 cell line. **B.** Inhibition confirmation for samples used to evaluate CD24, CD44, CD133 and CD166 expression, n=2. Results are shown as average ± SD.

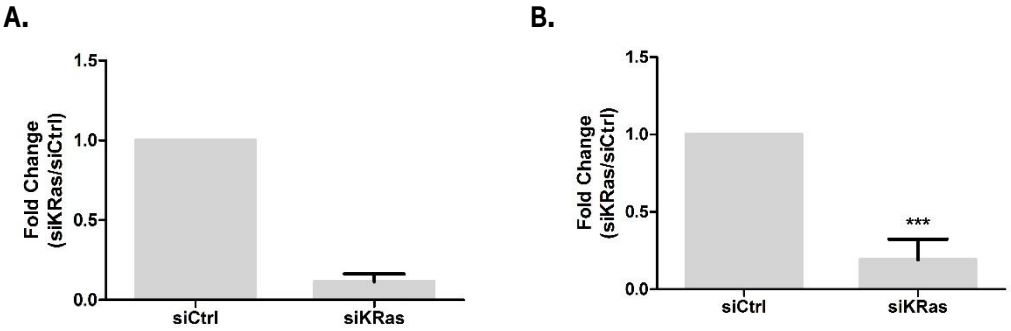

Figure S 4. Confirmation of *KRas* inhibition in SW480 cell line. A. Samples used for the evaluation of CD24 expression. n=2 **B.** Samples used for the evaluation of CD44, CD133 and CD166 expression. P= 0,0089, n=3. Results are shown as average ± SD

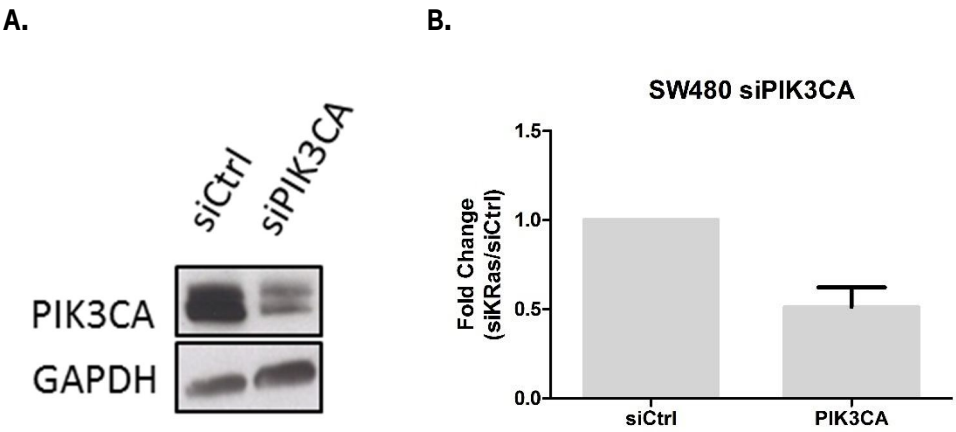

Figure S 5. Confirmation of *PIK3CA* inhibition in SW480 cell line. A. Western blot for PIK3CA in SW480 cell line. **B.** Inhibition confirmation for samples used to evaluate CD24, CD44, CD133 and CD166 expression, n=2. Results are shown as average ± SD.

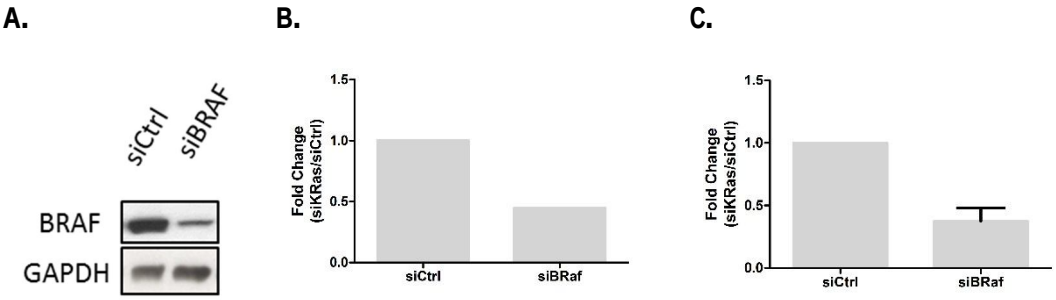

Figure S 6. Confirmation of *BRaf* inhibition in RKO cell line. A. Western blot for BRaf in RKO cell line. **B.** Inhibition confirmation for samples used to evaluate CD24 expression, n=1 **C.** Inhibition confirmation for samples used to evaluate CD44, CD133 and CD166 expression, n=2. Results are shown as average ± SD.

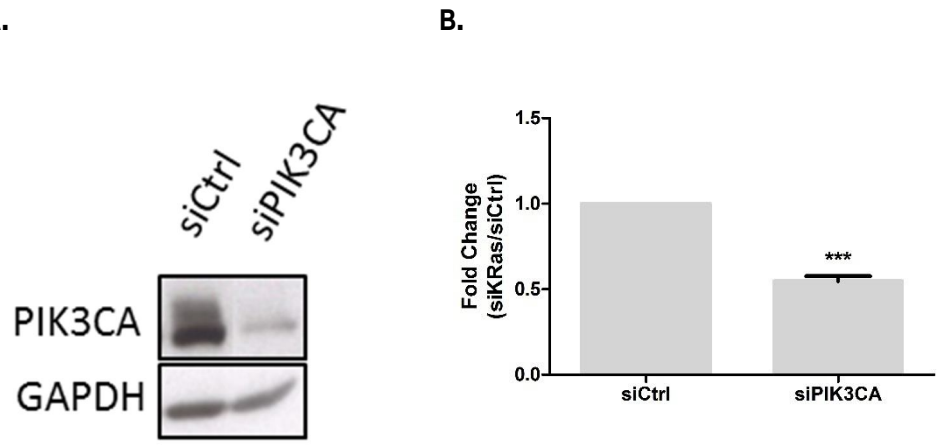

Figure S 7. Confirmation of *PIK3CA* inhibition in RKO cell line. A. Western blot for PIK3CA in RKO cell line. **B.** Inhibition confirmation for samples used to evaluate CD44, CD133 and CD166 expression, P=0,0015, n=3. Results are shown as average ± SD.

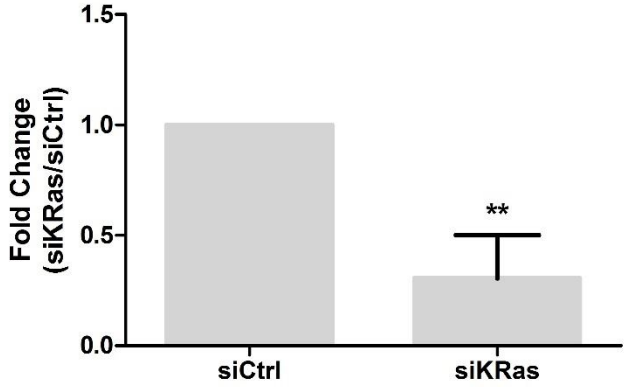

Figure S 8. Confirmation of *KRas* inhibition in HCT-15 cell line. Results are shown as average ± SD, n=3

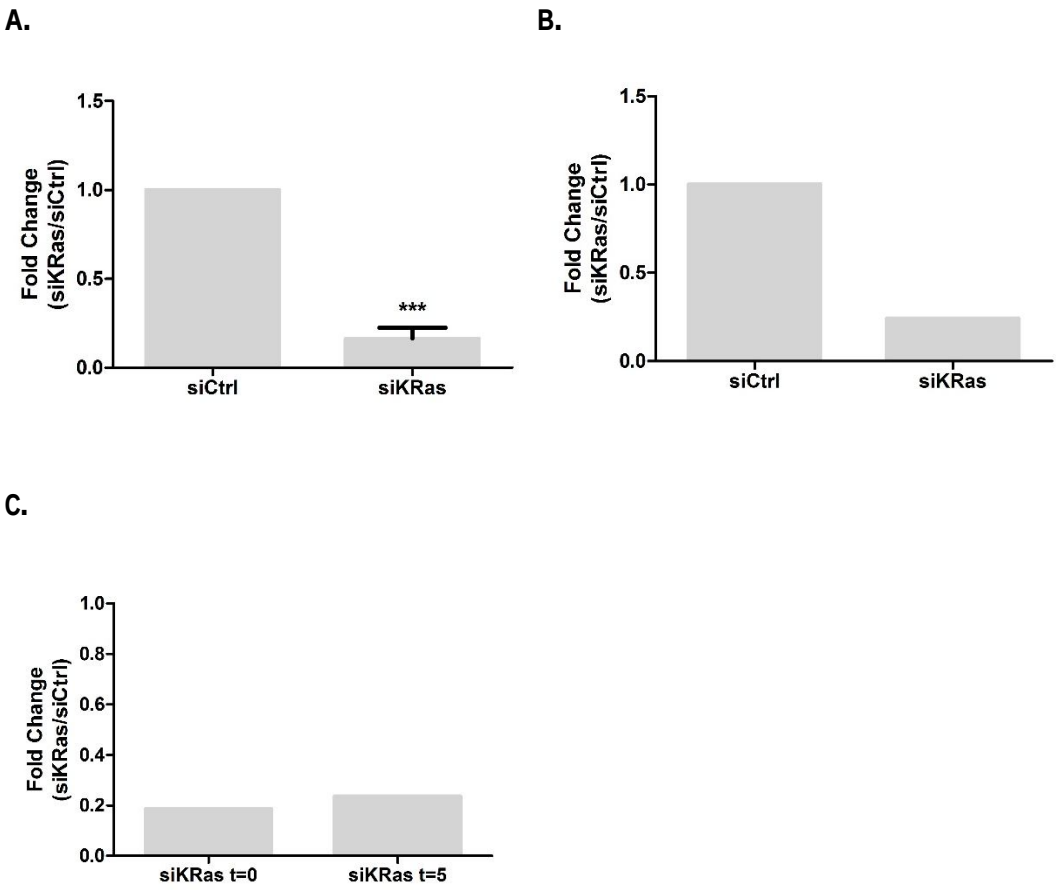

Figure S 9. Confirmation of *KRas* inhibition in HCT-116 and SW480 cell lines used for colosphere-forming assay. A. Confirmation of HCT-116 cell line inhibition on the day of plating, P=0,0001, n=3. **B.** Confirmation of HCT-116 cell line inhibition one day after plating, n=1. **C.** Confirmation of SW480 cell line inhibition on 2D cultured cells, isolated on the day of cell plating for sphere formation (t=0) and 5 days after plating (t=5), n=1. Results are shown as average ± SD.

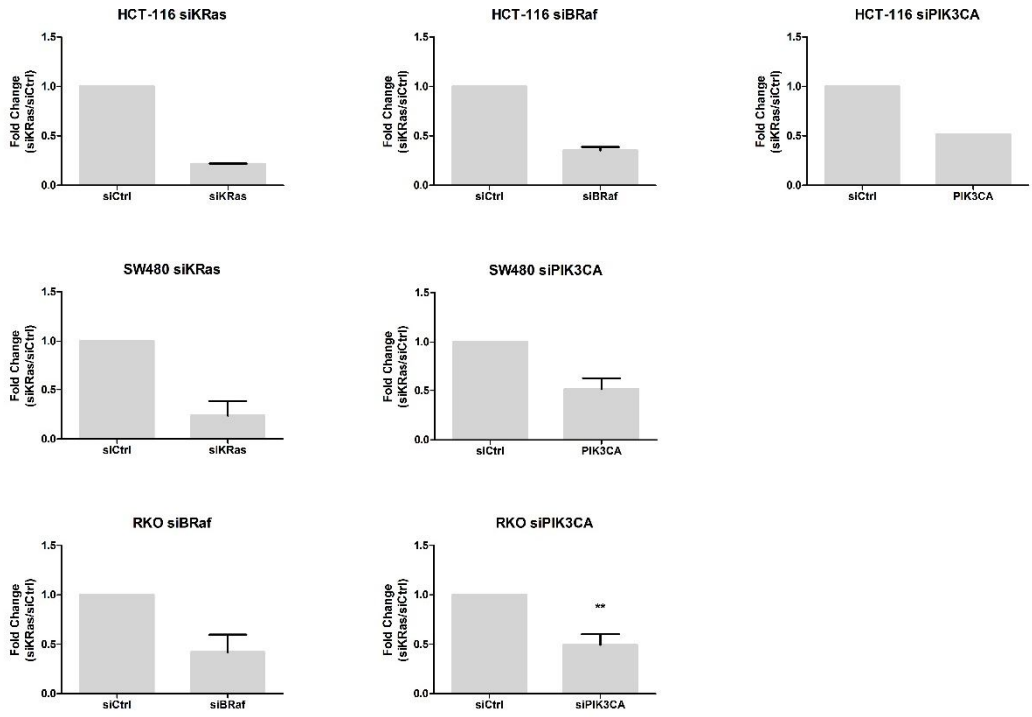

Figure S 10. Confirmation of inhibition of *KRas* (by real time PCR), *BRaf* and *PIK3CA* (by western blot) in HCT-116, SW480 and RKO cell lines for the samples used in E-cadherin expression. Results are shown as relative average to siCtrl-treated cells. Results are shown as average ± SD, n=2 except HCT-116 siPIK3CA n=1, SW480 siPIK3CA n=3 (P= 0,0260), RKO siPIK3CA n=4 (P= 0,0261).

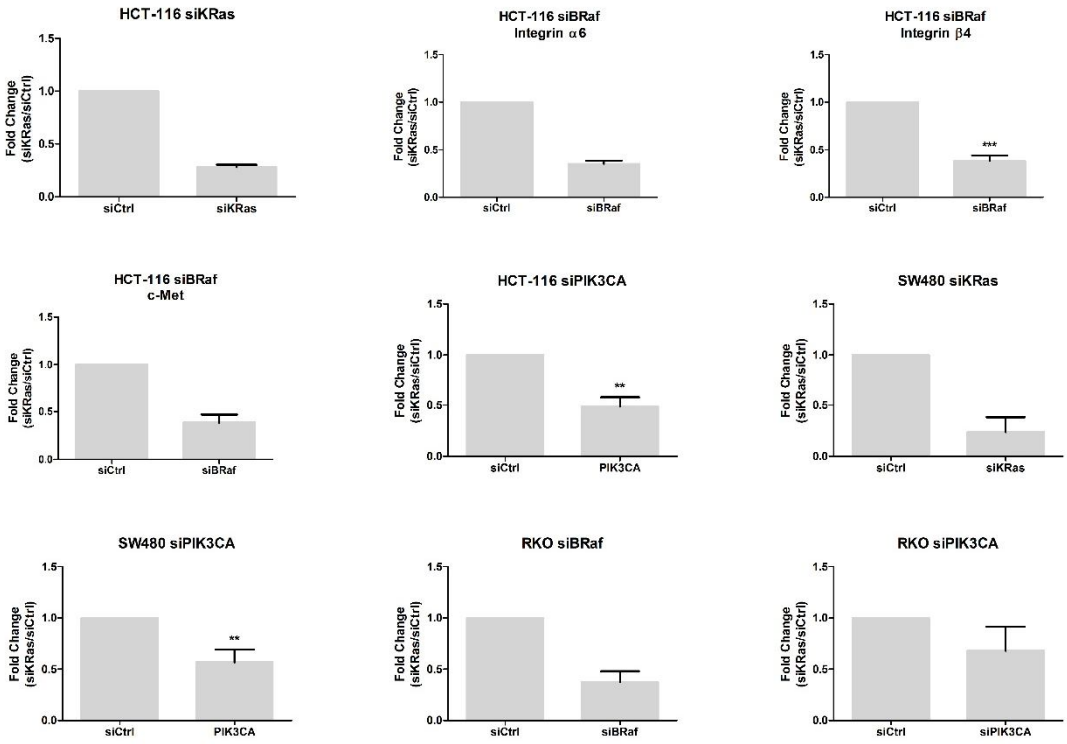

Figure S 11. Confirmation of inhibition of KRas (by real time PCR), BRaf and PIK3CA (by western blot) in HCT-116, SW480 and RKO cell lines for the samples used in c-Met, integrins α6 and β4 expression quantification. Results are shown as relative average to siCtrl-treated cells. n=3 except in n=3 except in HCT-116 siBRaf, RKO siBRaf and RKO siPIK3CA: c-Met and CD49f n=2; HCT-116 and SW480 siKRas n=2.

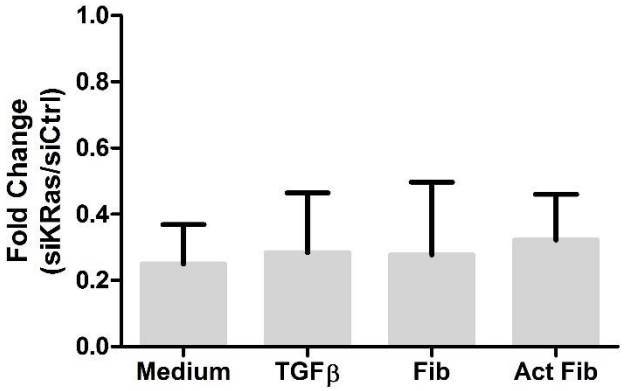

Figure S 12. Confirmation of KRas inhibition in HCT-116 cell line treated with conditioned media. Inhibition confirmation for samples used to evaluate CD44 and CD44v6 expression. Results are shown as average ± SD, n=2

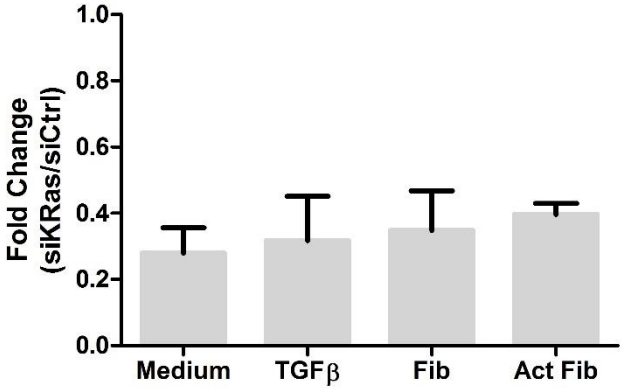

Figure S 13. Confirmation of *KRas* inhibition in HCT-116 cell line treated with conditioned media. Inhibition confirmation for samples used to evaluate E-Cadherin expression. Results are shown as average ± SD, n=2

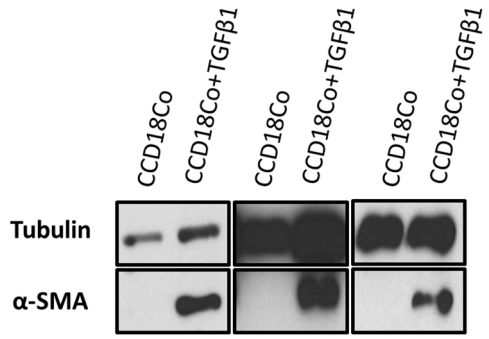

Figure S 14. Confirmation of activation of fibroblasts used in the microenvironment studies by the expression the α-SMA