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Effect of the microenvironment on alternative splicing in colorectal cells

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Doutoramento em Bioquímica
Especialidade de Genética Molecular

Joana Filipa de Sousa Pereira

Tese orientada por:
Doutor Peter Jordan

Documento especialmente elaborado para a obtenção do grau de doutor

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Júri:

Presidente:

- Doutor Manuel Eduardo Ribeiro Minas da Piedade, Professor Catedrático e Presidente do Departamento de Química e Bioquímica, da Faculdade de Ciências da Universidade de Lisboa

Vogais:

- Doutora Maria José Cardoso Oliveira, Investigadora Principal I3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto;
- Doutora Ana Arminda Lopes Preto de Almeida, Professora Auxiliar Escola de Ciências da Universidade do Minho;
- Doutor Nuno Luís Barbosa Morais, Professor Associado Convidado
- Instituto de Medicina Molecular João Lobo Antunes da Faculdade de Medicina da Universidade de Lisboa;
- Doutor Carlos Miguel Ribeiro da Silva Farinha, Professor Associado com Agregação Faculdade de Ciências da Universidade de Lisboa;
- Doutor Peter Jordan, Professor Associado Convidado Faculdade de Ciências da Universidade de Lisboa, Orientador.

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“Aprendi que mais vale tentar do que recuar...
Antes acreditar que duvidar,
o que vale na vida não é o ponto de partida
e sim a nossa caminhada.”

Cora Coralina

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Abstract

The mucosa of our gastrointestinal tract forms a barrier that separates the gut lumen from the surrounding tissues. Perturbation of the barrier function characterizes inflammatory bowel diseases, which constitute a risk factor for colon cancer development, because an inflammatory microenvironment is a tumor-promoting condition that provides survival signals to which cancer cells respond with changes in their gene expression. One key regulatory mechanism is alternative splicing. One example is RAC1B, a RAC1 alternative splicing variant, that the host laboratory previously identified in a subset of BRAF-mutated colorectal tumors, and was found increased in samples from inflammatory bowel disease or following experimentally-induced acute colitis in a mouse model.

The main goal of this work was to generate co-culture models to understand whether colorectal cancer cells respond to a pro-inflammatory microenvironment with changes in alternative splicing of RAC1B.

For this, culture conditions for polarized 2D and 3D models were established as physiologically more relevant colon cell models. Caco-2 colorectal cancer cells were grown as polarized cells on porous membranes and then co-cultured with stromal cells: fibroblasts, monocytes and macrophages. RAC1B expression was analyzed in Caco-2 by qRT-PCR, Western blot and confocal fluorescence microscopy. Co-culture experiments revealed that the presence of fibroblasts and/or M1 macrophages induced a transient increase in RAC1B protein levels in the colorectal cells, accompanied by a loss of epithelial organization. Through the use of a human inflammation array, we were able to identify three cytokines in co-culture media that associated with increased RAC1B: IL-1β, IL-6 and IL-8. In addition, the analysis of cytokine-stimulated signaling pathways allowed to identify the phosphorylation of ERK 1/2 and STAT3 as a major response to the external signals.

Overall, our data present evidence that a pro-inflammatory environment triggers an increase in RAC1B expression in colon epithelial cells. Since RAC1B was shown to sustain tumor cell survival and promote escape form oncogene-induced senescence, the data further strengthen the causal connection between inflammatory conditions and the development of colorectal cancer, and may indicate RAC1B as a potential biomarker.

Keywords: Colorectal cancer, RAC1B, Inflammatory microenvironment, Cytokines, Signaling pathways

Resumo

A mucosa do trato gastrointestinal é responsável pela formação de uma barreira que separa o lúmen intestinal dos tecidos circundantes. A perturbação da sua função de barreira, característica de doenças inflamatórias intestinais, constitui um fator de risco para o desenvolvimento do cancro colo-rectal. A alteração da flora intestinal e a forte presença de células com um elevado índice proliferativo nas criptas intestinais, também são aspetos que tornam a mucosa mais vulnerável à progressão tumoral.

Um microambiente inflamatório é uma condição promotora do desenvolvimento tumoral, que fornece sinais de sobrevivência aos quais as células respondem com alterações na sua expressão génica. Um dos mecanismos chave que permite às células esta regulação é o *splicing* alternativo. Um exemplo desta alteração, em resposta ao ambiente extracelular, é a variante de *splicing* alternativo do gene *RAC1*, RAC1B, que foi anteriormente identificada no laboratório de acolhimento, como estando aumentada num subtipo de tumores do cólon, caracterizados pela presença de mutação no gene *BRAF*, designada por via serreada. Além disso, esta variante foi também encontrada aumentada em amostras de pacientes com doença inflamatória do intestino e também após a indução experimental de colite aguda em modelos de ratinhos transgénicos. Contudo, sabe-se que o aumento de RAC1B não está associado com mutações no seu gene, pelo que ainda é fundamental compreender o que desencadeia o seu *splicing* alternativo.

Posto isto, o principal objetivo deste trabalho foi desenvolver modelos de co-cultura para estudar como as células do cólon respondem a um microambiente pró-inflamatório, através da análise do *splicing* alternativo de RAC1B.

O modelo celular Caco-2, que proveio de um adenocarcinoma de cólon humano, foi o escolhido para satisfazer o propósito deste trabalho, pela sua capacidade de formar uma camada epitelial com organização polarizada quando exposto a condições específicas de cultura. Sendo assim, estas células foram crescidas em filtros membranares porosos, que permitem a diferenciação e polarização celular, avaliada pela monotorização da resistência elétrica trans-epitelial e, em seguida, co-cultivadas com diferentes células do estroma (fibroblastos, monócitos e macrófagos), mimetizando o microambiente circundante do tumor. A expressão de RAC1B nas células Caco-2 foi analisada por qRT-PCR, *Western blot* e microscopia confocal de fluorescência. As células foram também

cultivadas em condições de formar esferóides, num plano 3D, com a formação de um lúmen interior.

Os resultados obtidos na co-cultura com células polarizadas revelaram que a presença de fibroblastos e/ou macrófagos M1 induzia um aumento transitório dos níveis de proteína RAC1B nas células Caco-2, acompanhado pela perda progressiva da organização epitelial obtida pela polarização celular.

A comparação dos resultados com outros modelos celulares não-polarizados permitiu perceber a importância que estes modelos 2D e 3D polarizados têm no estudo do cancro colorretal, nomeadamente como modelos fisiologicamente mais relevantes no estudo da camada epitelial do intestino no contexto do cancro. Contudo, as culturas 3D desenvolvidas em Matrigel, apesar da sua enorme relevância, mostraram algumas desvantagens técnicas, uma vez que são de difícil manutenção e não permitem a análise em separado dos diferentes tipos celulares que compõem as co-culturas.

Para determinar os sinais extracelulares provenientes das co-culturas, o seu meio condicionado foi analisado usando um *array* de anticorpos de citocinas, que permitiu identificar quatro citocinas com um possível papel no aumento da expressão da proteína RAC1B: GM-CSF, IL-1β, IL-6 e IL-8, todas estas citocinas associadas a quadros pró-inflamatórios. A adição individual destas citocinas purificadas às células Caco-2 polarizadas demonstrou que IL-1β, IL-6 e IL-8 levam a um aumento de RAC1B. Enquanto que o bloqueio da citocina IL-6, por adição de anticorpos específicos, leva a uma diminuição da quantidade de proteína RAC1B nas células Caco-2, tanto quando estão em co-cultura com células do estroma, como em ensaios com a adição de citocina purificada. Contudo, a adição de combinações de citocinas em simultâneo não mostrou ter um papel aditivo no aumento de RAC1B.

Os resultados identificaram também uma relação da citocina MIP-2α com o aumento de RAC1B, que apesar de não ser uma citocina presente no *array,* já tinha sido previamente descrita como estando associada à acumulação de RAC1B em modelos de ratinhos transgénicos.

Por outro lado, a análise de vias de sinalização estimuladas pelas citocinas da co-cultura, permitiu identificar a fosforilação de ERK 1/2 e STAT3 como tendo um papel fundamental na resposta a estímulos externos pelas células Caco-2, fazendo a ponte entre o microambiente pró-inflamatório e a maquinaria molecular que controla a variante RAC1B. A utilização de um inibidor da cinase MEK, que integra a via de sinalização ERK 1/2 e que se encontra imediatamente a montante da ativação da cinase ERK, leva

não só a uma diminuição da fosforilação de ERK, como a uma diminuição da expressão de RAC1B.

A análise do *array* de fosforilação permitiu ainda identificar outras alterações no padrão de fosforilação de proteínas, na condição em que RAC1B se encontra aumentado, que ainda requerem uma validação futura.

Por fim, a análise de amostras parafinadas de pacientes com doença inflamatória do intestino, por qRT-PCR, permitiu correlacionar a sobre-expressão de RAC1B com a progressão do estádio da doença e a sua severidade. Por seu lado, as amostras controlo, ou seja apenas de mucosa intestinal sem sinais de doença, apresentaram uma expressão de RAC1B normal. Todavia, este estudo teve acesso a um número reduzido de amostras, pelo que é necessário aumentar a quantidade de amostras analisadas para consolidar a associação observada.

No geral, os nossos resultados sugerem que o ambiente pró-inflamatório presente no intestino leva a um aumento da expressão de RAC1B nas células epiteliais do cólon. Uma vez que, já foi demonstrado que RAC1B tem a capacidade de sustentar a sobrevivência e promover a evasão à senescência que é induzida por oncogenes, estes dados fortalecem ainda mais a conexão causal entre doenças inflamatórias e o desenvolvimento de cancro colorretal, o que pode indicar RAC1B como um potencial biomarcador ou como um novo alvo terapêutico no tratamento de tumores do cólon do subtipo da via serreada.

Palavras-chave: Cancro colorretal, RAC1B, Microambiente inflamatório, Citocinas, Vias de sinalização

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Abbreviations

˚C	Degree Celsius
2D	Two-dimensional
3D	Tree-dimensional
3’ UTR	Three prime untranslated region
ABCC1	ATP binding cassette subfamily C member 1
AKT	Protein kinase B
APC	Adenomatous polyposis coli
AS	Alternative splicing
ATP	Adenosine triphosphate
BAD	Pro-apoptotic protein of the Bcl-2 family
BCL-xL	B-cell lymphoma-extra large
BCL-xS	B-cell lymphoma-extra small
BCL2	B-cell lymphoma 2
BEV	Bacterial extracellular vesicle
BLANK	Blank spots
bp	Base pair
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CASP9	Caspase-9
CBC	Crypt base columnar
CD44	CD44 molecule
CDC42	Cell division cycle 42
cDNA	Complementary DNA
CIMP	CpG island methylator phenotype
CIMP-H	CIMP-high
CIMP-L	CIMP-low
CIN	Chromosomal abnormalities
CLK1	Dual specificity protein kinase
CMS	Consensus molecular subgroups
CO_2	Carbon dioxide
CRC	Colorectal cancer

CRI	Cancer-related inflammation
CRIB	Cdc42/Rac interactive binding motif
CSC	Cancer stem cell
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCR2	C-X-C motif chemokine receptor 2
DAPI	4′,6-diamidine-2′-phenylindole dihydrochloride
DC	Dendritic cell
DCC	Deleted in colorectal carcinoma
DMEM	Dulbecco's modified Eagle medium
DMEM/F12	Dulbecco's modified Eagle medium/nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
F	Forward
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FTC	Follicular thyroid carcinoma
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Guanine dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate

h	Hour
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia-inducible factor-1 alpha
hMENA	ENAH actin regulator
HRAS	Harvey murine sarcoma virus oncogene
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interkeukin
INF-ɣ	Interferon gamma
JNK	c-Jun N-terminal kinase
KLF6	Krueppel-like factor 6
KRAS	Kirsten rat sarcoma viral oncogene homolog
L	Liter
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid-derived suppressor cell
MED24	Mediator complex subunit 24
MEK	Mitogen-activated protein kinase
MFI2	Melanotransferrin
MHL1	Human mutL homolog 1
min	Minute
MIP-2α	Macrophage inflammatory protein 2- alpha
miRNA	Micro RNA
mL	Milliliter
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MSI	Microsatellite instability
MSI-H	High microsatellite instability
MSI-L	Low microsatellite instability
MSS	Microsatellite stability
mTORC1	Mammalian target of rapamycin complex 1

NBCS	Heat inactivated newborn calf serum
NEG	Negative control spots
NF-κB	Nuclear factor-kappa B
ng	Nanogram
NIH	National Institutes of Health
NK	Natural killer
NLR	Nucleotide-binding and oligomerization domain-like receptor
NLRP3	NLR pyrin domain-containing protein 3
nm	Nanometer
NRAS	Neuroblastoma RAS viral oncogene homolog
NRF2	Nuclear factor erythroid 2–related factor 2
NSCLC	Non-small cell lung cancer
OIS	Oncogene-induce senescence
p	Phospho
PAK	p21-activated kinase
PBS	Phosphate-buffered Saline
PBS-T	PBS + 0.01% Tween
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L	Programmed death ligand
PDAC	Pancreatic adenocarcinoma
PDGF	Platelet-derived growth factor
PEN/STREP	Penicillin/streptomycin
PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PKM	Pyruvate kinase M
PMA	Phorbol-12-myristate 13-acetate
POS	Positive control spots
PTC	Papillary thyroid carcinoma

PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qRT-PCR	Real-time quantitative reverse transcribed-polymerase chain reaction
R	Reverse
RAC1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras homolog family member A
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RON	Macrophage-stimulating protein receptor
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute 1640 medium
RTK	Receptor tyrosine kinase
S	Serine
SAM68	KH domain containing, RNA binding, signal transduction associated 1
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
sec	Second
sIL-6R	Soluble IL-6 receptor
SMAD4	SMAD family member 4
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
SR	Serine/arginine-rich
SRC	Proto-oncogene tyrosine-protein kinase Src
SRPK1	SRSF protein kinase 1
SRRT	Serrate, RNA effector molecule
SRSF1	Serine/arginine-rich splicing factor 1
SRSF3	Serine/arginine-rich splicing factor 3
STAT3	Signal transducer and activator of transcription 3
T	Threonine
TAM	Tumor-associated macrophage
TBS	Tris-buffered saline
TCF	T cell factor
TEC	Tumor endothelial cell

TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor beta
Th	T-helper cell
TJ	Tight junctions
TME	Tumor microenvironment
TNF-α	Tumour necrosis factor alpha
TP53	Tumor protein p53
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
WB	Western blot
Y	Tyrosine
YAP	Yes-associated protein
µL	Microliter
µm	Micrometer
µM	Micromolar

Chapter 1 - General Introduction

In the human body, the complex physiological processes need to be coordinated at cellular level. Circulating cytokines, hormones, and growth factors control aspects such as cell proliferation, differentiation, metabolism, angiogenesis, apoptosis, and senescence. Cells respond to such signals from their environment through sensors at the cell surface, receptor proteins that propagate their activation to intracellular proteins via sequential protein kinase signaling, including in the nucleus, where transcription factors become activated resulting in changes in gene expression and subsequent biological response by the cell.

Tumor cells develop several well-defined features that cause dysregulation of cellular signal transduction pathways, leading to increased cell proliferation, resistance to apoptosis, metabolic changes, genetic instability, induction of angiogenesis and increased migratory capacity. This dysregulation involves genetic mutations in the tumor cells but also a complex interplay and exchange of signals with surrounding non-neoplastic cells and the extracellular matrix (ECM), designated as the tumor microenvironment (TME).

1. 1 Cancer

Cancer is the second leading cause of mortality worldwide and manifests as a variety of tissue-specific diseases and this variability is a major challenge for its specific diagnosis and treatment (Ferlay *et al.* 2019). In a general way, cancer is a disease of uncontrolled proliferation whereby cells have escaped the normal growth control mechanisms and gained the ability to divide indefinitely. It is a multi-step process that requires the acquisition of mutations in key regulatory genes, which arise as a consequence of genome damage. So, cancer is much more than a simple genetic disease (Bertram 2001; Hassanpour and Dehghani 2017; Korgaonkar and Yadav 2019).

Hanahan and Weinberg (2000, 2011), proposed a classification of ten key aspects of cancer pathophysiology, designated hallmarks of cancer, that incorporates eight distinct functional capabilities and two enabling facilitators (see Figure 1.1): sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics and metabolism, avoiding immune destruction, genome instability and tumor-promoting immune cell infiltration (inflammation) (Hanahan and Weinberg 2000, 2011).

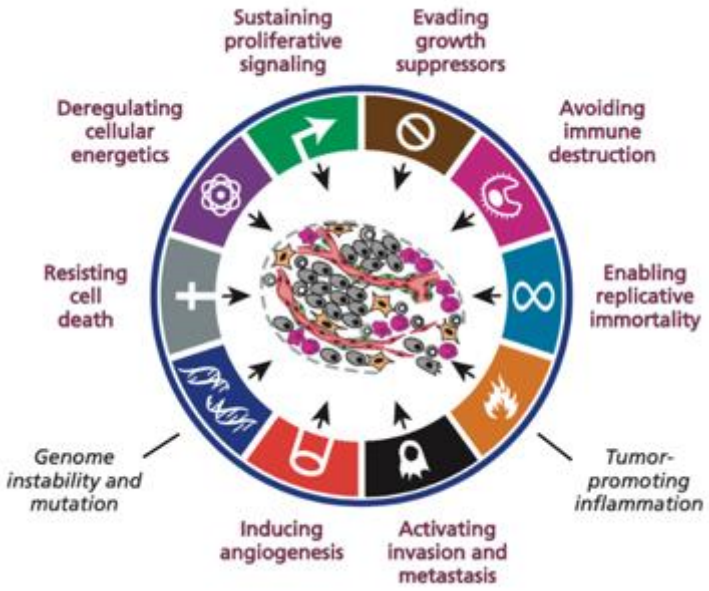

Figure 1.1 - **The biological hallmarks of cancer**. The scheme illustrates typical characteristics that manifest in malignant disease - the hallmarks of cancer, comprising eight distinct and complementary functional capabilities and two facilitators (in black italics) of their acquisition. Adapted from Hanahan and Weinberg, 2011.

In a simplified view of solid tumors, these alterations typically promote progression from an initially benign group of proliferating cells (hyperplasia) to a mass of cells with abnormal morphology, appearance and cellular organization. After a tumor expands, the cells at the tumor core lose access to oxygen and nutrients, often inducing the growth of new blood vessels (angiogenesis), which restore access to nutrients and oxygen. Subsequently, tumor cells can develop the ability to invade by leaving the tissue of origin, enter the circulation, and seed new tumors at other locations and different organs (metastasis). However, this simplified view of solid tumors does not apply to all, as there are tumors, such as diffuse type gastric cancer and some breast tumors, that typically show an infiltrative and poorly cohesive appearance, due to loss of expression of adhesion molecules, so at a very early stage in their development they start to disseminate metastases (Iyer *et al.* 2020).

Metastasis is the defining feature of malignancy and the principal cause of cancer lethality (Kalluri 2016; Suhail *et al.* 2019).

Investigation of cancer biology has provided a prosperity of knowledge on cancer initiation and propagation and has enabled the development of new and more specific treatment strategies against cancer.

1. 2 Cancer cell signaling

Cancer is a disease in which cells have acquired the ability to divide and grow uncontrollably, usually through genetic alterations in specific genes involved in signaling pathways that control key processes associated with tumorigenesis and classified by the above cancer hallmarks (Garraway and Lander 2013; Vogelstein *et al.* 2013). Cell proliferation and differentiation are regulated by a number of hormones, growth factors and cytokines. These molecules interact with cellular receptors and communicate with the nucleus of the cell through a network of intracellular signaling pathways. The majority of these molecules are protein kinases, although other signaling proteins are involved in metabolic pathways, protein transport and protein degradation (Adjei and Hidalgo 2005).

In cancer cells, oncogenic mutations in the affected genes can originate their overexpression or production of mutated proteins whose activity is dysregulated. Alternatively, deletions and other mutations can inactivate critical negative regulators of signaling that normally function as tumor suppressors. Definitely, one of the most commonly mutated genes in cancer is the tumor suppressor p53 (Sever and Brugge 2015).

Signal transduction defines the precise series of molecular events that occur to convert an external stimulus into a cellular response. Individual tumors typically have multiple functional alterations affecting more than a single pathway.

There is considerable variation in the genes and pathways altered across different tumor types and individual tumor samples; however, the most frequently affected proteins and signaling pathways conferring tumor-promoting properties are growth factor receptor tyrosine kinases (RTKs), for example the epidermal growth factor receptor (EGFR), small GTPases (e.g., RAS), serine/threonine kinases [e.g., protein kinase B (AKT)], cytoplasmic tyrosine kinases [e.g., proto-oncogene tyrosine-protein kinase Src (SRC)], lipid kinases [e.g., phosphoinositide 3-kinases (PI3Ks)], as well as nuclear receptors (e.g., the estrogen receptor). Components of developmental signaling pathways, such as WNT, Hedgehog and NOTCH can also be affected. Downstream nuclear targets of signaling pathways such as transcription factors [e.g., MYC and nuclear factor-kappa B (NF-κB)] or cell cycle effectors (e.g., cyclins) can also be disturbed (a simplified scheme of pathways in Figure 1.2) (Vogelstein and Kinzler 2004; Sever and Brugge 2015; Sanchez-Vega *et al.* 2018; Samadani *et al.* 2018; Harvey 2019).

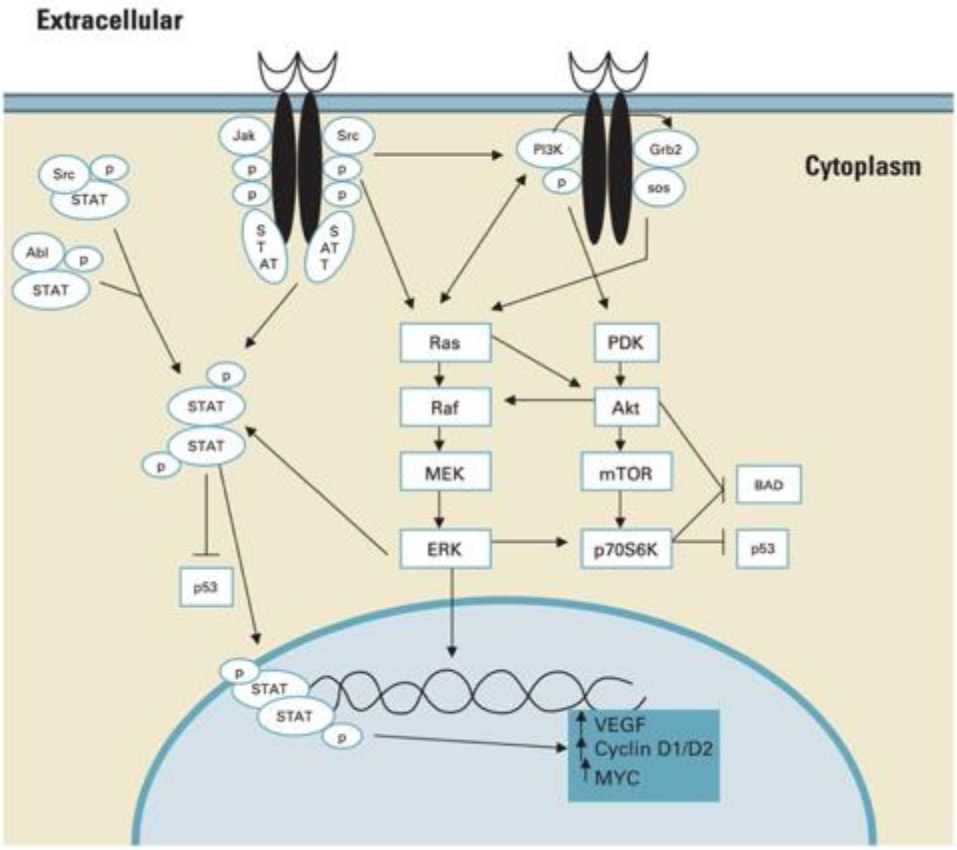

Figure 1.2 - **Simplified scheme of intracellular signal transduction pathways often deregulated in cancer.** Ligand binds to the extracellular domain of membrane receptors, which are then phosphorylated, leading to activation of cytoplasmic second messengers, which sequentially activate transcription factors in the nucleus (e.g., PI3K, phosphoinositide-3-kinase; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase, also known as MAP kinase; BAD, pro-apoptotic protein of the Bcl-2 family; SRC; p70S6 kinase, 40S ribosomal protein S6 kinase; STAT, signal transducer and activator of transcription; AKT, RAC-alpha serine/threonine-protein kinase or protein kinase B; mTOR, mammalian target of rapamycin (adapted from (Adjei and Hidalgo 2005).

The RAS pathway is the most common target for mutations in cancer. Overall, up to 30% of all human tumors harbor mutations in the 3 canonical *RAS* genes [Kirsten rat sarcoma viral oncogene homolog (*KRAS*), Harvey murine sarcoma virus oncogene (*HRAS*) and neuroblastoma RAS viral oncogene homolog (*NRAS*)] (Forbes *et al.* 2010). Remarkably, oncogenic KRAS mutations are being detected in 25–30% of all screened solid tumor samples. Members of the RAS family are crucial for the connection between signal-activated receptors and downstream effector pathways involving most cell functions, such us cell cycle progression, cell growth and survival, migration, cytoskeletal changes and senescence.

In tumor cells, activation of mutated *RAS* can induce several intracellular signaling pathways, including the RAF/MEK/ERK kinase cascade, the PI3K-AKT pathway and the Rho-family GTPases (RHOA, RAC1, CDC42, ARF6) (Raftopoulou and Hall 2004; Downward 2009).

The hyperactivation of RAS-ERK can lead to excessive proliferation in cells. One important target of the RAS-ERK pathway is the transcription factor MYC, which is phosphorylated by ERK. MYC stimulates cell proliferation by inducing numerous genes that promote cell proliferation, including those encoding G1/S cyclins, CDKs and the E2F-family transcription factors that drive the cell cycle (Duronio and Xiong 2013), and same time by repressing expression of various cell cycle inhibitors (Sever and Brugge 2015). MYC pathway alterations are most common in breast and ovarian cancer (Schaub *et al.* 2018).

Another example is aberrations in the PI3K pathway with activating events in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) [less commonly in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB)] and inactivating events in phosphatase and tensin homolog (PTEN) or phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1). PI3K activation is present in a range of solid tumors and hematological cancers, such as head and neck cancer, breast cancer, gastrointestinal, leukemia and gynecological tumors (Sanchez-Vega *et al.* 2018). Aberrant activation of PI3K signaling contributes to most hallmarks of cancer, including increased cell proliferation, metabolic changes, cell survival and motility. Moreover, PI3K pathway promotes tumor metastasis *via* activation of matrix metalloproteinases which degrade the ECM (Jiang and Liu 2009).

PI3K-AKT regulates cell growth during cell cycle progression by controlling mammalian target of rapamycin complex 1 (mTORC1), which promotes increased protein synthesis (Vara *et al.* 2004; Yang *et al.* 2019). AKT also phosphorylates the glycogen synthase kinase 3 beta (GSK3β), inhibiting its catalytic activity, that causes stabilization of important cell cycle regulators, such us cyclin D and MYC. Phosphorylation and consequent inhibition of GSK3β by AKT may, in certain contexts, lead to stabilization and nuclear translocation of β-catenin and induction of β-catenin targets.

The strongest co-occurrence among pathways was found between alterations of the PI3K and the nuclear factor erythroid 2-related factor (NRF2) pathways. Here, gain of function mutations and amplifications of the *NRF2* gene significantly co-occurred with

PIK3CA amplification (Zimta *et al.* 2019). Another example of intersection of pathways is the inactivation of tumor-suppressive Hippo pathway by RAS-ERK and PI3K-AKT pathways. The Hippo pathway plays a critical role in regulating inhibition of proliferation (Harvey and Hariharan 2012), and disruption of this pathway, which suppresses the transcriptional co-activator yes-associated protein (YAP), is emerging as a key tumor suppressor pathway in many cancers (Harvey *et al.* 2013; Lin *et al.* 2018).

These abnormal signaling pathways, which are specific to neoplastic cells, represent potential selective targets for new anticancer therapies, including ligands (typically growth factors), cellular receptors, intracellular pathway components or second messengers, and nuclear transcription factors (Adjei and Hidalgo 2005).

Genetic analysis of tumors revealed patterns of mutual exclusivity between alterations affecting the same pathways indicating that once one occurred and is selected, a second alteration will only happen if it provides a further selective advantage instead of functional redundancy (Etemadmoghadam *et al.* 2013; Mina *et al.* 2017). Co-occurrence of altered pathways in many tumor samples, on the other hand, indicate functional synergies and, importantly, may reflect resistance to therapy when targeting only one alteration (Nissan *et al.* 2014).

1. 3 Colorectal cancer (CRC)

Worldwide, cancer of the colon and rectum corresponds to the fourth most common incidence (behind lung, breast and prostate cancer) and the second cause of cancer mortality in both sexes, while in Europe it reaches the second highest incidence, according to the new global cancer data – GLOBOCAN 2018 (Ferlay *et al.* 2019).

Colorectal cancer accounts for approximately 10% of all annually diagnosed cancers and cancer-related deaths worldwide (Dekker *et al.* 2019). CRC incidence continues to rise especially in low and middle income countries (Jordan 2018).

The initial paradigmatic model for CRC tumorigenesis was based on the loss of the tumor suppressor gene adenomatous polyposis coli (*APC*) and stepwise accumulation of mutations in critical genes including *KRAS*, deleted in colorectal carcinoma (*DCC*) and tumor protein p53 (*TP53*) (Fearon and Vogelstein 1990). However, subsequent pathological or molecular analyses have discovered the existence of several CRC subtypes instead of one uniform disease. First, sporadic CRC tumors were classified into two main groups.

One group (with approximately 70% of the cases) developed tumors mostly in the distal colon, that appeared to follow an adenoma-carcinoma sequence involving somatic mutations in the *APC* and *TP53* tumor suppressor genes or in the oncogene *KRAS*. These type of tumors usually exhibit chromosomal abnormalities (CIN), and derive from adenomatous polyps (Jordan 2018).

The second major group of sporadic CRC included about 15% of patients and occurred preferentially in the proximal colon, presenting a high rate of sequence mutations, called the microsatellite instability phenotype (MSI), but a stable chromosome number. This phenotype develops following the somatic silencing, by DNA methylation of the promoter of the human mutL homolog 1 *(MLH1*) gene that encodes a protein required for a functional DNA mismatch repair system. The majority of these tumors derive from precursor polyps with a serrated morphology, called serrated adenoma, and present activating mutations in the oncogene v-raf murine sarcoma viral oncogene homolog B (*BRAF*) (Bettington *et al.* 2013; Matos and Jordan 2018).

Subsequent studies have shown further heterogeneity within these two groups of tumors. For example, about 8% of sporadic cases have a mutation in *BRAF* but are not MSI. Another 10% have mutation in *KRAS* but occur in the proximal colon and derive from a type of serrated polyp called traditional serrated adenoma. In addition, many adenomatous-derived polyps in the distal colon lead to carcinomas with CIN, but without the presence of mutated *KRAS* (Jass 2007; Jordan 2018).

Morphological classification is sometimes difficult to achieve and contributes to different frequency values for different types of CRC described in the literature. More recently, a variety of studies based on whole-genome gene expression profiling were published and resulted together in the definition of at least four consensus molecular subgroups (CMS) for CRC: CMS1 (MSI Immune), CMS2 (Canonical), CMS3 (Metabolic), and CMS4 (Mesenchymal) (Guinney *et al.* 2015). A resume of the most relevant characteristics of each CMS is given in Table 1.1 (Jordan 2018).

Table 1.1 - **Relevant characteristics of each consensus molecular subgroups (CMSs).** Comparison of the pathological, molecular and genomic features that distinguish the four CMS of colorectal tumors defined by gene expression-based clustering. CIMP, CpG island methylator phenotype; -H, high; -L, low; MSI, microsatellite instability; MSS, microsatellite stability; CIN, chromosomal instability (Jordan 2018).

	CMS1	CMS2	CMS3	CMS4
Frequency	14%	37%	13%	23%
Tumour location	proximal	distal	proximal or distal	distal
Precursor polyp	sessile serrated	adenomatous	serrated or adenomatous	adenomatous
DNA sequence stability	MSI	MSS	MSS or MSI	MSS
DNA methylation	CIMP-H	no CIMP	CIMP-L	no CIMP
Chromsome number	stable	CIN	stable or CIN	CIN
Mutated genes	BRAF	APC, TP53	KRAS	
Pathway signature	immune activation	WNT and MYC	metabolic deregulation	TGF-β, mesenchymal

1. 4 Serrated pathway in CRC

The tumors of this pathway display a typical morphology with a serrated folding of the crypt epithelium that likely results from increased epithelial mass. They can form in all segments of the colon, however, serrated carcinomas grow mostly in the proximal colon (Snover 2011; Sanz-Garcia *et al.* 2017; De Palma *et al.* 2019).

There are three major molecular features that distinguish the serrated pathway: mitogen-activated protein kinase (MAPK) pathway activation, CpG island methylator phenotype (CIMP) and microsatellite instability (MSI).

Two early tumor-initiating events are mutations in either the *KRAS* or *BRAF* genes that both stimulate proliferation downstream of the EGFR and along the same MAPK pathway. These mutations revealed to be alternative genetic events (Sandmeier *et al.* 2009; Bettington *et al.* 2013; Matos *et al.* 2016).

Another tumor phenotype is genome-wide DNA hypermethylation at or around the CpG islands present in the majority of human gene promoters, called CpG island methylator phenotype.

Two distinct CIMP phenotypes have been recognized in the serrated pathway: CIMP-high (CIMP-H) was defined by methylation in the majority of five selected CIMP marker gene promoters and is a characteristic feature of sporadic tumors with *BRAF* mutation and a high level of microsatellite instability (MSI-H). By contrast, a CIMP-low (CIMP-L) phenotype shows methylation in only few markers and is associated with *KRAS* mutation and either microsatellite stability (MSS) or low level of microsatellite instability (MSI-L) status.

Altogether, the serrated pathway is best characterized by the association between the MAPK activation and CIMP phenotypes, where MAPK activation can be via gene mutation in *BRAF* or *KRAS* and CIMP can be high or low, with patient prognosis varying with these features (Matos et al., 2016; Matos and Jordan, 2018). The best prognosis is observed in patients with tumors of the MSI-H- CIMP-H- mutant *BRAF* subtype and this is attributed to the high infiltration with immune cells that are believed to recognize neo-antigens resulting from the genome-wide mutational effect of the MSI-H phenotype (Jordan 2018).

1. 5 Colorectal cancer cell models

Epithelial tissues are composed of cell sheets with strong intercellular bonds to form physical barriers that line the cavities of major organs (lung, skin, intestine, etc.) and protect them from external physical, chemical, and microbial insults (Torras *et al.* 2018).

The entire intestinal epithelium is exposed to mechanical attrition and requires constant renewal. The intestinal epithelium (scheme of Figure 1.3) is organized into millions of crypt–villus units, where adult stem cells continuously generate replacement cells at the crypt bottom, which first pass through a highly proliferative zone and then differentiate on their way to the epithelium surface into enterocytes or colonocytes (Gehart and Clevers 2019). The WNT pathway is tightly linked with stem cell maintenance, differentiation and, upon deregulation, cancer in the intestinal epithelium. WNT ligands bind to the Frizzled–LRP5–LRP6 receptor complex, which inhibits continuous destruction of β-catenin by the cytoplasmic APC destruction complex. The resulting accumulation of β-catenin leads to its translocation to the nucleus, where it binds T cell factors (TCFs) and directly regulates gene expression (Nusse and Clevers 2017).

WNT signaling has emerged as a key effector in both normal intestinal stem cell homeostasis and in disease (Czerwinski *et al.* 2018; Flanagan *et al.* 2018).

In the colon, this proliferative environment is susceptible to malignant transformation and most of the available cell line models were derived from human tumor samples.

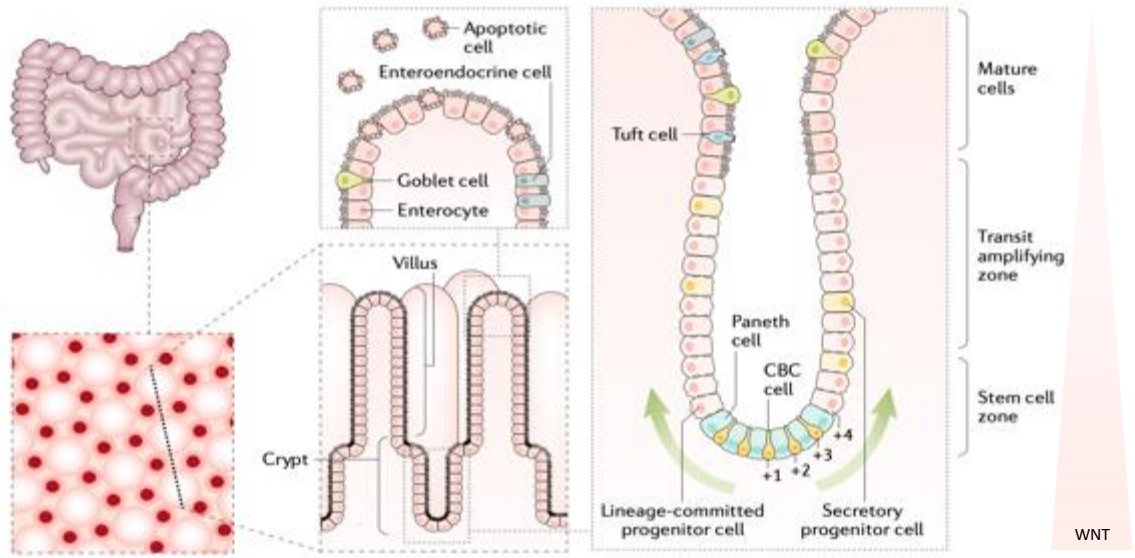

Figure 1.3 - **Intestinal structure.** The intestinal epithelium is organized into crypts and villi. One villus is surrounded by multiple crypts. The crypt generates a continuous stream of new cells that differentiate and migrate upwards towards the villus. Crypt base columnar (CBC) cells divide continuously, acting as intestinal stem cells and sit at position +1 to +3 at the bottom of the crypt wedged between Paneth cells, which protect and nurture stem cells. The proliferation is related with WNT signaling, whose gradient decreases along the crypt to villus. Above the stem cell zone, in the transit amplifying zone, lineage-committed progenitor cells divide rapidly to fuel the rapid intestinal cell turnover. Fully mature epithelial cells emerge from the crypt and move up towards the villus tip. Finally, cells undergo apoptosis close to the villus tip and are rapidly replaced by advancing cells (adapted from Gehart and Clevers, 2019).

For many research applications, intestinal cells are cultivated as two-dimensional (2D) flat monolayers on plastic surfaces. This allows rapid growth under relatively cost-effective conditions, and the cells can be easily handled, manipulated or transfected, and harvested for analysis. However, when grown on plastic surface, many of the functionally important epithelial cell properties are not developed and may thus yield results of limited physiological relevance. For example, the undifferentiated state of cells without tight junctions that is observed in monolayer cell cultures does not exist in the tissue (Pereira *et al.* 2016). In addition, the lack of signals contributed by other stroma cell types may affect the morphological organization or response of intestinal cells (Dolznig *et al.* 2011) or render sensitive tumor cell lines resistant to targeted drugs (Straussman *et al.* 2012; Wilson *et al.* 2012).

A significant improvement in physiological relevance of the 2D cell models is achieved upon their growth on microporous membrane inserts that allow free access of ions and nutrients to either the apical or the basolateral sides of the cell monolayer. These

conditions induce full cell polarization. This is a process requiring several days to weeks and implies a striking functional separation between the apical and basolateral plasma membrane domains. As a result, an apical domain (corresponding to the intestinal lumen) generates a multitude of actin bundle-supported microvilli forming the morphological structure known as brush border that confers massively increase in the apical cell surface and guarantee the uptake of luminal nutrients through cell surface enzymes and transport proteins. In addition, a basolateral membrane domain establishes cell-cell adhesion complexes and ECM interactions, and exposes receptors to growth factors or hormones. Cell polarization also implies differential sorting of proteins to each membrane domain, including specific sets of ion channels, receptors, and solute transporters.

The experimental limitations and disadvantages of growing an epithelial cell monolayer on filter membranes are first the long period required for establishment of a fully polarized organization (10–14 days) before experiments can be performed. Second, the required transwell filter inserts represent additional costs, and the cell number is limited. These systems appear thus to reach an interface between 2D and three-dimensional (3D) growth conditions.

3D cultures are nowadays frequently used for studying the biology of epithelial tissues, since they better represent the 3D organization and functional properties of cells within tissues (Shamir and Ewald 2014; Kawai *et al.* 2020), when compared to 2D monolayer cells. There is no doubt that immortalized colorectal tumor cell lines grown in 2D culture have contributed tremendously to the knowledge about the molecular pathways involved in malignant cell transformation, but they cannot represent adequate model systems for complex tumor biology (Hickman *et al.* 2014) and drug development (Hay *et al.* 2014). At the cellular level, 3D means that the cell is exposed to stimuli on all sides and this factor influences the cell signaling. One example of this change is in the cytoskeleton, because in a 3D environment, it is usually not uniform in shape and composition and interferes with signaling gradients (Rubashkin *et al.* 2014).

Presently, there are several 3D culture methods, including the scaffold-free platforms for spheroid growth and the scaffold-based models (hydrogels or solid biomaterials) (Burdett *et al.* 2010; Achilli *et al.* 2012; Knight and Przyborski 2015; Pereira *et al.* 2016).

1.5.1. Caco-2 cells

Caco-2 cells are the most widely used cellular cancer model over the last 30 years. Cells grow into a confluent culture and undergo differentiation with several morphological and functional characteristics of small intestine enterocytes, although cells were initially obtained from a human colorectal adenocarcinoma (Sambuy *et al.* 2005). When grown on filter membranes, these cells have the ability to form a confluent cell layer with a polarized organization, in particular, the sealing of the lateral intercellular space through tight junctions (TJ) (Suzuki 2013). The integrity of the Caco-2 monolayer can be measured as transepithelial electrical resistance (TEER) and is a useful tool in the assessment of drug permeability, transport mechanism, and gene regulation of transporters and enzymes (Sun *et al.* 2008).

Caco-2 cells also have the ability, under specific and favorable culture conditions, to form polarized spheroids or cysts with a central lumen in a 3D context.

Caco-2 are well-characterized and show a MSS phenotype and harbor mutations in the tumor-suppressing proteins APC, p53, and SMAD family member 4 (SMAD4) but not in the oncogenes *KRAS*, *BRAF*, or *PIK3CA* (Bosscher and Hill 2004; Forbes *et al.* 2010; Ahmed *et al.* 2013; Pereira *et al.* 2016)

The parental Caco-2 cell line has revealed some morphological heterogeneity and a mosaic expression pattern of intestinal marker enzymes during the cellular differentiation process. Alternatively, a proportion of the cells may retain stem-cell like properties or phenotypic plasticity and diverge in their features during differentiation. Interestingly, Caco-2 cells cultured under continuous flow conditions can form a crypt-villus organization and also differentiate into other cell types, such as goblet and enteroendocrine cells (Kim and Ingber 2013; Sakharov *et al.* 2019).

1.5.2. Other cell line models

HT29 is a colorectal adenocarcinoma cell line with epithelial morphology but does not form a fully differentiated cell layer. They contain a small proportion of goblet cells with mucin secretion and carry a mutation in the *BRAF* oncogene. One useful selected clone, designated HT29-MTX, displays the properties of mucus-secreting goblet cells, the second major cell type of the intestinal epithelium. Consequently, for many research purposes the co-culture of Caco-2/HT29-MTX is used as a model that mimics the human

intestinal epithelium better than the simple Caco-2 monolayers (Chen *et al.* 2010), for example, the study of the mucus role in the transport of drugs through the intestinal tract (Béduneau *et al.* 2014; Lozoya-Agullo *et al.* 2017) or the effect of food-borne nanomaterial additives on the epithelium (Verhoeckx *et al.* 2015).

SW480 are colorectal adenocarcinoma cells with more mesenchymal phenotype and a high proliferation rate, which do not form a polarized monolayer. They are mostly used for research on WNT-related oncogenic signaling pathways or assays validating anti-cancer drugs with nanomaterials (Freeman *et al.* 2012; Abbott Chalew and Schwab 2013; Hirsch *et al.* 2014; Elimrani *et al.* 2015; Buhrmann *et al.* 2015; Matsuda *et al.* 2016).

T84 cells were derived from a lung metastasis of a colon carcinoma and can spontaneously differentiate in culture forming apical microvilli and tight junctions (Lee *et al.* 2006; Hurley *et al.* 2016).

Properties and main applications of some cell lines are summarized in Table 1.2, but there are many other colon cancer-derived cell lines that serve as models for specific genetic alterations found in CRC and are grown as standard monolayer cultures (Ahmed *et al.* 2013; Mouradov *et al.* 2014; Pereira *et al.* 2016).

Table 1.2 - **Summary of properties of the some of the most commonly used colorectal-derived cell lines** (adapted from Pereira et al., 2016).

Cell line	Mutated genes	Origin	Main characteristics	Main applications
Caco-2	*APC, TP53, SMAD4*	Colorectal adenocarcinoma	Functional characteristics of small intestine enterocytes; can differentiate into a polarized monolayer	Electrophysiology; absorptive and pathogen-defensive properties of the intestinal barrier; innate immune response; drug resistance
SW480	*APC, TP53, KRAS, PIK3CA*	Duke's type B colorectal adenocarcinoma	Do not form polarized monolayer; high proliferation rate; more mesenchymal phenotype	Research on WNT and other oncogenic signaling pathways; drug resistance; toxicity of nanoparticles
T84	*APC, KRAS, PIK3CA*	Lung metastasis of colon carcinoma	Differentiate into a polarized monolayer; mixed differentiation into both, chloride-secreting enterocytes and mucin-secreting goblet- like cells	Electrophysiology; pathogen- epithelium interactions; barrier function

DLD-1	*KRAS, PIK3CA, TP53*	Colorectal adenocarcinoma	Epithelial characteristics	Cancer research; study of polar solvents on cell characteristics
LS174T	*CTNNB1, KRAS*	Duke's type B colorectal adenocarcinoma	Goblet-like cells with secretion of mucins MUC2, MUC5A/C and MUC6; do not form polarized monolayer	Mucin expression studies; cancer research; drug resistance
HT29	*APC, TP53, BRAF*	Colorectal adenocarcinoma	Epithelial morphology; grow as a nonpolarized, undifferentiated multilayer	Absorption, transport, and secretion studies; drug resistance
HT29-MTX	*APC, TP53, BRAF*	Selected subclone of adenocarcinoma derived HT29 cells	Display mucus producing goblet-cell properties; do not form polarized monolayer; HT29-MTX co-cultured with Caco-2 generate a confluent cell layer model covered by a protective mucus layer at the expense of reduced barrier function	Effect of food-borne nanomaterial additives on the epithelium; bioavailability of drugs in the presence of mucus; drug resistance
NCM460	Cytogenetic changes; lack *CDKN2A* expression	Immortalized normal colonocytes	Metabolic characteristics of normal mucosa, with low glycolytic rate and pentose phosphate synthesis but high tricarboxylic acid cycle activity	Interaction with microbial pathogens; control cells for studying oncogenic signaling pathways

1. 6 Tumor microenvironment (TME)

In healthy tissue, the supporting connective tissue or stroma functions as an important barrier against tumorigenesis; however, the presence of transformed tumor cells initiates crucial changes that can convert this microenvironment into one that supports cancer progression.

Interactions between malignant and non-transformed cells define the TME and are nowadays recognized as being of vital importance in the tumorigenic process. Intercellular communication is driven by a complex and dynamic network of cytokines, chemokines, growth factors, and inflammatory and matrix remodeling enzymes, which all respond to perturbations to the physical and chemical properties of the tissue (Balkwill *et al.* 2012). Cells within the TME are highly plastic, continuously changing their phenotypic and functional characteristics (Greten and Grivennikov 2019).

Besides these soluble factors, TME consists of ECM as well as cellular players, such as fibroblasts and cancer-associated fibroblasts (CAFs), neuroendocrine cells, adipose cells, immune-inflammatory cells such as myeloid-derived suppressor cells (MDSCs), mast cells, monocytes, neutrophils, $CD8^+$ and $CD4^+$ T cells, dendritic cells (DCs), natural killer (NK) cells and tumor-associated macrophages (TAMs), and the blood vessel with their endothelial cells (ECs) and a lymphatic vascular networks. This TME can provide a complex functional support to potentiate cancer progression and metastasis (Li, Fan *et al.* 2007; Chen *et al.* 2015; Wang *et al.* 2017). Cancer arises from mutations accumulating within cancer cells, but both disease progression and responses to therapy are strongly modulated by surrounding non-transformed cells of TME (Figure 1.4) (Balkwill *et al.* 2012).

Indeed, over the past decade, solid tumors have increasingly been recognized as organs that consist not only of cancer cells but also a variety of morphologically distinct cells. In a simplified way, these TME components can be classified into three main groups: cells of haematopoietic origin, cells of mesenchymal origin and non-cellular components (Pattabiraman and Weinberg 2014).

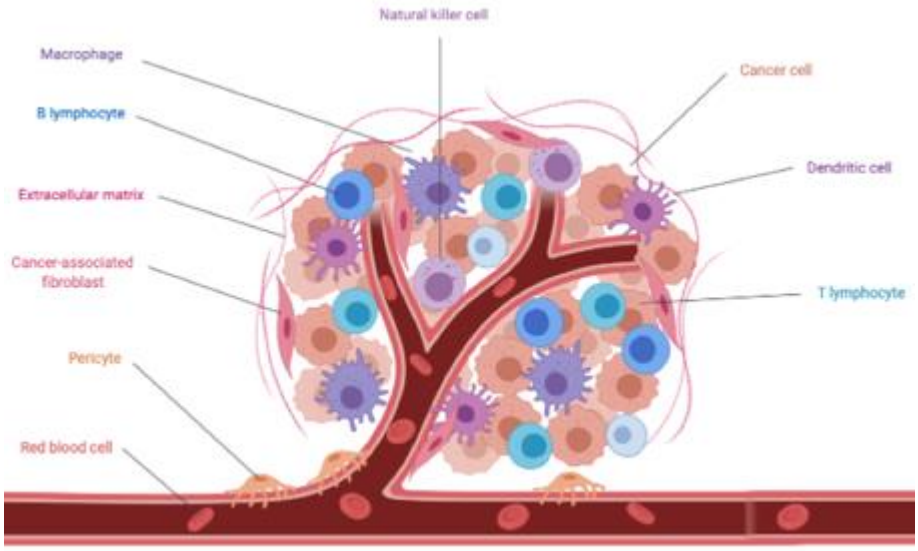

Figure 1.4 - **A schematic and simplified view of the tumor microenvironment.** The tumor microenvironment consists of complex cellular and molecular constituents. The cellular constituents consist of immune cells of hematopoietic origin and stromal cells of non-hematopoietic origin. The immune cell compartment comprises tumor-infiltrating lymphocytes of T, B, and natural killer cells and tumor-associated myeloid populations of dendritic cells and macrophages. The stromal compartment consists of cancer-associated fibroblasts and endothelial cells of the lymphatic and blood vasculature.

1.6.1. Cancer cells

Cancer cells are at the basis of the disease: they initiate tumor formation and drive tumor progression forward, carrying the oncogenic and tumor suppressor mutations that define cancer as a genetic disease. Traditionally, the cancer cells within tumors have been portrayed as cell populations of clonal origin, but in the course of tumor progression, hyperproliferation combined with increased genetic instability spawn distinct clonal subpopulations (Melo *et al.* 2013). Reflecting such clonal heterogeneity, many human tumors are histopathologically diverse, containing regions demarcated by various degrees of differentiation, proliferation, vascularity, inflammation and invasiveness. Tumor heterogeneity may be responsible for tumor progression, metastasis, resistance to therapy, and relapse (Almendro *et al.* 2013; Melo *et al.* 2013).

In recent years, the evidence was emerging of the existence of a new dimension of intratumor heterogeneity and a hitherto-unappreciated subclass of neoplastic cells within tumors, termed cancer stem cells (CSCs). CSCs share similar properties with normal stem cells, including the ability to self-renew and differentiation that give rise to heterogeneous, differentiated cancer cells making up the bulk of the tumor. Due to this similarity, CSCs are commonly characterized by the expression of surface markers associated with stem cells, such as CD133, CD44, CD90 (Beck and Blanpain 2013).

CSCs reside in particular TME niches that play an important role in regulating their proliferation, renewal, differentiation, and stemness. CSC regulation by their niche operates through cell-cell interaction, secreted factors, cell-matrix interaction, and the biophysical properties of the niche, such as hypoxia. CSCs may also be inherently resistant to medical therapy and contribute to tumor relapse (Clevers 2011; Marjanovic *et al.* 2013; Nassar and Blanpain 2016).

1.6.2. Cells of mesenchymal origin

1.6.2.1 Cancer-associated fibroblasts (CAFs)

Fibroblasts are non-epithelial, non-immune cells with a likely mesenchymal lineage origin, and are part of the diverse connective tissue components (Tarin and Croft 1970; Desmouliere *et al.* 2014). Fibroblasts in normal tissues are generally single cells present in the interstitial space or occasionally near a capillary, without any association with a basement membrane but embedded within fibrillar ECM of the interstitium.

Without question, fibroblasts are the most versatile and extensively studied cells *in vitro* owing to their ease of isolation and culture. Tissue-resident fibroblasts are usually quiescent and become activated in a wound healing response, regulating the proliferation and differentiation of epithelial tissues (Kalluri 2016).

After activation by the presence of tumor cells, fibroblasts are known as CAFs or myofibroblasts. These cells have a significant impact on cancer progression through synthesis and remodeling of the ECM and direct stimulation of cancer cell proliferation via the secretion of growth factors, recruitment of inflammatory cells, induction of angiogenesis and immune suppressive cytokines. CAFS also influence mesenchymal-epithelial cell interactions, tumor mechanics, drug access and therapy responses (Kalluri and Zeisberg 2006; Kalluri 2016; Wang *et al.* 2017; Sahai *et al.* 2020).

CAFs can derive from multiple resident precursors, such as ECs, smooth muscle cells and myoepithelial cells, or mesenchymal stem cells (Balkwill *et al.* 2012). It has been postulated that about 40% of CAFs are formed from endothelial cells (Kalluri and Zeisberg 2006).

Targeting CAFs, by altering their numbers, subtype or functionality, is being explored as an avenue to improve cancer therapies. However, research in this area faces numerous challenges because CAFs can have both pro-tumorigenic and anti-tumorigenic effects (Sahai *et al.* 2020). Various studies suggest that under certain stimuli, CAFs can acquire a pro-inflammatory signature characterized by the expression of immunomodulatory molecules [e.g., transforming growth factor beta (TGF-β) or programmed death ligand 1/2 (PD-L1/L2)], as well as chemokines that promote recruitment of immunosuppressive myeloid cells (Bhowmick 2004; Giraldo *et al.* 2019).

Many patient studies have documented how either CAF number or CAF function is linked to outcome (Calon *et al.* 2015; Franco-Barraza *et al.* 2017), and thus being able to target CAFs would represent an appealing addition to the suite of anticancer therapies. Further targeting mechanisms, such as TGF-β signaling, that activate CAFs or emanate from CAFs to modulate the tumor phenotype are being intensively explored in CRC (Tauriello *et al.* 2018). More recently, there has been a growing appreciation of the ability of CAFs to modulate the immune response.

1.6.2.2 Endothelial cells (ECs)

The vascular endothelium is a versatile structure that separates the circulating blood from tissues. Moreover, apart from regulation and maintenance of blood fluidity, it plays multifunctional roles in the delivery of water and nutrients, maintenance of metabolic homeostasis, trafficking of immune cells, activation of innate and acquired immune responses, as well as angiogenesis (Hida *et al.* 2018; Birbrair 2020).

In the initial stage, in order to survive and proliferate, tumor cells take oxygen and nutrients by diffusion. The environment in which it develops undergoes hypoxia and acidification as a result of excess metabolic products. When tumor volume exceeds 1–2 mm^3, the tumor must become angiogenic and recruit their vasculature to grow. Cancer cells, together with host/niche cells, stimulate the development of new blood vessels, using various mechanisms of tumor angiogenesis (Döme *et al.* 2007), which are targeted by many new cancer therapies since solid tumors require blood vessels for growth.

Tumor cells play a crucial role in initiation and regulation of cancer angiogenesis (Birbrair 2020), since blood vessels are formed by ECs recruited from the surrounding tissue as tumor endothelial cells (TECs). Many soluble factors present in the TME, such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs) and chemokines stimulate ECs and their associated pericytes during the neovascularization that is needed for cancer growth. Hypoxia-inducible factor-1 alpha (HIF-1α) is the main factor that initiates sprouting. When a quiescent blood vessel senses an angiogenic signal from malignant or inflammatory cells, or owing to hypoxic conditions in the TME, new vessels sprout from the existing vasculature (Carmeliet and Jain 2011). The tumor vasculature, in contrast to well-differentiated normal vessels, is abnormal in almost every aspect of its structure and function (Jain 2005).

1.6.3. Cells of hematopoietic origin

1.6.3.1 Tumor-associated macrophages (TAMs)

Macrophages are tissue sentinels that maintain tissue integrity by eliminating/repairing damaged cells and matrices (Italiani and Boraschi 2014).

Despite the initial hypothesis that macrophages are involved in antitumor immunity, there is now abundant evidence from human and experimental mouse cancer

models that their activities in the majority of cases are pro-tumorigenic and enhance tumor progression to malignancy (Qian and Pollard 2010; Balkwill *et al.* 2012).

The tumor promoting functions of macrophages at the primary site include supporting tumor-associated angiogenesis, promotion of tumor cell invasion, migration and intravasation, as well as suppression of antitumor immune responses. Excellent examples of these functions are displayed by the macrophage-derived osteoclasts that remodel bone for metastatic cell colonization and for macrophages in mammary tumor development (Lin *et al.* 2001).

There is pre-clinical and clinical evidence that an abundance of TAMs in the TME is associated with poor prognosis for breast cancer (Leek *et al.* 1996; Volodko *et al.* 1998; Lee *et al.* 2006) and for lung cancer (Koukourakis *et al.* 1998). Additionally, gene array studies in follicular lymphoma demonstrate that the expression of genes that are associated with a strong 'macrophage' signature confer a poor prognosis, independent of other clinical variables (Dave *et al.* 2004; Balkwill *et al.* 2012). However there are examples of TAMs correlated with good prognosis in cancer (Bingle *et al.* 2002).

In the literature, there are several terms and definitions to describe the macrophage activation and polarization. One of the most used nomenclatures, introduced in 2000, divides macrophages in M1 and M2 (Mills *et al.* 2000). M1 or classical macrophages, activated with lipopolysaccharide (LPS) and/or interferon gamma (INF-γ), have a pro-inflammatory phenotype with pathogen-killing abilities, whereas M2 or alternative macrophages, activated with interleukin (IL)-4, promote cell proliferation and tissue repair and can also promote tumor growth (Orecchioni *et al.* 2019). In this linear view of classification, M1 macrophages represent one extreme and M2 macrophages represent the other (Mosser and Edwards 2008). However, the M2 designation has rapidly expanded to include essentially all other types of macrophages, with dramatic differences in their biochemistry and physiology, arising the class of regulatory macrophages. Nevertheless, currently the definition of macrophage populations continues to be defined by the different expression of marker proteins (Edwards *et al.* 2006; Rőszer 2015).

The role of macrophages in cancer has been controversial and many aspects remain unresolved, so macrophages can have opposing roles depending on their phenotype (Klimp *et al.* 2002).

While classically activated macrophages would act anti-tumorigenic in later tumor stages, they have the potential to contribute to the earliest stages of neoplasia (Swann *et al.* 2008), primarily because the free radicals that they produce can lead to DNA damage

and cause mutations in surrounding cells that can promote transformation. However, as tumors progress and grow, the TME markedly influences TAMs. These macrophages change their physiology and take on a phenotype that is more closely to regulatory macrophages of M2-class.

Many observations indicate that TAM express several M2-associated pro-tumoral functions, including promotion of angiogenesis, matrix remodeling and suppression of adaptive immunity. The pro-tumoral role of TAM in cancer is further supported by clinical studies that found long-term use of non-steroidal anti-inflammatory drugs reduces the risk of several cancers (Mantovani *et al.* 2006).

1.6.3.2 T lymphocytes

There are many different T cell populations within the TME that infiltrate the tumor areas at the invasive tumor margin and in draining lymphoid organs. Cytotoxic $CD8^+$ memory T cells, which are normally antigen 'experienced' and capable of killing tumor cells, and $CD4^+$ T helper 1 cells, which are characterized by the production of the cytokines IL-2 and IFN-γ, are strongly associated with a good prognosis in a lot of cancer types, such as: melanoma (Clark *et al.* 1989; Mackensen *et al.* 1993; Clemente *et al.* 1996), breast cancer (Marrogi *et al.* 1997; Teschendorff *et al.* 2010; Mahmoud *et al.* 2011; Fridman *et al.* 2012), colorectal cancer (Jass 1986; Baier *et al.* 1998; Galon *et al.* 2006; Camus *et al.* 2009; Pagès *et al.* 2009; Mlecnik *et al.* 2011; Dahlin *et al.* 2011), lung carcinoma (Ito *et al.* 2005; Kawai *et al.* 2008; Dieu-Nosjean *et al.* 2008) and renal cancer (Nakano *et al.* 2001). In these last two types there is also contradictory evidence associating the presence of the $CD8^+$ lymphocytes with a poor prognosis (Nakano *et al.* 2001; Wakabayashi *et al.* 2003).

Another type of T cells, such as TH2 or TH17 and immunosuppressive regulatory T cell (Treg) are generally thought to promote tumor growth and correlate with worse prognosis in many types of cancer (Curiel *et al.* 2004; Hiraoka *et al.* 2006; Bates *et al.* 2006). Tregs can also be tumor suppressive, their presence in Hodgkin's lymphoma correlates with a good prognosis, presumably through a direct suppression of tumor cell growth (Tzankov *et al.* 2008; Koreishi *et al.* 2010).

Regulatory pathways that limit the immune response to cancer are becoming increasingly well characterized and targets for immunotherapy. One good example is Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), which is an immune

checkpoint molecule that down-regulates pathways of T-cell activation (Junttila and de Sauvage 2013). CTLA-4 directed immunotherapy is used in a range of solid tumors, such as melanoma (Hodi *et al.* 2010). Another example is programmed cell death protein 1 (PD-1) and PD-L1, since these proteins play a key role in physiological immune homeostasis. PD-1 signaling negatively regulates T cell-mediated immune responses and serves as a mechanism for tumors to evade an antigen-specific T cell immunologic response, so that cancer cells evade the immune system. The development and application of immune checkpoint inhibitors that block PD-1/PD-L1 interaction result in very durable responses and prolong survival in patients with a wide range of cancers. There are a lot of different Food and Drug Administration (FDA) approved drugs for this immunotherapy (Akinleye and Rasool 2019).

1.6.3.3 B lymphocytes

B cells can be found at the invasive margin of tumors, but are more common in lymphoid structures adjacent to the TME and are associated with good prognosis in some breast and ovarian cancers (Coronella *et al.* 2001; Milne *et al.* 2009); however, in mouse models, in which B cells inhibit tumor-specific cytotoxic T cell responses (Qin *et al.* 1998), the role of B cells is tumor-promoting (Visser *et al.* 2005; Andreu *et al.* 2010).

B lymphocytes affect other immune cells in the surrounding lymphoid tissue (Schioppa *et al.* 2011), as well as modulate the activity of myeloid cells (Andreu *et al.* 2010).

1.6.3.4 Other immune cells

NK cells and natural killer T (NKT) cells (which share the characteristics of both T and natural killer cells) also infiltrate the tumor stroma, but are not found in contact with tumor cells. For many cancers, such as colorectal, gastric, lung, renal and liver, they appear to predict a good prognosis (Cui *et al.* 1997; Kawano *et al.* 1999; Smyth *et al.* 2000; Terabe *et al.* 2000; Tachibana 2005).

DCs have important functions in antigen processing and presentation. The DCs that are found in the TME are thought to be defective, that is, they cannot adequately stimulate an immune response to tumor-associated antigens, maybe due to the hypoxic

and inflammatory conditions in the TME that inhibit their activation (Meredith *et al.* 2012).

The immune cell populations infiltrating human tumors are highly complex and due to their synergistic or opposing effects it is difficult to predict their activity. They may influence tumors differently depending on their histological and molecular type, their stage, the microenvironment of the organ in which they grow, or the nature of the primary tumor or its metastases (Junttila and de Sauvage 2013).

1.6.4. Non-cellular components

1.6.4.1 The extracellular matrix (ECM)

The ECM provides structure and support for the cellular components in the extracellular space of tissues and organs, but also contains cell-secreted growth factors that can be released during remodeling and contribute to paracrine cellular signaling. The ECM is comprised of highly organized interactions of fibrous molecules, proteoglycans, glycoproteins, glycosaminoglycans, and other macromolecules. It is a dynamic structure that is constantly remodeled by cells to control tissue homeostasis and is composed of around 300 proteins, in mammals (Bonnans *et al.* 2014; Valkenburg *et al.* 2018).

Although long viewed as a stable structure that plays a mainly supportive role in maintaining tissue morphology, the ECM is an essential part of the milieu of a cell that is surprisingly dynamic and versatile, and influences fundamental key aspects of cell biology (Hynes 2009). ECM composition ranges from soft and compliant to stiff and rigid and determine how a cell senses and perceives external forces (Paszek *et al.* 2005; Lopez *et al.* 2008; Gehler *et al.* 2009) and thus provides a major environmental cue that determines cell comportment and contributes to development and disease (Lu *et al.* 2012).

Components of the ECM constantly interact with epithelial cells by serving as ligands for cell receptors such as integrins or growth factors, thereby transmitting signals that regulate adhesion, migration, proliferation, apoptosis, survival or differentiation. Disruption to such control mechanisms deregulates and disorganizes the ECM, leading to abnormal behaviors of cells residing in the niche and ultimately failure of organ homeostasis and function (Lu *et al.* 2012). Indeed, abnormal ECM dynamics are one of the most ostensible clinical outcomes in diseases, such as fibrosis and cancer (Cox and Erler 2011) and are considered a hallmark of cancer (Lu *et al.* 2012).

Tumor-derived ECM is biochemically distinct in its composition compared with normal ECM. Furthermore, reports have demonstrated that the tumor stroma is typically stiffer than normal stroma, for example in breast cancer (Kass *et al.* 2007; Levental *et al.* 2009). Consistent with these changes, expression of many ECM remodeling enzymes is often deregulated in human cancers, for example: heparanases, sulfatases, cysteine cathepsins, urokinases and many matrix metalloproteinases (MMPs) are frequently overexpressed in different cancers (Ilan *et al.* 2006; Kessenbrock *et al.* 2010).

In addition, there is evidence that ECM is involved in angiogenesis and metastasis (Lu *et al.* 2012), and growing evidence suggests that the ECM is an essential non-cellular component of the adult stem cell niche. For example, ECM receptors allow stem cells to anchor to the special local niche environment where stem cell properties can be maintained. Such an anchorage physically constrains stem cells to make direct contact with niche cells, which produce paracrine signaling molecules that are essential for maintaining stem cell properties (Li and Xie 2005). Moreover, anchorage allows stem cells to maintain cell polarity, orient their mitotic spindles, and undergo asymmetric cell division (Lu *et al.* 2012).

1. 7 Inflammation and tumor development

Under normal conditions, acute inflammation is a desirable, strictly regulated response to infection and tissue damage. Normal inflammation is usually associated with tissue healing processes and self-limiting because the production of pro-inflammatory cytokines is followed by the production of anti-inflammatory cytokines. The dysregulation of this controlled process can lead to pathogenesis, as is the case with neoplastic transformation (Chen *et al.* 2015; Wang *et al.* 2017).

A persistent or chronic inflammatory environment is considered today to be a risk factor for cancer development because cells infiltrating the TME provide tumor-supporting molecules such as cytokines and growth factors, cell survival signals, angiogenic factors and other carcinogenesis mediators (Hanahan and Weinberg 2011; Landskron *et al.* 2014). Numerous studies provide evidence that chronic inflammation increases the risk of cancer, promotes tumor progression, and supports metastatic spread (Kawanishi *et al.* 2006; Mantovani *et al.* 2008; Feagins *et al.* 2009; Aggarwal and Gehlot 2009; Murata *et al.* 2012).

Chronic inflammation is linked to cancer development in a number of organs. Around 15%–20% of all cancer cases are preceded by infection, chronic inflammation, or auto-immunity at the same tissue or organ site (Mantovani *et al.* 2008; Grivennikov *et al.* 2010).

Patients with inflammatory bowel diseases (IBDs), such as ulcerative colitis and Crohn's diseases, stimulated by both genetic and environmental factors are at increased risk of developing colorectal cancer (Eaden *et al.* 2001; Rutter *et al.* 2004; Canavan *et al.* 2006; Jess *et al.* 2006; Fakhoury *et al.* 2014). A number of other examples are: purely environmental-related inflammation caused by asbestos, smoking, and silica-associated with lung cancer (Vainio and Boffetta 1994), chronic gastritis caused by bacteria such as *Helicobacter pylori* associated with gastric cancer (Yoshida *et al.* 2014), *Escherichia coli* infection of the prostate correlated with prostate cancer (Krieger *et al.* 2000), a viral infection with hepatitis virus B/C associated with hepatocellular carcinoma (El-Serag 2012), or human papilloma virus linked with many different cancers, such as cervical and anogenital cancers, head and neck squamous cell carcinoma, esophageal carcinoma, and even ophthalmologic and breast cancers (Voronov *et al.* 2003; Cohen 2017; Araldi *et al.* 2018), Barrett's esophagitis associated with esophageal cancer (Lekakos *et al.* 2011), liver fluke and primary sclerosing cholangitis correlated with cholangiocarcinoma (Zabron *et al.* 2013), UV irradiation-associated skin inflammation related with melanoma (Singh *et al.* 1995), endometriosis linked with endometrial carcinoma (Bats *et al.* 2008), and gall bladder stone-associated chronic cholecystitis associated with gall bladder carcinoma (Fox *et al.* 1998; Levin 1999).

One mechanism whereby inflammation may contribute to the development of cancer is through the production of reactive oxygen and nitrogen species that can cause oxidative damage to DNA, proteins, and lipids. Cancer-related inflammation (CRI) requires the presence and activation of inflammatory cells such as macrophages and granulocytes in the tumor microenvironment, formation of inflammatory mediators by tumor and stromal cells, tumor remodeling, and angiogenesis (Kundu and Surh 2008; Colotta *et al.* 2009). Some cytokines, together with different chemokines and growth factors, are thought to contribute to CRI by altering the adaptive immunity, responses to hormones, angiogenesis, tumor growth and progression, invasion and metastasis.

In the panoply of molecular players involved in cancer-related inflammation, one can identify prime drivers, which include transcription factors such as NF-κB and signal transducer and activator of transcription 3 (STAT3) and primary inflammatory cytokines,

such as interleukin 1 beta (IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α) (Colotta *et al.* 2009).

A number of studies provided unequivocal evidence that NF-κB is involved in tumor initiation and progression in tissues in which CRI typically occurs, such as the gastrointestinal tract and the liver (Greten *et al.* 2004; Maeda *et al.* 2005). Specific inactivation of NF-κB in tumor-infiltrating leukocytes, by a strategy targeting the inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), inhibited colitis-associated cancer, thus providing unequivocal genetic evidence for the role of NF-κB and inflammatory cells in intestinal carcinogenesis (Greten *et al.* 2004). Meylan *et al.* demonstrated that inhibition of the NF-κB pathway in lung tumors resulted in significantly reduced tumor growth (Meylan *et al.* 2009).

IL-1 family is one of the prominent tumorigenic inflammatory cytokines, and novel IL-1 blockers have been developed for cancer therapy (Litmanovich *et al.* 2018). Elevated levels of IL-1 have been identified in several human tumor entities such as melanoma, head and neck, colon, lung, and breast cancer (Chen *et al.* 1999; Gemma *et al.* 2001; Elaraj 2006). Overall, patients harboring IL-1-positive tumors have markedly worse prognoses (Lewis *et al.* 2006). IL-1 induces expression of metastatic genes such as MMP and stimulates nearby cells to produce angiogenic proteins and growth factors such as VEGF, interleukin 8 (IL-8), IL-6, TNF-α and TGF-β (Barille and Harousseau 1997; Akagi *et al.* 1999; Konishi *et al.* 2005). Also, Voronov and colleagues (2003) have determined the necessity of IL-1 in tumor growth, metastasis and angiogenesis.

A major effector molecule of NF-κB activation and also linked to STAT3 signaling is IL-6, a multifunctional cytokine with growth-promoting and anti-apoptotic activity in intestinal context (Bollrath *et al.* 2009). The link between IL-6 and cancer was described in multiple myeloma (Annunziata *et al.* 2007; Keats *et al.* 2008) and in liver carcinogenesis (Naugler *et al.* 2007).

Associated with an inflammatory TME are also bacterial extracellular vesicles (BEVs). With recent technology, their potential involvement in carcinogenesis has been proposed as new future research area. Indeed, microbial dysbiosis is one of major contributing factor in oncogenesis and tumor progression for a number of gastro-intestinal-tract-related malignancies, including gastric, colorectal, liver, and pancreatic cancer, and might even influence the treatment response to chemotherapy and immunotherapy (Peek and Blaser 2002; Mima *et al.* 2016; Gopalakrishnan *et al.* 2018;

Riquelme *et al.* 2019; Aykut *et al.* 2019). There are already some studies, such as the work of Poore and colaborators, that exposed the possibility of using microbial-based liquid biopsies for early detection of certain cancers (Poore *et al.* 2020). This potential microbiome-based oncology diagnostic tool warrants further exploration (Chronopoulos and Kalluri 2020).

The growing understanding of the complex interplay between the tumor and the TME has emphasized the impact of an inflammatory microenvironment in cancer development (Gupta *et al.* 2008; Feagins *et al.* 2009).

1. 8 Alternative splicing (AS)

Alternative splicing (AS) is a mechanism by which a single gene can give rise to multiple transcript products enhancing the gene expression diversity (Nilsen and Graveley 2010; Roy *et al.* 2013). The first example of AS was discovered 40 years ago in immunoglobulin genes (Alt *et al.* 1980; Early *et al.* 1980).

AS can occur in approximately 94% of human genes (Wang *et al.* 2008; Pan *et al.* 2008), and it represents a major source of the human transcriptome diversity (Sette *et al.* 2013). The splicing reaction that assembles a mature eukaryotic messenger RNA (mRNA) from the much longer primary transcript, or pre-mRNA, provides a uniquely versatile means of genetic regulation. Alterations in splice site choice can result in different effects on the mRNA, and two main outcomes need to be distinguished: 1) the alternative transcript is translated into a protein product with altered functional properties, or 2) the alternative transcript generates a pre-mature stop codon and gets degraded, thus downregulating the levels of functional mRNA produced from a given gene.

In the pre-mRNA, the borders of exons and introns are delineated by highly conserved signal sequences, the 5′ splice site at the upstream end of the intron and the 3′ splice site at the downstream end. AS involves changes in the choice of splice sites by the splicing apparatus and in the definition of introns. Subsequently, introns are excised and exons ligated by the spliceosome, a sophisticated ribonucleoprotein machine comprising five small nuclear RNAs (snRNAs; U1, U2, U4, U5 and U6 snRNA) and approximately 200 proteins (Wahl *et al.* 2009; Desterro *et al.* 2020).

Most exons are constitutive; they are always spliced and included in the final mRNA but in a typical multiexon pre-mRNA, the splicing pattern can be altered in many ways. Currently, eight main types of AS were described, as schematized in Figure 1.5.

An exon that is sometimes included and sometimes excluded from the mRNA is called a cassette exon. In certain cases, multiple cassette exons are mutually exclusive and producing mRNAs that always include only one of several possible exon choices. Exons can also be scrambled leading to a change in their order. Moreover, exons can be shortened by choosing one of two possible splice sites. The 5'-terminal exons of an mRNA can be switched through AS and define the use of alternative promoters, that primarily affect transcriptional control. Similarly, the 3'-terminal exons can be switched by AS providing alternative polyadenylation sites, which has consequences for mRNA stability due to different availability of microRNA (miRNA) target sequences. Finally, some important regulatory events are controlled by the failure to remove an intron, a splicing pattern called intron retention (Chen and Weiss 2015).

Of these, the cassette-type alternative exon accounts for approximately one-third of all AS events. The alternative 5' or 3' splice sites commonly occur together, forming approximately 25% of all AS events and are proficient enough to bring changes in the coding sequence in as little as one codon. All AS events summarized may occur in the translated as well as the untranslated regions of the transcripts (Black 2003; Li, Lee *et al.* 2007; Frankiw *et al.* 2019).

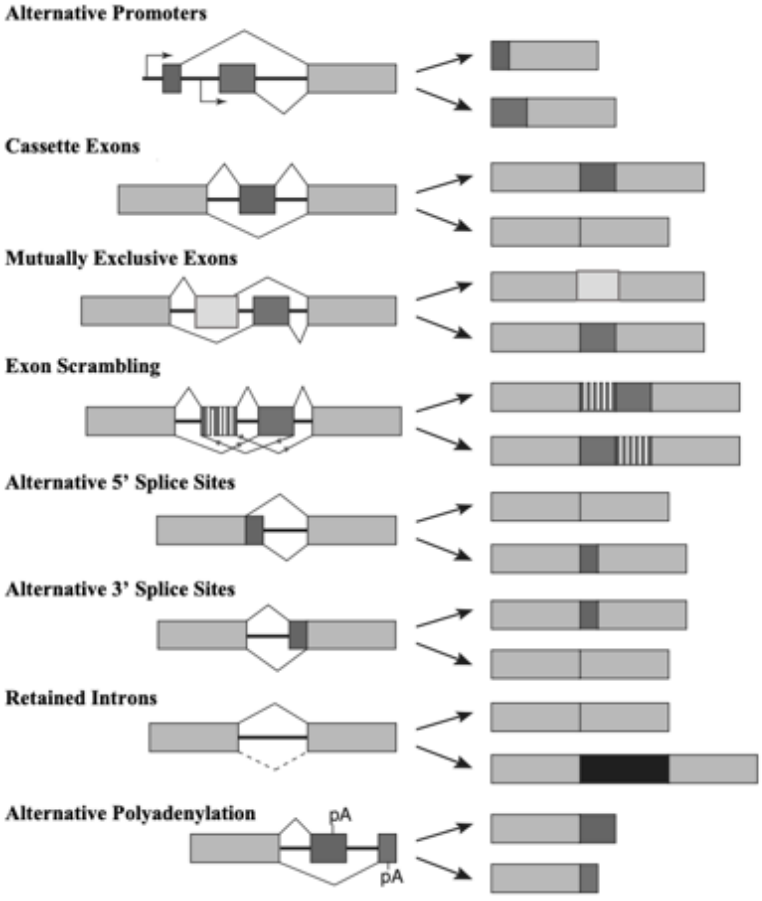

Figure 1.5 - **Common mechanisms of alternative splicing.** Alternative splicing can occur through a number of different patterns and give rise to different mature transcripts (right). Exons and final transcripts are illustrated as boxes while lines represent introns. Constitutively expressed exons are depicted in light grey, and alternatively spliced exons are depicted in dark grey. Retained introns occur when they are not recognized during splicing, so that the intervening intron (black) is included in the final transcript (adapted Chen and Weiss, 2015).

1. 9 AS and cancer

Changes in AS patterns are observed in normal development, tissue-specific differentiation, and in response to physiological stimuli, but also in diseases. Tumor-related aberrant patterns feature variants that contribute to multiple aspects of tumor establishment and progression, or to resistance to therapeutic treatments (Ghigna *et al.* 2008; David and Manley 2010; Biamonti *et al.* 2012)

Aberrant profiles observed in tumors mostly reflect the selection of endogenous AS variants with different functional properties that allow the malignant progression of initiated tumor cells. Mainly, the selected functions relate to sustained proliferation, evasion of apoptosis, metabolic adaptation, or angiogenesis (Pagliarini *et al.* 2015; Gonçalves *et al.* 2017).

For example, escape from cell death is critical for tumorigenesis. One of the alternatively spliced genes modulating apoptosis is B-cell lymphoma 2 (*BCL2*). This gene owns an alternative 5' splice site after exon 2 that produces either long or short isoforms that are translated into the BCL-xL and BCL-xS proteins, respectively. BCL-xS promotes apoptosis whereas BCL-xL has anti-apoptotic effects, and cancers show predominant expression of the BCL-xL isoform (Chen and Weiss 2015).

Metabolic pathways are also frequently altered in cancer, leading to a shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect) (Liberti and Locasale 2016; Burns and Manda 2017) and this is partly driven by AS of the pyruvate kinase M (PKM) gene. Exons 9 and 10 are mutually exclusive, giving rise to PKM1 (exon 9) or PKM2 (exon 10). PKM2 is expressed widely in cancer and replacement of PKM2 with PKM1 reverses the Warburg effect and increases oxidative phosphorylation (Chen and Weiss 2015). PKM1 and PKM2 differ by 22 amino acids (Noguchi and Inoue 1986), which confer crucial differences between PKM1- and PKM2-mediated catalysis and cellular metabolism: PKM1 exists as a tetramer with high substrate affinity and its main biological function is to provide pyruvate for the generation of adenosine triphosphate (ATP) *via* oxidative phosphorylation. In contrast, PKM2 can switch to the less active

dimeric form that leads to accumulation of several glycolytic intermediates, which can be used as building blocks for the biosynthesis of amino acids, lipids and nucleotides. Nowadays, further non-canonical roles of PKM2 were recognized, such as regulating gene transcription through its nuclear translocation and protein kinase activity (Yang and Lu 2015).

Genome-wide studies have long revealed the existence of cancer-specific splicing patterns (Klinck *et al.* 2008; Venables *et al.* 2009; Bemmo *et al.* 2010; Menon and Omenn 2011; Misquitta-Ali *et al.* 2011; Chen and Weiss 2015). For example, the splicing profile of ATP binding cassette subfamily C member 1 (ABCC1), mouse double minute 2 homolog (MDM2), and fibronectin transcripts has been used to distinguish normal ovary from epithelial ovarian cancer (Klinck *et al.* 2008), whereas altered splicing of mediator complex subunit 24 (MED24), melanotransferrin (MFI2), serrate RNA effector molecule (SRRT), CD44 molecule (CD44) and dual specificity protein kinase (CLK1) has been associated with metastatic phenotype in breast cancer and poor prognosis in patients (Bemmo *et al.* 2010). Notably, splicing of protein ENAH actin regulator (hMENA) an actin regulatory protein may improve the early diagnosis of breast cancer and clinical decision (Di Modugno *et al.* 2012), whereas the balance between splicing variants of Krueppel-like factor 6 (*KLF6*) and caspase-9 (*CASP9*) genes could be useful to predict the susceptibility of cancer cells to chemotherapy (Sangodkar *et al.* 2009; Shultz *et al.* 2010). Thus, for diagnostic purposes, changes in splicing patterns can be used as markers of the cell linked with disease (Roy *et al.* 2013).

1. 10 The Rho family of small GTPases

GTPases are proteins that cycle between two conformational states, an inactive guanosine diphosphate (GDP)-bound form and an active guanosine triphosphate (GTP)-bound form. Examples are the heterotrimeric G proteins that operate downstream of G-protein coupled receptors, and the small GTPases (~ 21 kDa) of the Ras superfamily. The Rho family of small GTPases belongs to this superfamily and consists of at least 20 members (Figure 1.6). The best studied Rho GTPases are Ras homolog family member A (RHOA), Ras-related C3 botulinum toxin substrate 1 (RAC1) and cell division cycle 42 (CDC42), highly conserved across eukaryotic species (Boureux *et al.* 2007; Vega and Ridley 2008; Wittinghofer 2014; Cardama *et al.* 2018).

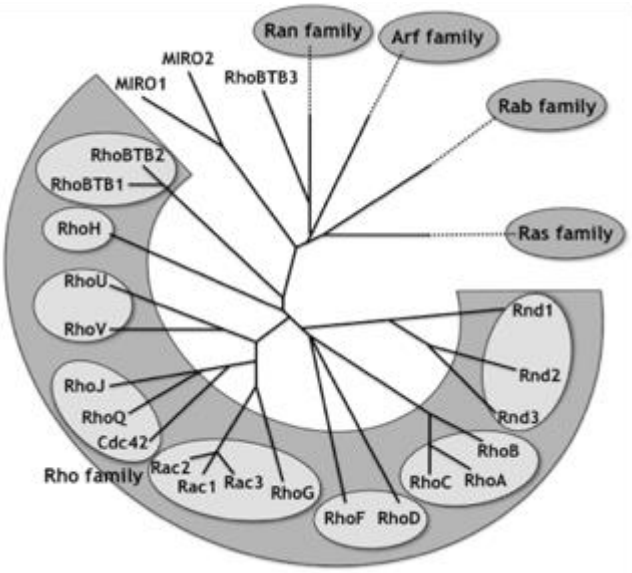

Figure 1.6 - **Phylogenetic tree of the mammalian RAS superfamily of small GTPases.** The 20 Rho family members are grouped into 8 subfamilies (adapted from Vega and Ridley, 2008).

RAC1 is ubiquitously expressed and acts as a binary switch by cycling between the inactive GDP-bound form and the active GTP-bound conformation (Jaffe and Hall 2005). The exchange of GDP with GTP induces a conformational change that allows it to activate specific signaling pathways. (Etienne-Manneville and Hall 2002; Cherfils and Zeghouf 2013; Hodge and Ridley 2016; Song *et al.* 2019)

The conformational changes are particularly evident in two short and flexible loop structures designated as the switch I (RAC1 residues 30–38) and switch II (RAC1 residues 60–76) regions (Alfred Wittinghofer, 2014; Lam and Hordijk, 2013, Wennerberg, 2005). In the GTP-bound conformation, these regions provide a binding domain for both regulatory and effector proteins and also orientate amino acid residue 61 required to catalyze the hydrolysis of GTP.

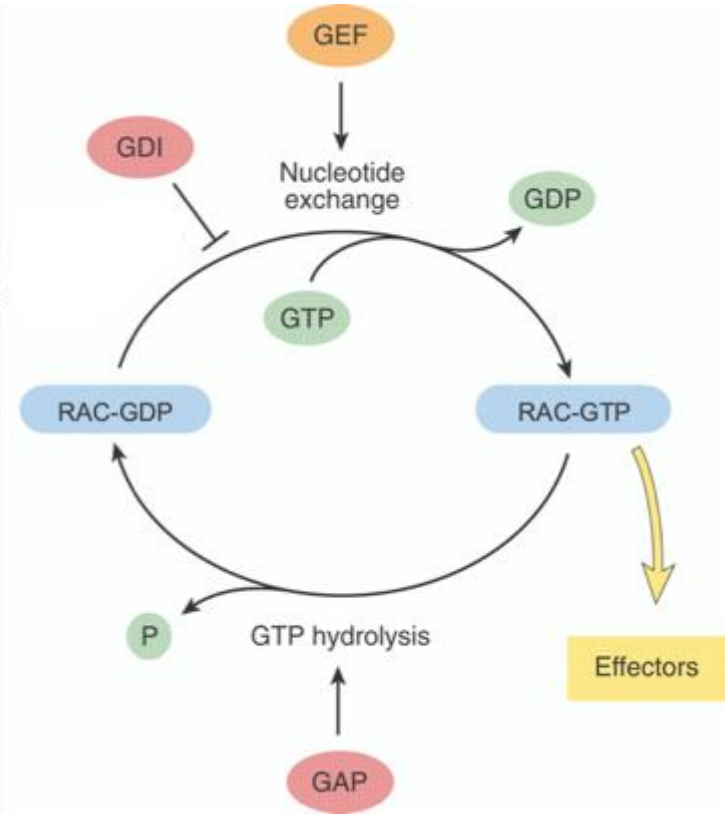

Figure 1.7 - **RAC regulators of nucleotide cycling, between the GTP- and GDP-bound forms.** The shift to the GTP-bound form is achieved by nucleotide exchange, whereas the change to the GDP-bound form is accomplished by GTP hydrolysis. In orange, factors that stimulate RAC conversion to the GTP-bound "on" form (GEF). In red, factors that stimulate RAC conversion to the GDP-bound "off" form (GAP and GDI). Once in the "on," GTP-bound state, RAC transmits the signal through interaction with specific downstream effectors (adapted from Segev, 2001).

The transition between the active and inactive states of RAC1 occurs at the plasma membrane following appropriate cellular signals. This is tightly controlled and spatially regulated by guanine nucleotide exchange factors (GEFs), which promote exchange of GDP for GTP, by GTPase activating proteins (GAPs), which enhance the intrinsic GTPase activity, and by Rho guanine dissociation inhibitors (Rho-GDIs), which bind to and remove RAC1 from the plasma membrane, keeping it inactive in the cytoplasm and blocking its activation by GEFs (Figure 1.7). External stimuli, such as activation of growth factor receptors or engagement of cell adhesion molecules, determine the activity of GEFs, GAPs and GDIs and thus the activation status of RAC1 (Bernards, 2003).

Rho GTPases have traditionally been described as the main regulators of actin cytoskeleton reorganization, in particular the formation of stress fibers, *lamellipodia* or *filopodia* (Hall 1998). RAC1 mainly affects endocytosis and trafficking, cell cycle progression, adhesion and migration, with the latter involving control of *lamellipodia*

formation and membrane ruffles after stimulation by extracellular ligands such as epidermal growth factor (EGF), PDGF or insulin (Bustelo *et al.* 2007; Bosco *et al.* 2009; Cardama *et al.* 2018).

One important effector that interacts directly with GTP-RAC1 is the p21-activated kinase (PAK) (Manser *et al.* 1994). PAKs are a highly conserved group of serine/threonine kinases represented, in mammals, by six isoforms (PAK1 to PAK6). The binding of active RAC1 to an N-terminal Cdc42/Rac interactive binding motif (CRIB) leads to phosphorylation and activation of PAK. Activated PAKs can phosphorylate multiple substrates or interact with other proteins to modulate a range of biological activities, including the regulation of cytoskeletal dynamics and cell motility, stimulation of cell proliferation, pro- and anti-apoptotic signals and regulation of gene expression (Zhao and Manser 2012).

In addition, active RAC1 activates the WASP family member WAVE and adapter proteins, such as non-catalytic region of tyrosine kinase (NCK), which promote the assembly of a meshwork of actin filaments at the cell periphery to produce *lamellipodia* and membrane ruffles (Hall 1998; Eden *et al.* 2002).

Activated RAC1 also stimulates transcription factors and gene expression, for example through the activation of the c-Jun N-terminal kinase (JNK) cascade (Coso *et al.* 1995), or the transcription factor NF-κB (Perona *et al.* 1997). The pathway that links RAC1 to NF-κB involves the production of reactive oxygen species (ROS) and occurs in epithelial cells *via* direct interaction between GTP-RAC1 and the NADPH-dependent oxidase NOX1 complex (Sulciner *et al.* 1996; Joneson and Bar-Sagi 1998; Park *et al.* 2004; Cheng *et al.* 2006). RAC1-stimulated NF-κB leads to increased cyclin D1 expression and subsequent cell cycle progression (Guttridge *et al.* 1999; Hinz *et al.* 1999; Joyce *et al.* 1999; Klein and Assoian 2008), independent of the ERK or JNK kinase cascade (Lamarche *et al.* 1996).

In contrast to the ubiquitously expressed RAC1, two other family members, RAC2 and RAC3 have tissue-specific expression patterns, and cannot encode an alternative splicing isoform, designated RAC1B.

1. 11 RAC1B

As referred above, the RAC1 gene encodes a second isoform, designated RAC1B, through an alternative cassette-exon splicing event. At the molecular level, RAC1B is

translated into protein and results from the inclusion of a 57 base pair (bp) long alternative exon 3b, located between exons 3 and 4 of RAC1 gene. Exon 3b provides an *in-frame* insertion of 19 new amino acids between codons 75 and 76 of RAC1B, immediately behind the switch II region (Figure 1.8) (Jordan *et al.* 1999).

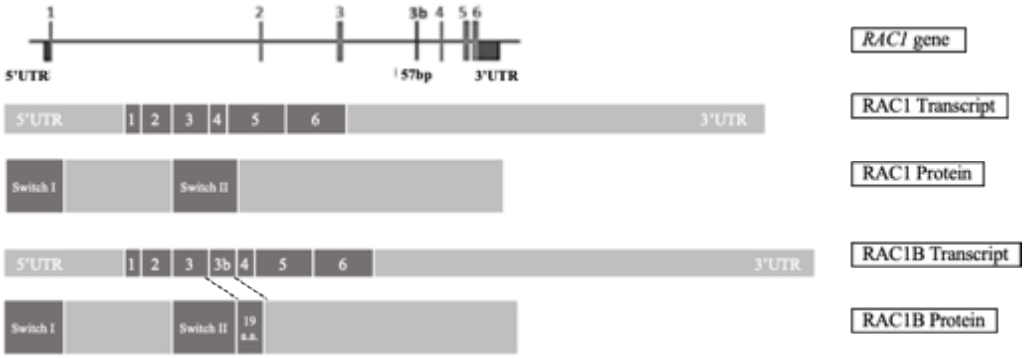

Figure 1.8 - **Diagram of the *RAC1* gene and the encoded proteins and alternative transcripts**. 1, 2, 3, 3b, 4, 5, 6, represent exons; a.a. amino acids (adapted from Silva e*t al*. 2016).

The AS event that generates RAC1B was found to be regulated by two antagonistic serine/arginine-rich (SR) splicing factors that bind directly to exon 3b sequences: SR splicing factor 1 (SRSF1) enhanced, while SRSF3 suppressed inclusion of exon 3b (Gonçalves *et al.* 2014).

RAC1B was shown to be a highly activated variant. The analysis of the total endogenous level of RAC1B protein *versus* the activated GTP-bound fraction revealed that, although present in small amounts in cells, the amount of active RAC1B is surprisingly high, and exceeds the amount of active RAC1. Thus, RAC1B was found to be predominantly in the signaling-competent GTP-bound conformation in cells (Schnelzer *et al.* 2000; Matos *et al.* 2003; Fiegen *et al.* 2004; Radisky *et al.* 2005), so that small changes in its expression level yield significant cellular responses.

The high activation level of RAC1B is due to several differences that originate from the extra 19 amino acids. First, RAC1B is unable to interact with Rho-GDI and consequently to cycle between the plasma membrane and the cytoplasm, which leaves it persistently associated with membranes, in a favored position to become activated (Matos *et al.* 2003). Additionally, RAC1B shows impaired intrinsic GTPase activity *in vitro* (Schnelzer *et al.* 2000), yet maintaining GAP responsiveness *in vivo* (Matos *et al.* 2003) and *in vitro* (Fiegen *et al.* 2004; Singh *et al.* 2004). Finally, it also revealed an increased intrinsic nucleotide exchange rate (GDP to GTP) *in vitro* (Schnelzer *et al.* 2000).

Also, in RAC1B, the already flexible switch 2 region is followed by the 19 additional amino acids, which may allow a different pattern of effector protein association. RAC1B binds less effectively to proteins that act as signal pathway inhibitors and more effectively to proteins that can promote loss of epithelial cell structure and increased cell proliferation, for example p120-catenin (Orlichenko *et al.* 2010).

Interestingly, RAC1B was found to fail to activate several classical RAC1 pathways, as the formation of *lamellipodia*, the activation of the protein kinase PAK, or the stimulation of JNK pathway. However, RAC1B retained the ability to stimulate the classical NF-κB pathway. So, RAC1B seems to be selective in downstream signaling properties, with a preferential to cell survival stimulation (Matos *et al.* 2003).

1. 12 RAC1B and cancer

RAC1B expression was predominantly identified in skin and epithelial tissues from the intestinal tract and in breast tissues but is normally less abundant than RAC1. Curiously, RAC1B was found overexpressed in colorectal (Jordan *et al.* 1999; Matos *et al.* 2000), breast (Schnelzer *et al.* 2000), lung (Stallings-Mann *et al.* 2012; Zhou *et al.* 2013), pancreas (Ungefroren *et al.* 2018) and thyroid tumors (Silva *et al.* 2013).

Initial studies found that RAC1B increased G1/S progression, survival and transformation of NIH3T3 fibroblasts (Singh *et al.* 2004; Matos and Jordan 2005, 2006), promoted epithelial-mesenchymal (EMT) transition of mouse mammary epithelial cells (Radisky *et al.* 2005), and contributed to further activate WNT signaling in HCT116 colorectal cells (Esufali *et al.* 2007). However, the molecular details of how the overexpression of RAC1B can promote the progression of tumors remain unclear.

1.12.1. RAC1B and CRC

RAC1B is overexpressed in some colorectal tumors (Matos and Jordan 2008). More detailed analysis revealed that about 80% of BRAF-V600E-positive (the most common BRAF mutation), but not the KRAS-mutated, colorectal tumors overexpress RAC1B (Jordan *et al.* 1999; Matos *et al.* 2008). This indicates that RAC1B is overrepresented in the CMS1 group of colorectal cancer (i.e., with recurring MSI and BRAF-mutation), representing about 15% of the sporadic CRC cases. CMS1 is characterized by a gene expression profile associated with a diffuse immune infiltrate,

strong activation of immune evasion pathways, defective DNA mismatch repair, overexpression of proteins involved in DNA damage repair, and hypermethylation. Patients with CMS1 tumors have initially good prognosis, but very poor survival after relapse CRC tumors (Guinney *et al.* 2015).

On the basis of this association, a separate genetic pathway to CRC was proposed, which becomes initiated by a BRAF-V600E mutation but later selects for overexpression of hyperactive RAC1B, allowing further tumor progression through activation of the transcription factor NF-κB. RAC1B signaling has a direct role in maintaining the viability of CRC cells, particularly regarding the prevention of apoptosis, by activating the NF-κB pathway (Matos and Jordan 2008) without stimulating other classic RAC1 signaling targets including JNK (Matos *et al.* 2003; Fiegen *et al.* 2004).

Furthermore, colorectal tumor cells expressing BRAF-V600E and RAC1B were highly sensitive to combined depletion of both transcripts, leading to massive cell death (Matos and Jordan 2008; Matos *et al.* 2008). Thus, RAC1B overexpression and BRAF-V600E functionally cooperate to sustain tumor cell survival. This cooperation complements the observation that BRAF-V600E revealed a 50-fold lower oncogenic potential than mutant KRAS protein in cell transformation assays using fibroblasts (Davies *et al.* 2002; Wan *et al.* 2004), being dependent on additional genetic changes.

In a study of 153 spanish CRC patients treated with first-line FOLFOX/ XELOX therapy, RAC1B overexpression was detected in 40% of the BRAF mutated tumors, and RAC1B overexpression constituted a marker of poor prognosis (Alonso-Espinaco *et al.* 2014).

Activating mutations in the *BRAF* gene have been clearly found to induce oncogene-induced senescence (OIS) response, which leads to growth arrest following an initial proliferative phase, both in melanoma (Michaloglou *et al.* 2005; Dhomen *et al.* 2009) and colon cancer (Carragher *et al.* 2010). Unclear remains, how senescent cells eventually escape from the growth arrest state and progress to more malignant tumor cells (Collado *et al.* 2005; Michaloglou *et al.* 2005; Carragher *et al.* 2010; Kriegl *et al.* 2011). At least in colorectal cancer cells with BRAF-V600E, evidence exists that the overexpression of RAC1B can contribute to this escape from OIS, although the presence of the mutation was not sufficient to induce RAC1B (Henriques *et al.* 2015).

Colon inflammation can be a trigger for increased RAC1B expression. This was observed during experimentally induced acute colitis in mice but also in surgical samples from patients with inflammatory diseases of the colon (Matos *et al.* 2013), which is a

known risk factor for CRC development (O'Connor et al., 2010; Ullman and Itzkowitz, 2011a). The underlying signaling cascades remain to be determined.

1.12.2. RAC1B and other tumors

RAC1B is expressed in normal thyroid tissue and overexpressed in papillary thyroid carcinomas (PTCs): 59% of classical variant and in 25% of follicular variant. RAC1B overexpression showed a striking association with both BRAF-V600E mutation and poor clinical outcome. This suggests an association and synergy of RAC1B with BRAF-V600E to sustain the proliferation and survival of thyroid cancer cells, such as documented for colorectal cancer cells (Silva *et al.* 2013). Moreover, NF-κB activation has been implicated as one of the molecular mechanisms associated with the pro-tumorigenic advantage of RAC1B overexpression in thyroid carcinomas (Faria *et al.* 2017).

In follicular thyroid carcinomas (FTCs), RAC1B was found to be overexpressed in 33% of carcinomas. RAC1B overexpression was significantly associated with both the presence of distant metastases and poorer clinical outcome. Furthermore, the lack of RAC1B overexpression in follicular adenomas reveals its potential as a molecular marker for preoperative differential diagnosis of thyroid follicular lesions (Faria *et al.* 2016).

High expression levels of RAC1 was reported in neoplastic breast tissue of ductal carcinoma *in-situ*, primary breast cancer and lymph node metastases, while low levels in benign breast tissue. However, there was no differences in RAC1B expression levels between benign and malignant breast tissue (Schnelzer *et al.* 2000). In contrast, a transgenic breast cancer mouse model revealed RAC1B to promote EMT following transgenic activation of matrix metalloproteinase 3 (MMP3) (Radisky *et al.* 2005), a finding confirmed in lung epithelial cells (Stallings-Mann *et al.* 2012). In pancreatic adenocarcinoma (PDAC), MMP3-mediated RAC1B expression increased cellular invasiveness and tumorigenic potential (Mehner *et al.* 2014, 2015).

MMPs are upregulated in almost all types of human cancer and associated with poor survival (Egeblad and Werb 2002).

In conflict with a role as an EMT inducer, RAC1B was found as a negative regulator of TGF-β1 and its induction of cell cycle inhibitor p21 in breast cancer cells, which supports proliferative effects of RAC1B through cell cycle progression (Melzer *et al.* 2017). In pancreatic cancer cells, RAC1B also blocks growth inhibition by TGF-β1, a

paradigmatic induced of EMT (Witte *et al.* 2017), so RAC1B was described as a protector against EMT (Zinn *et al.* 2019). RAC1B overexpression was identified in the ductal epithelial cells of stage III PDAC tumors and, in addition, in chronic pancreatitis tissues, in which the levels of RAC1B protein even exceeded those in PDAC (Ungefroren *et al.* 2018).

In non-small cell lung cancer (NSCLC), either RAC1 and RAC1B were found to be significantly increased compare to healthy controls, independent of the cancer stage and RAC1 and RAC1B protein were postulated to be a diagnostic serum marker for NSCLC (Sahu *et al.* 2016). In a lung cancer mouse model, RAC1B was apparently required for *KRAS*-driven tumorigenesis (Zhou *et al.* 2013) and, thus, opposite to what was observed in colon.

Knockdown of RAC1B significantly impaired cell migration and invasion capacities of multipotent neural crest cells and metastatic melanomas (Wu *et al.* 2018). In hepatocellular carcinoma (HCC), Rho GTPase activating protein 11A (ARHGAP11A) promoted a malignant phenotype by facilitating cell proliferation, EMT, invasion and migration in a RAC1B-dependet manner. This Rho-GAP increases total RAC1B protein levels and RAC1B activity in HCC cell lines, although the precise mechanism of this regulation remains to be determined (Dai *et al.* 2018).

RAC1B might exert protective or malignant effects depending of context. In a transgenic mouse model, increased expression of RAC1B alone was not tumorigenic and contributed to intestinal homeostasis and tissue repair after injury; however, in the pro-tumoral context of the Apc^{min} mouse, or dysregulation in the crosstalk between epithelial intestinal, stromal, and immune cells, RAC1B might promote neoplastic transformation (Kotelevets *et al.* 2018).

Therapeutic targeting of RAC1B to inhibit its expression or to downregulate its activity or shifting the endogenous RAC1B/RAC1 balance toward RAC1 could represent a promising approach in several types of tumors (Melzer *et al.* 2019). Unclear remains how the overexpression of RAC1B is triggered.

1. 13 Objectives

As a brief rationale, RAC1B, a RAC1 alternative splicing variant, that the host laboratory previously identified, is overexpressed in a subset of BRAF-mutated colorectal tumors. Evidence exists that the overexpression of RAC1B can contribute to the escape

of tumor cells from oncogene-induced senescence caused by *BRAF* mutation, although the presence of the mutation alone was not sufficient to induce RAC1B overexpression. Moreover, it is known that the increase in RAC1B is not associated with mutations in the RAC1 gene. RAC1B expression was also found increased in samples from inflammatory bowel disease patients or following experimentally-induced acute colitis in a mouse model, suggesting inflammation as a possible trigger for its alternative splicing.

Therefore, the main objective of the present work was to understand how tumor cells respond to a pro-inflammatory microenvironment with changes in the alternative splicing of RAC1B, representative of plasticity in their gene expression. For this, the work described in this thesis had the following tasks:

- Implement co-cultures of colorectal and stromal cell types and determine the conditions that lead to increased RAC1B expression in colorectal cells;
- Identify the secreted cytokines affecting RAC1B expression in colorectal cells;
- Analyze cytokine-stimulated signaling pathways to understand which are involved in the regulation of RAC1B overexpression.

Through these tasks, this PhD project was expected to identify how the pro-inflammatory microenvironment can alter the transcriptome of colorectal cells through the modulation of alternative splicing, which may indicate new therapeutic targets in colorectal cancer.

Chapter 2 - Materials and Methods

2.1. Cell culture

Caco-2, THP-1, NCM460 and HT29 cells were maintained in Roswell Park Memorial Institute 1640 Medium (RPMI), while DLD-1 and T84 colorectal cells were cultured in Dulbecco's modified Eagle medium (DMEM) or DMEM/nutrient mixture F-12 (DMEM/F-12), respectively, all supplemented with 10% (v/v) of heat inactivated fetal bovine serum (FBS) (all reagents from Gibco). NIH3T3 cells were maintained in DMEM supplemented with 10% (v/v) of heat inactivated newborn calf serum (NBCS) (Gibco).

CT5.3 cancer-associated fibroblasts (gift from O. Wever, Ghent, Belgium) were grown in DMEM supplemented with 10% (v/v) FBS, 2.5 μg/mL puromycin (Invitrogen) and 100 U/ml penicillin/streptomycin (PEN/STREP, Gibco).

Cells were maintained at 37˚C with 5% CO_2, and regularly checked for absence of mycoplasm infection by PCR amplification of a 16S ribossomal DNA fragment [primers forward (F) 5' ACTCCTACGGGAGGCAGCAGTA 3' and reverse (R) 5' TGCACCATCTGTCACTCTGTTAACCTC 3'] from lysates of cells harvested from the culture medium.

2.2. THP-1 differentiation

THP-1 monocyte cells were differentiated with 50 ng/mL phorbol-12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 24 h, to obtain a M0 macrophage morphology. Cell differentiation was verified by evaluating cell adhesion and spreading under an optical microscope. After this, the M0 cells were refed for 24 h with fresh medium containing either 10 ng/mL LPS (Sigma) and 10 ng/mL INF-γ (Gibco), or 10 ng/mL IL-4 (R&D systems) to differentiate into M1 and M2 macrophages, respectively. The scheme of THP-1 differentiation is shown in Figure 2.1.

THP-1 differentiation was evaluated by PCR amplification of marker genes (see section 2.6) (Chanput *et al.* 2013; Littlefield 2014; Genin *et al.* 2015; Wheeler *et al.* 2018; Li *et al.* 2018).

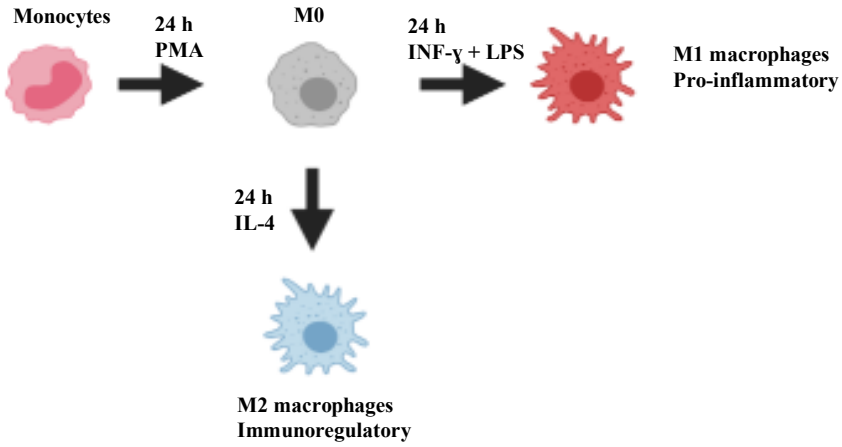

Figure 2.1 – **Scheme of THP-1 differentiation into M0, M1- and M2-like macrophages**.

2.3. Cell polarization and co-culture assays

For cell polarization, Caco-2 cells were grown on porous (1 μm) transwell PET filter inserts (24-well size, 6.4 mm diameter and 0.3 cm^2 area, Corning) in RPMI medium supplemented with 5% (v/v) FBS for 10–15 days, until they reached a TEER of 1000 to 1200 Ω, as measured with a chopstick electrode STX2 (World Precision Instruments).

T84 cells were polarized in DMEM/F12 medium supplemented with 5% (v/v) FBS for 10–15 days, until they reached a TEER of 1500 to 2000 Ω.

For co-culture assays, epithelial and stromal cells were first grown in 24-well plates for 24 h, then the filters with polarized Caco-2 or T84 cells, or filters with non-polarized cells were added. The co-cultures were maintained during different time periods (24 h, 48 h, 72 h, 96 h or 120 h).

The conditioned basolateral media from co-cultures were recovered, frozen at -80˚C and then analyzed with a human inflammatory antibody array (see section 2.10).

2.4. Cell treatments

For treatment with purified cytokines, polarized Caco-2 cells were incubated in basolateral side, for 48 h, with either the control vehicle phosphate-buffered saline (PBS, Gibco) + 0.01% bovine serum albumin (BSA, Sigma-Aldrich) or with 1 ng/mL, 10 ng/mL or 100 ng/mL of the following cytokines: IL-1β, IL-6, IL-8, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage inflammatory protein

2-alpha (MIP-2α) (R&D Systems). The cytokine stock solutions were prepared at least 1000-fold in PBS + 0.01% BSA and stored at -20˚C.

For cytokine neutralization, polarized Caco-2 with purified IL-6 at 10 ng/mL or co-cultures of polarized Caco-2 with Caco-2 or with CT5.3 and M1 were incubated in basolateral side, for 48 h, with the vehicle PBS or with polyclonal goat anti-human IL-6 antibody (R&D Systems) at a final concentration of 500 ng/mL and 1000 ng/mL. The stock solutions were prepared at least 1000-fold in PBS and stored at -20˚C.

For drug treatments, polarized Caco-2 cells from co-culture with Caco-2 or CT5.3 and M1 were incubated in apical side, for 24 h, with the vehicle dimethyl sulfoxide (DMSO) (Sigma-Aldrich) or with the MEK inhibitor AZD6244 (Selumetinib; APExBio) at a final concentration of 10 μM. The stock solutions were prepared at least 1000-fold in DMSO and stored at -20˚C.

2.5. 3D spheroids in Matrigel

Caco-2 cell maintained in 2D cell cultures as described in section 2.1, were cultured between passages 5 and 20 in 8-well glass chamber slides (Ibidi) coated with Matrigel (Corning) at 1.5×10^5 cells per well. Cells were cultured in RPMI with 10% FBS containing 2% of Matrigel at 37˚C in 5% CO_2 and medium was changed every 2 days. The cells formed 3D clusters by day 5 or 7 and subsequently started forming a cyst-like structure with a central hollow lumen.

2.6. Western blot (WB) procedures

Cells were lysed in 50 μl of lysis buffer [50 mM Tris/HCl (pH 7.5), 2 mM $MgCl_2$, 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP40] and total proteins from co-culture assays were separated in 10 or 12% (w/v) SDS-PAGE gels. For subsequent phospho-array analysis, lysates of cells were separated on 10% (w/v) SDS-PAGE with 1% (v/v) glycerol gels and run at 4˚C. Following electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). WB membranes were blocked in 5% (w/v) milk powder in TBS with 0.5% (v/v) Triton X-100 and specific proteins probed using the indicated primary antibodies (Table 2.1) overnight (o/n), followed by three wash steps and incubation with a secondary peroxidase-conjugated antibody (Bio-Rad). Protein bands were visualized by chemiluminescence on X-rays films and

quantified on digitalized images by densitometric analysis with ImageJ software (National Institutes of Health – NIH).

Table 2.1 - **Table of antibodies used in WB assays and/or immunofluorescence assays.**

Primary antibody	Clone; Catalog Number	Brand/Supplier	Secondary antibody
mouse anti-β-actin	AC-15	Sigma-Aldrich	Goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (#170-6516; Bio-Rad)
mouse anti-β-catenin	14; 610154	BD Transduction Laboratories	
mouse anti-E-cadherin	36; 610181	BD Transduction Laboratories	
mouse anti-p-ERK 1/2 (Thr202/ Tyr204)	M8159	Sigma-Aldrich	
mouse anti-α-tubulin	B-5-1-2; T5168	Sigma-Aldrich	
mouse anti-RAC1	23A8; #05-389	Merk Millipore	
rabbit anti-AKT	#9272	Cell Signaling Technology	Goat anti-rabbit IgG HRP conjugate (#170-6515; Bio-Rad)
rabbit anti-p-AKT (S473)	#9271	Cell Signaling Technology	
rabbit anti-ERK 1/2	M5670	Sigma-Aldrich	
rabbit anti-GSK3β	27C10; #9315	Cell Signaling Technology	
rabbit anti-p-GSK3β (S9)	D3A4; #9322	Cell Signaling Technology	
rabbit anti-p-STAT3 (Y705)	D3A7	Cell Signaling Technology	
rabbit anti-p-STAT3 (S727)	#9134	Cell Signaling Technology	
rabbit anti-STAT3	#30835	Cell Signaling Technology	
rabbit anti-RAC1B	#09-271	Merk Millipore	
rabbit anti-ZO-1	H-300; sc-10804	Santa Cruz Biotechnology	

2.7. Total RNA extraction from formalin-fixed paraffin-embedded (FFPE) blocks

A total of 32 blocks of FFPE tissue from patients with inflammatory bowel disease were obtained from the archives of the biobank of Centro Hospitalar Universitário de S. João, Porto. RNA extraction was performed with Rneasy FFPE (Qiagen). For this, the samples were first deparaffinized by addition of 1.0 mL xylene (Invitrogen), followed by centrifugation for 2 min at maximum speed. Next, the supernatant was discarded, and the pellet was washed twice with 1.0 mL absolute ethanol, followed by incubation for 10 min at 37˚C. The proteins were degraded with 150 µL digestion buffer and 10 µL proteinase K, followed by incubation for 15 min at 56˚C and for 15 min at 80˚C. Subsequently, RNA was isolated by adding 720 µL of buffer containing absolute ethanol, along with passage through a purification column. The column was then washed twice with a buffer from the kit, and Dnase treatment was performed, followed by two additional washings. Finally, RNA was eluted in 30 µL of Rnase-free water from the kit at room temperature, according to the manufacturer's instructions.

RNA was reverse transcribed to complementary DNA (cDNA) using random primers (Invitrogen) and Ready-to-Go You-Prime Beads (GE Healthcare).

2.8. Semi-quantitative reverse transcribed-PCR (RT-PCR)

Total RNA was extracted from THP-1, M1 and M2 cells with the RNA isolation kit (Macharey-Nagel) and reverse transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime Beads (GE Healthcare). Amplification reactions were performed using Go Taq G2 Flexi DNA polymerase (Promega). The primers forward (F) and reverse (R) and amplification conditions for each gene were summarized in Table 2.2. All reactions included an initial denaturation step of 5 min at 94˚C and a final extension step of 10 min at 72˚C. To allow a semi-quantitative analysis of transcript levels, all amplification conditions were experimentally optimized to correspond to the linear amplification phase, using serial dilutions of control cDNAs. The products were separated on 2% agarose gels containing ethidium bromide and band intensities were quantified on digitalized images using ImageJ software (NIH) followed by normalization to GAPDH expression levels. Each PCR was repeated at least 3 times from independent experiments.

Table 2.2 - **Table of primers for RT-PCR used for characterization of macrophage phenotype.** (F - forward, R-reverse).

	Transcript	Sequence primer	Annealing temperature
M1 markers	IL-1β	F: 5' CCACAGACCTTCCAGGAGAATG 3'	66˚C
		R: 5' GTGCAGTTCAGTGATCGTACAGG 3'	
	TNF-α	F: 5' CTCTTCTGCCTGCTGCACTTTG 3'	68˚C
		R: 5' ATGGGCTACAGGCTTGTCACTC 3'	
	CXCL10	F: 5' GAAAGCAGTTAGCAAGGAAAGGTC 3'	66˚C
		R: 5' ATGTAGGGAAGTGATGGGAGAGG 3'	
	CCR7	F: 5' TGGTGGTGGCTCTCCTTGTC 3'	66˚C
		R: 5' TGTGGTGTTGTCTCCGATGTAATC 3'	
	CXCL16	F: 5' ACTACACGAGGTTCCAGCTCC 3'	64˚C
		R: 5' CTTTGTCCGAGGACAGTGATC 3'	
M2 markers	CD163	F: 5' GTCGCTCATCCCGTCAGTCATC 3'	68˚C
		R: 5' GCCGCTGTCTCTGTCTTCGC 3'	
	CD206	F: 5' TACCCCTGCTCCTGGTTTTT 3'	68˚C
		R: 5' CAGCGCTTGTGATCTTCATT 3'	
	CCL17	F: 5' CGGGACTACCTGGGACCTC 3'	66˚C
		R: 5' CCTCACTGTGGCTCTTCTTCG 3'	
	CD36	F: 5' GAGAACTGTTATGGGGCTAT 3'	60˚C
		R: 5' TTCAACTGGAGAGGCAAAGG 3'	
Control	GAPDH	F: 5' GTCTCCTCTGACTTCAACAGCG 3'	66˚C
		R: 5' ACCACCCTGTTGCTGTAGCCAA 3'	

2.9. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from Caco-2 cells with the RNA isolation kit (Macherey-Nagel) or from blocks of FFPE tissue and reverse transcribed using random primers (Invitrogen) and Ready-to-Go You- Prime Beads (GE Healthcare). Real-time quantitative RT-PCR (qRT-PCR) was performed on an ABI Prism 7000 Sequence Detection System. Primers were designed using the ABI Primer Express software that amplified amplicons specific for endogenous RAC1B (78 bp) (F: 5' GGGCAAAGACAAGCCGATTG 3' and R: 5' CGGACATTTTCAAATGATGCAGG 3') or total RAC1 transcripts [(RAC1+RAC1B; 75 bp) (F: 5' CCTGCATCATTTGAAAATGTCCG 3' and R: 5' CCCACTAGGATGATGGGAGTGT 3')]. Each cDNA sample was diluted 5-fold to guarantee accurate pipetting and 5 μL added to 300 nmol/L primers and SYBR Green Master Mix (Applied Biosystems). The cycling conditions comprised 10 min polymerase

activation at 95˚C and 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. For standardization, all samples were analyzed against Caco-2 cDNA as a reference sample using the 7000 SDS 1.1 RQ Software (Applied Biosystems), using the comparative Ct method (ΔΔCt). Each amplification was performed in triplicate reactions and the PCR repeated at least 3 times from independent experiments.

2.10. Cytokine array procedure

Cytokine array, namely human inflammation antibody array C3 (AAH-INF-3-8; RayBiotech Inc.) consisted of 40 human antibodies against inflammatory factors spotted in duplicate onto a semiquantitative membrane (Table 2.3) (Lin *et al.* 2003; Mahlknecht *et al.* 2012; Drolia *et al.* 2019).

The membranes were blocked with blocking buffer and processed according to the instructions of the manufacturer. One milliliter of conditioned basolateral medium from co-cultures was added to each membrane in separate wells of an 8-well incubation tray. The membranes were shaken overnight at 4˚C and washed in the incubation tray. One milliliter of a 1:500 dilution of biotinylated antibody cocktail was added to each membrane, and the mixture was incubated on a shaker, overnight at 4˚C. Following the washing steps, the membranes were incubated with 2 mL of 1x streptavidin-conjugated peroxidase for 2 h at room temperature. Following a thorough wash, the membranes were exposed to a peroxidase substrate (detection buffer mixture) for 1 min in the dark, before exposure to X-ray films. Membranes were placed side-by-side in a plastic protective folder, sealed and exposed for times ranging from 5 sec to 5 min. X-rays films were digitalized, and images analyzed with ImageJ software (NIH).

The 6 positive-control spots (POS) were used for normalization of signal intensities between arrays, the 6 negative-control spots (NEG) to measure the non-specific baseline signal, and blank spots (BLANK) to measure the background signal.

For each spot, the remaining density gray level was obtained by subtracting the background gray levels from the total raw density gray level values. Then, the mean value was calculated from the duplicates of each spot. The relative fold-difference in each cytokine signal was then determined by dividing the mean value from the co-culture condition through the mean value from the control culture condition.

Table 2.3 - **List of cytokines analyzed in the human inflammation antibody array.**

Eotaxin-1 (CCL11)	IL-7	M-CSF
Eotaxin-2 (MPIF-2/CCL24)	IL-8 (CXCL8)	MIG (CXCL9)
GCSF	IL-10	MIP-1α (CCL3)
GM-CSF	IL-11	MIP-1β (CCL4)
ICAM-1 (CD54)	IL-12 p40	MIP-1Δ (CCL15)
IFN-ɣ	IL-12 p70	RANTES (CCL5)
I-309 (TCA-3/CCL1)	IL-13	TGF-β1
IL-1α (IL-1 F1)	IL-15	TNF-α
IL-1β (IL-1 F2)	IL-16	TNF-β (TNFSF1B)
IL-2	IL-17α	TNF RI (TNFRSF1A)
IL-3	IP-10 (CXCL10)	TNF RII (TNFRSF1B)
IL-4	MCP-1 (CCL2)	PDGF-BB
IL-6	MCP-2 (CCL8)	TIMP-2
IL-6 RI		

2.11. Proteome profiler human phospho-kinase antibody array procedure

Proteome profiler human phospho-kinase antibody array (ARY003B; R&D Systems, Bio-techne) was utilized according to the manufacturer's protocol. It simultaneously detects the phosphorylation status of 43 human kinases plus the total amounts of the signaling-related proteins HSP60 and β-catenin (Table 2.4). All antibodies are spotted in duplicate on nitrocellulose membranes.

Table 2.4 - **List of proteins analyzed in the human phospho-kinase antibody array.** S corresponds to the amino acid serine, T corresponds to threonine and Y to tyrosine.

AKT 1/2/3 S473	Fgr Y412	p38α T180/Y182	Src Y419
AKT 1/2/3 T308	Fyn Y420	P53 S15	STAT2 Y689
AMPKα1 T183	GSK3α/β S21/S9	P53 S392	STAT3 S727
AMPKα2 T172	Hck Y411	P53 S46	STAT3 Y705
c-Jun S63	HSP27 S78/S82	P70 S6 Kinase T389	STAT5a Y694
Chk-2 T68	HSP60	P70 S6 Kinase	STAT5a/b
CREB S133	JNK 1/2/3	T421/S424	Y694/Y699
	T183/Y185,T221/Y223	PDGF Rβ Y751	STAT5b Y699

EGFR Y1086	Lck Y394	PLC-ɣ1 Y783	STAT6 Y641
eNOS S1177	Lyn Y397	PRAS40 T246	TOR S2448
ERK 1/2	MSK 1/2 S376/S360	PYK2 Y402	WNK1 T60
T202/Y204,T185/Y187	P27 T198	RSK 1/2/3	Yes Y426
FAK Y397		S380/S386/S377	β-catenin

Caco-2 cells from 24 h and 48 h co-cultures were lysed using the provided lysis buffer. The lysates were incubated overnight with the array membranes, and then washed to remove any unbound proteins. Further incubation was performed with a cocktail of biotinylated detection antibodies for 2 h at room temperature. The membranes were then incubated with streptavidin-HRP for 30 min. After a final wash, the cellular proteins bound to the antibodies on the membrane were detected by chemiluminescence for 1 min. Membranes were placed side-by-side in a plastic protective folder and sealed. Chemiluminescent signals were captured on X-ray films within exposure times ranging from 1 to 10 min. X-rays films were digitalized, and images analyzed with ImageJ software (NIH).

To compare different membranes, the values were normalized to those of the positive controls on each membrane, and the protein expression levels were then quantified.

2.12. Confocal immunofluorescence microscopy

Cells were grown on PET transwell filters or in Matrigel, washed twice in PBS, immediately fixed with 4% (v/v) formaldehyde in PBS for 20 min at room temperature, and subsequently permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min at room temperature. Cells were then labeled for 2 h with antibodies (see Table 2.1) against RAC1B (1:250), β-actin (1:200), E-cadherin (1:200) or ZO-1 (1:250), washed 3x in PBS-T (PBS + 0.01% Tx-100) for 5 min with gentle shaking, followed by 30 min incubation with a 1:250 dilution of mouse or rabbit Alexa Fluor 488 or mouse Alexa Fluor 546 (Invitrogen) and phalloidin-TRITC (Sigma-Aldrich). Cells were washed 3x in PBS, briefly stained with 1.25 µg/mL 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma-Aldrich), washed again, post-fixed with 4% (v/v) formaldehyde in PBS for 10 min at room temperature. Then cover slips were mounted on glass slides in VectaShield

(Vector Laboratories) and sealed with nail polish. The 405 nm, 488 nm and 532 nm laser lines of a Leica TCS-SPE confocal microscope were used to acquire one Airy thick XZ, and Z-stacks of XY images from, respectively, polarized monolayers in transwell filters and spheroids cultured in Matrigel. Recorded images were processed with Leica in-built software and assembled in figures with Adobe Photoshop software.

2.13. Statistical analysis

Data were analyzed using Welsh test or one-way ANOVA test followed by a Dunnett post-test, with $P < 0.05$ accepted as the statistical significance level. Shown data reflect the mean ± SEM from at least 3 independent experiments.

Chapter 3 - Results

3.1. RAC1B and RAC1 protein levels in different cell models

Previous work had shown that some colorectal cells express the RAC1B splice variant, besides the canonical RAC1 protein (Matos *et al.* 2003). In order to assess their RAC1B and RAC1 protein levels by Western blot, we compared a normal mucosa cell line, NCM460, and four colorectal cancer cell lines with different features and origins (Figure 3.1).

We determined that HT29 cells expressed the highest levels of RAC1B protein, followed by T84 and Caco-2 cells, while DLD-1 and NCM460 showed the lowest levels of RAC1B expression. RAC1B can be detected either together with conventional RAC1, using an anti-RAC1 antibody, or independently, using a RAC1B-specific antibody, which increases the sensitivity of detection allowing a better estimation of RAC1B, although both methods gave proportional results (Figure 3.1).

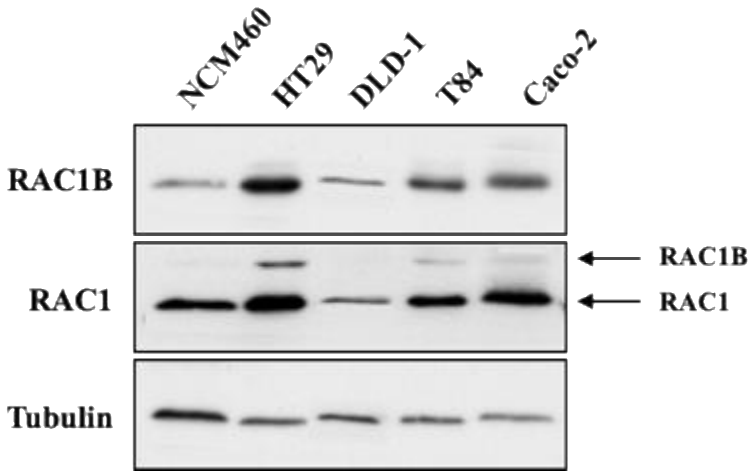

Figure 3.1 - **Expression of RAC1B and RAC1 in colorectal cell lines.** Proteins were analyzed by SDS-PAGE and WB and the indicated proteins detected in the whole-cell lysates. Tubulin is used as loading control.

On the other hand, analyzing the level of expression of RAC1B and RAC1 in stromal cell types, such as THP-1 monocytes and mouse NIH3T3 or human CT5.3 fibroblasts, revealed no expression of RAC1B neither in monocytes nor in fibroblasts, not even with the RAC1B-specific antibody (Figure 3.2).

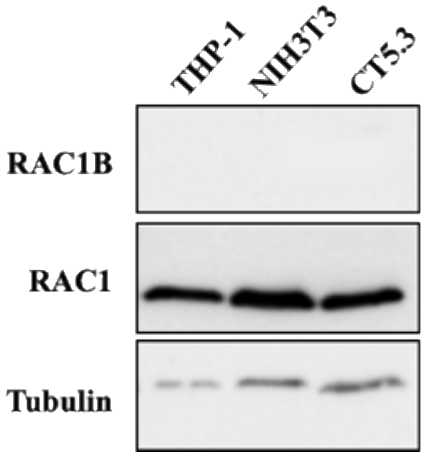

Figure 3.2 - **Expression of RAC1B and RAC1 in stromal cell lines.** Proteins were analyzed by SDS-PAGE and WB and the indicated proteins detected in the whole-cell lysates. Tubulin is used as loading control.

3.2. Implementation of cellular and co-culture conditions to identify stimuli that lead to an increase in RAC1B

A significant improvement in physiological relevance of the 2D cell models is achieved upon their growth on microporous membrane inserts that allow free access of ions and nutrients to either the apical or the basolateral sides of the cell monolayer. These conditions induce full cell polarization and require several days to weeks and Caco-2 cells have this capacity to form polarized layer (Pereira *et al.* 2016). As a result, an apical domain (corresponding to the intestinal lumen) generates microvilli and the lateral intercellular space is sealed through tight junctions so that differential sorting of proteins to each membrane domain can occur, including specific sets of ion channels, receptors, and solute transporters (Nelson 2003; Saaf *et al.* 2007; Mellman and Nelson 2008).

In order to validate our study model, Caco-2 colorectal cells, which express endogenous RAC1B (Figure 3.1), were seeded on filter inserts for monolayer formation and cell polarization was monitored by measurement of transepithelial electrical resistance (TEER) during 17 days (Figure 3.3).

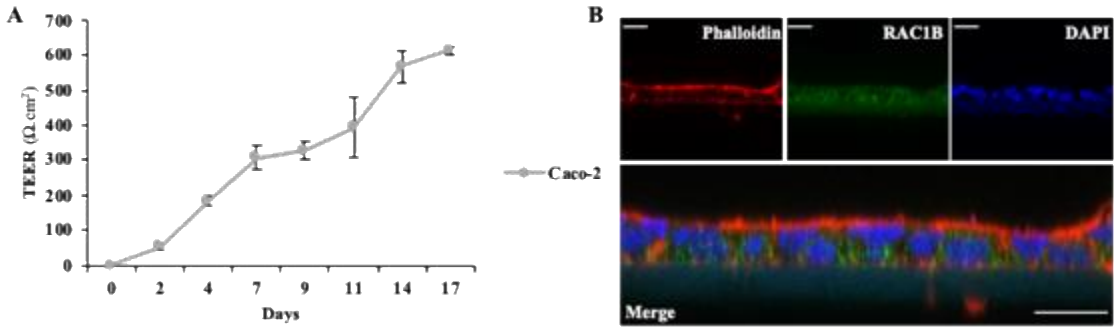

Figure 3.3 - **Polarization of Caco-2 cells.** Caco-2 cells were seeded on filter inserts for monolayer formation and cell polarization. (**A**) Polarization was monitored by TEER measurement with a Chopstick Electrode STX2, during 17 days. Data are expressed as mean ± SEM from 5 independent experiments (**B**) Cell polarization and RAC1B localization were analyzed by confocal immunofluorescence microscopy at day 14 of culture. Merge is the colored overlay of three confocal immunofluorescence XZ images, which detected cell nuclei in blue (DAPI), the localization of endogenous RAC1B protein in green (polyclonal anti-RAC1B antibody), followed by anti-rabbit Alexa488 and the actin filament marker phalloidin in red.

As shown in Figure 3.3A, TEER of Caco-2 epithelial cells increased over the days in culture and reached the reference value described for Caco-2 (400 to 600 $\Omega.cm^2$) between the 11^{th} and the 17^{th} day of culture, yet, on day 14 a stabilization in resistance was observed. Usually, monolayers with TEER values of over 1000 $\Omega.cm^2$ are considered as tight, with values of 300–400 $\Omega.cm^2$ as intermediate, and as leaky with values of 50–100 $\Omega.cm^2$ (Pereira *et al.* 2016). However, TEER values ranging from 62 to 1290 $\Omega.cm^2$ have been reported for Caco-2 cells and also depend on cell line variants and culture conditions (Mathias *et al.* 2010; Srinivasan *et al.* 2015; Pereira *et al.* 2016).

In order to confirm the polarization of cells at day 14 of culture, we used confocal microscopy to visualize the epithelial organization. Staining for actin revealed a strong microvilli signal on the apical side, while RAC1B staining localized mostly to the basolateral side of polarized Caco-2 cells (Figure 3.3B).

With the aim to understand whether the expression of RAC1B differed between non-polarized and polarized cells, WB analysis was performed with specific antibodies for RAC1B and RAC1. As we can perceive in Figure 3.4A, there were no major changes in these proteins. On the other hand, when comparing E-cadherin levels between the two models, a great increase in its expression was detected in polarized cells, as expected by increased formation of adherens junctions.

By confocal microscopy, we confirmed the localization of E-cadherin at the basolateral membrane of the polarized epithelium (Figure 3.4B).

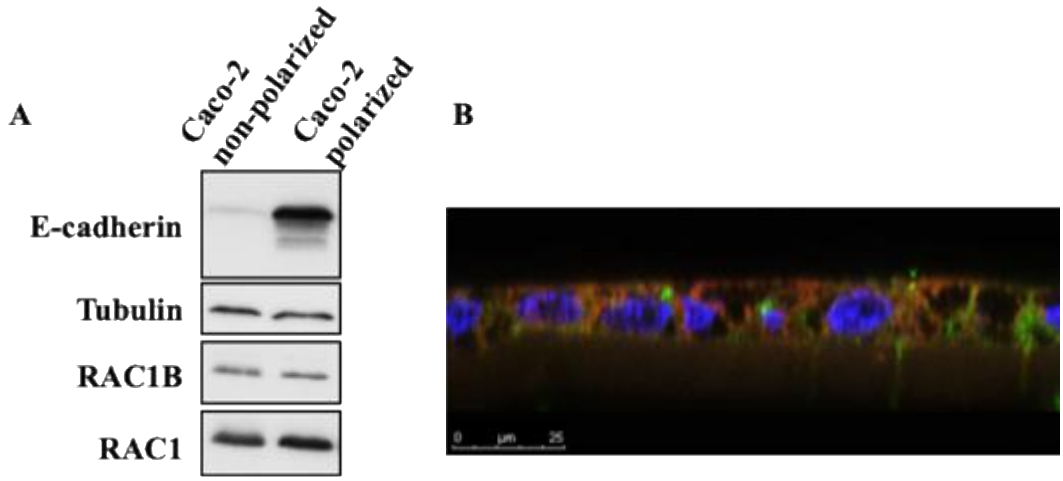

Figure 3.4 - **Comparison of RAC1B, RAC1 and E-cadherin expression in non-polarized and polarized Caco-2 cells. (A)** Proteins were analyzed by SDS-PAGE and WB and the indicated proteins detected in the whole-cell lysates. Tubulin is used as loading control. (**B**) Cell organization and E-cadherin localization were analyzed by confocal immunofluorescence microscopy at day 14 of culture of polarized cells. Shown is a representative XZ image by merging of three confocal immunofluorescence images, which detected cell nuclei in blue (DAPI), the localization of E-cadherin protein in green (monoclonal anti-E-cadherin antibody), followed by anti-mouse Alexa488, and the actin filament marker phalloidin in red.

An inflammatory TME has an important role in tumor promotion and Matos *et al.* (2013) described that colon inflammation can generate increased RAC1B expression in acute colitis mouse models and also in surgical samples from patients with inflammatory bowel diseases. These data suggested that stroma-derived signals can induce changes in the generation or stability of the alternative spliced RAC1B variant.

In order to test the hypothesis that microenvironmental stimuli could be a trigger for changes in the abundance of the splice variant RAC1B in tumor cells, filter inserts with polarized Caco-2 cell monolayers were placed into culture dishes pre-seeded with either Caco-2 (control), NIH3T3 fibroblasts or undifferentiated THP-1 monocytes, to understand whether these co-culture conditions would influence RAC1B abundance. Cell types are physically separated by the filter's porous membrane, which allows only free medium exchange, so that colorectal cells can unambiguously be analyzed for changes in RAC1B expression.

Following co-culture times of 0 h, 24 h, 48 h, 72 h, and 96 h, the TEER of the upper polarized Caco-2 layer was determined and then cells were lysed to compare total RAC1B protein levels by WB. As revealed in Figure 3.5A, co-culture with NIH3T3 fibroblasts led to an increase in RAC1B, after 24 h, which became statistically significant after 48 h, when compared to the control condition Caco-2 with Caco-2. On the other

hand, Caco-2 cells co-cultured with THP-1 monocytes exhibited a behavior similar to the control condition, allowing to conclude that undifferentiated monocytes do not promote any increase in the RAC1B variant.

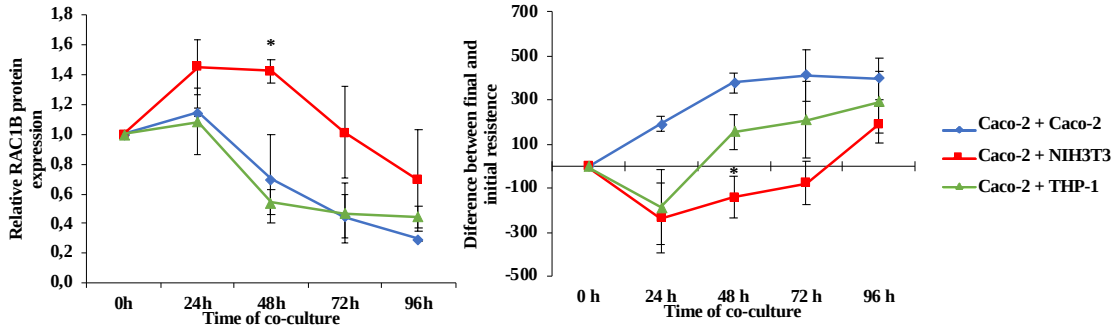

Figure 3.5 - **RAC1B levels and changes in polarization of Caco-2 cells under co-culture with stromal cells.** Filter inserts with fully polarized Caco-2 monolayers were placed into pre-seeded 24-well plates with Caco-2 (control), NIH3T3 fibroblasts, or THP-1 monocytes. The Caco-2 cells were exposed to different times of co-culture: 0 h, 24 h, 48 h, 72 h, and 96 h. (**A**) RAC1B protein levels in lysates from polarized Caco-2 cells were analyzed by SDS-PAGE and WB techniques after different co-cultures times. (**B**) Changes in the polarization state were assessed by the differences between final and initial TEER measurement of Caco-2 after different co-culture times. The data represent the mean ± SEM of 3 independent experiments. Statistical analysis was carried out with Welsh test. * significantly different from the corresponding control (Caco-2 + Caco-2) with $P < 0.05$.

When comparing the initial and final TEER for each type of co-culture and time, it became evident that the increase in RAC1B was associated with a decrease in TEER (Figure 3.5B).

To better understand the decrease in TEER observed in co-culture with fibroblasts, we analyzed these three co-culture conditions by confocal microscopy. As shown in Figure 3.6, the fibroblast co-culture led to a disorganization of the Caco-2 epithelial layer consistent with a loss in differentiation and polarization that manifested in the observed decrease in TEER (Figure 3.5B)

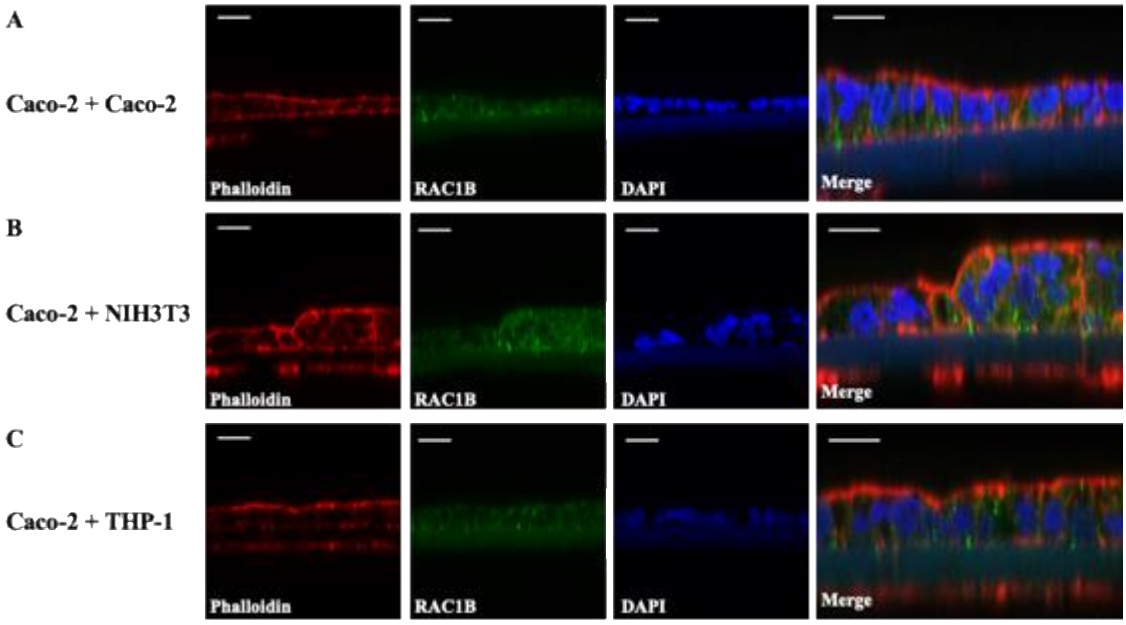

Figure 3.6 - **Morphology of polarized Caco-2 cell layers after 48 h of co-culture with stromal cells.** Cell polarization and RAC1B expression and localization were analyzed by confocal immunofluorescence microscopy. Merge is the colored overlay of three confocal immunofluorescence XZ images, which detected cell nuclei in blue (DAPI), the localization of endogenous RAC1B protein in green (polyclonal anti-RAC1B antibody), followed by anti-rabbit Alexa488, and actin (phalloidin) in red. Co-culture of Caco-2 with Caco-2 (control) (**A**), with NIH3T3 (**B**) or with THP-1 (**C**).

3.3. Differentiation of monocytes in macrophages

Macrophages are immune cells known for their phagocytic capacity and most tissues contain the mononuclear phagocytic system, a population of long-lived tissue-resident macrophages. In addition, monocytes from the bloodstream can differentiate into macrophages in response to inflammatory conditions (Ginhoux and Jung 2014; Prenen and Mazzone 2019). Different macrophage population have been recognized. The most broad classification divides macrophages in two functionally distinct subtypes: M1 macrophages secrete pro-inflammatory mediators and are activated *in vitro* by IFN-γ and LPS; M2 macrophages, on the other hand, secrete anti-inflammatory cytokines, are activated *in vitro* by IL-4 and are involved in processes like wound healing and tissue repair (Sica and Mantovani 2012; Italiani and Boraschi 2014). Notably, while the M1 and M2 designation has been traditionally used to classify macrophages, these phenotypes are nowadays considered part of a wider spectrum, revealing intrinsic plasticity and cross-regulation (Mantovani *et al.* 2004).

To clarify whether macrophages could influence RAC1B splicing in Caco-2 cells, we proceeded by differentiating human THP-1 monocytes into macrophages prior to their use in co-culture experiments similar to those described above. For this, we used a two-step process. First, an incubation with 50 ng/mL of PMA during 24 h, after which the cells became adherent (designated as M0 macrophages). Second, the M0 cells were placed for 24 h in fresh medium containing either 10 ng/mL LPS and 10 ng/mL INF-γ, or 10 ng/mL IL-4 to differentiate into M1 and M2 macrophages, respectively (as depicted in Figure 2.1 of Chapter 2).

As shown in Figure 3.7, M0 (Figure 3.7A), M1 (Figure 3.7B) and M2 (Figure 3.7C) macrophage cells are adherent, in contrast to monocytes, and differ from each other in morphology when analyzed by optical microscopy, since M0 has a round shape, M1 shows a more spread out shape and M2 has physical characteristics closer to M0.

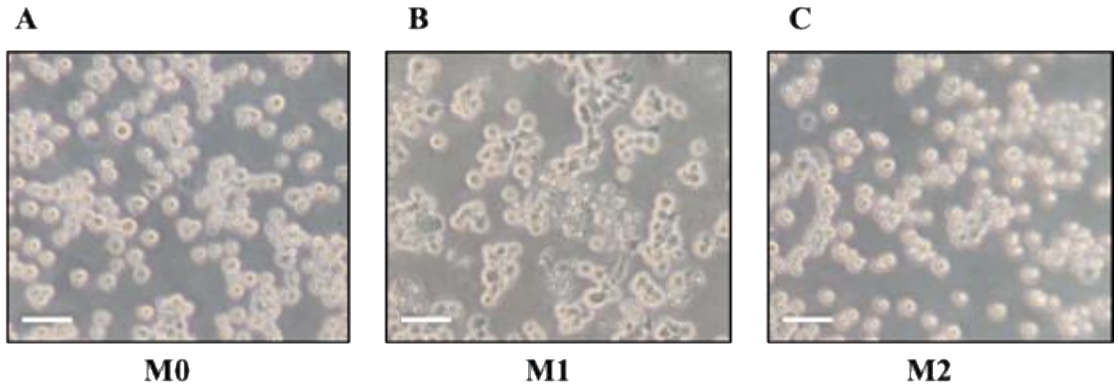

Figure 3.7 - **Differentiation of THP-1 cells induces morphological alterations.** (**A**) THP-1 cells after 24 h with 50 ng/mL of PMA, designated M0 cells. (**B**) M0 cells after 24 h with 10 ng/mL LPS and 10 ng/mL INF-γ, designated M1 cells. (**C**) M0 cells after 24 h with 10 ng/mL IL-4, designated M2 cells. Photographs were taken with a digital camera coupled to a bright-field microscope with a 40x magnification.

THP-1 differentiation into macrophages was confirmed, as previously described (Daigneault *et al.* 2010; Genin *et al.* 2015; Forrester *et al.* 2018), by amplification of specific marker gene expression using semi-quantitative RT-PCR, with GAPDH as an internal housekeeping gene control (Figure 3.8). Successful M1 subtype differentiation was validated by detecting the expression of several classical M1 markers: IL-1β, TNF-α, CXCL10 and CXCL16, which are pro-inflammatory cytokines, or of CCR7 expression, a membrane receptor. As expected, these markers were elevated in M1-differentiated macrophages but not in THP-1 monocytes or M2-like cells (Figure 3.8A).

Conversely, the expression of M2-specific markers: CD163, CCL17 and CD206, was clearly increased in macrophages differentiated into the M2-subtype, when compared with THP-1 monocytes or M1-differentiated cells (Figure 3.8B).

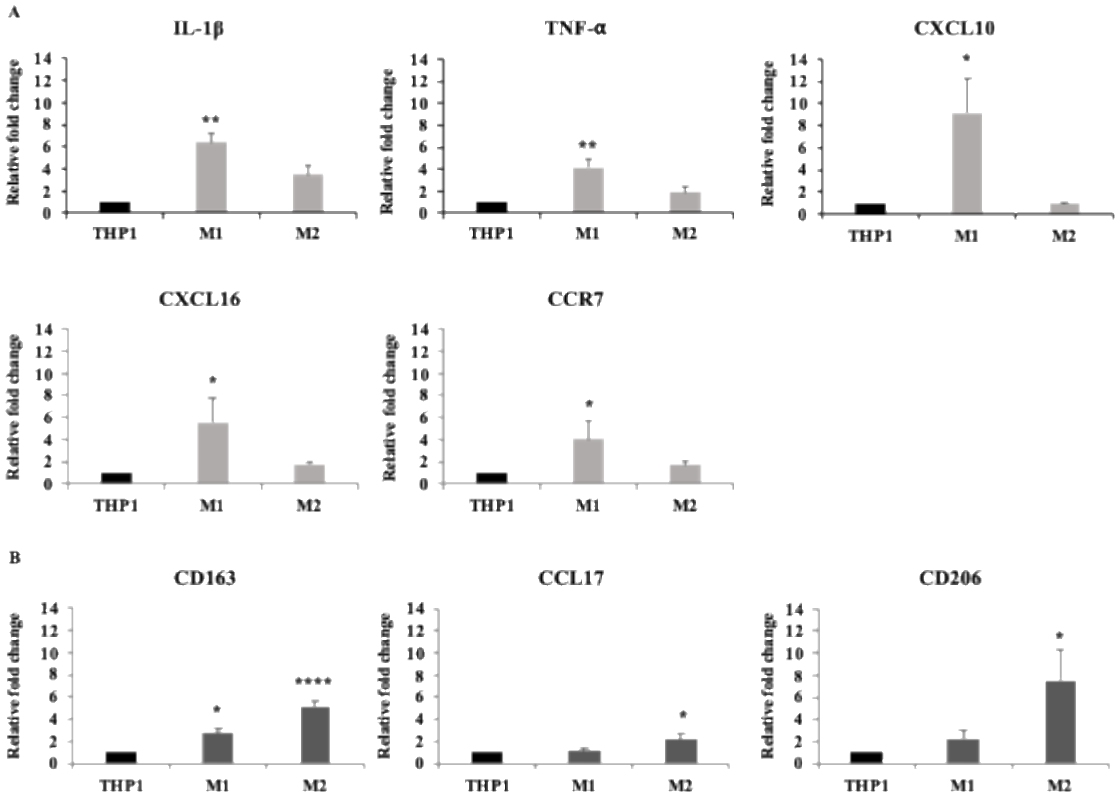

Figure 3.8 - **Expression of classical markers on THP-1 monocytes, M1 and M2 macrophage subsets.** Total RNA was isolated from monocytes and macrophages of each phenotype, reverse transcribed and amplified by PCR with GAPDH as an internal standard. **(A)** M1 markers: expression of IL-1β, TNF-α, CXCL10, CXCL16 and CCR7; **(B)** M2 markers: expression CD163, CCL17 and CD206. The data represent the mean ± SEM of 3 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. * significantly different from the corresponding control (THP-1), respectively, with $P < 0.05$, ** with $P < 0.01$ or **** $P < 0.0001$.

Following the successful *in vitro* differentiation of macrophages into two subtypes with distinct markers and characteristics, their effect on RAC1B expression in Caco-2 cells was studied in co-culture experiments.

3.4. Co-culture with CAFs and macrophages increases RAC1B expression

Our preliminary co-culture experiments showed that THP-1 monocytes did not influence RAC1B splicing but NIH3T3 fibroblasts did. To further investigate the contribution of stromal cells to RAC1B splicing, we repeated the co-culture experiments now using specialized and physiologically more relevant cell types, namely, immortalized CT5.3-hTERT colon cancer-associated fibroblasts (CAFs) (gift from O Wever, Ghent University), and the above described macrophages (M0, M1 and M2) differentiated from THP-1 monocytes.

As shown in Figure 3.9, an increase in RAC1B was observed after 48 h of co-culture of Caco-2 with both types of fibroblasts, CT5.3 and NIH3T3, as well as with M0 and M1 macrophages. On the other hand, co-culture with M2 macrophages did not translate into a RAC1B protein increase, similar to the co-culture with undifferentiated monocytes (THP-1). Moreover, when we increased the complexity of the co-cultures and combined fibroblasts and macrophages (triple co-cultures), the increase in RAC1B was more significant, especially in cultures of polarized Caco-2 with CT5.3 and M1 macrophages.

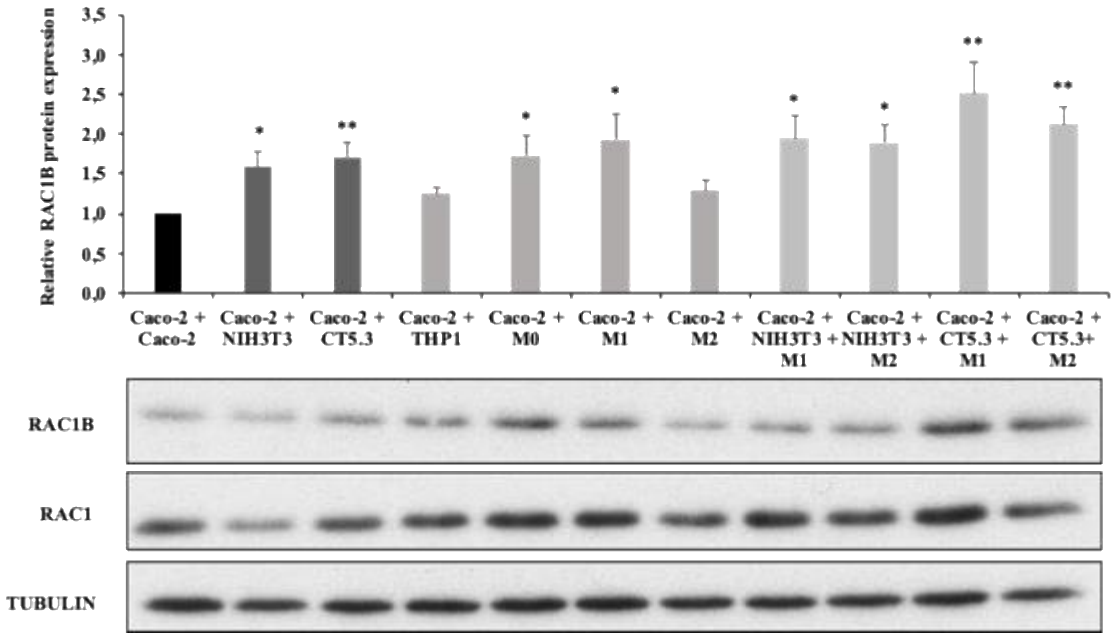

Figure 3.9 - **Effect of different co-cultures on RAC1B protein levels in Caco-2 cells.** Polarized Caco-2 cells were co-cultured with the indicated stromal cells and lysed after 48 h of culture. Proteins were analyzed by SDS-PAGE and WB, and the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of Caco-2 with Caco-2 and the tubulin protein served as a loading control. Corresponding quantification of RAC1B

levels in Caco-2 obtained from at least three independent biological replicate experiments. Band intensities were measured, then normalized to tubulin levels, followed by normalization to total RAC1 levels. Then the RAC1B/RAC1 ratio was calculated (as both are derived from the same pre-mRNA transcript). Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM, of 5 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. * significantly different from the corresponding control (Caco-2 + Caco-2), respectively, with $P < 0.05$ or ** with $P < 0.01$.

In short, the presence of CAFs and M1 macrophages most significantly increased RAC1B protein levels in Caco-2 cells.

In parallel, the levels of the two transcripts of the RAC1 gene were evaluated by qRT-PCR in RNA extracted from Caco-2 cells under the co-cultures conditions mentioned above. The results of the qRT-PCR, in Figure 3.10, showed that the variations in transcript expression levels were considerably more tenuous than at the protein level.

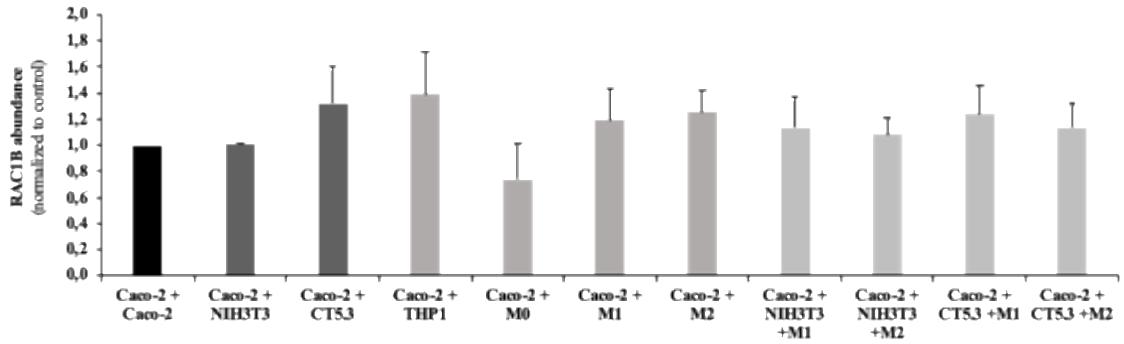

Figure 3.10 - **Effect of co-culture on RAC1B mRNA levels in Caco-2 cells.** Polarized Caco-2 cells were co-cultured with the indicated stromal cells and lysed after 48 h of culture. Total RNA was extracted, cDNA synthetized and RAC1 and RAC1B were amplified by qRT-PCR. Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM, of 4 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test.

These results may mean that small variations in the levels of AS that give rise to the RAC1B transcript result in more significant changes in the amount of the respective protein present in the cells.

On the other hand, the time point of 48 h of co-culture chosen to visualize changes in the amount of RAC1B protein, may not be appropriate to detect changes in RAC1B/RAC1 mRNA levels, which might occur before this time-point. To confirm this, we analyzed two key co-culture conditions: Caco-2 + Caco-2 (control) and Caco-2 + CT5.3 + M1, at 24 and 48 h, and compared the levels of the two transcripts in Caco-2 by RT-PCR. In Figure 3.11 we observe that the culture of CAFs with M1-like macrophages

induced an increase in the expression of mRNA levels of RAC1B as early as 24 h, while at 48 h there is already a reversion of the mRNA levels of this variant. In other words, the change in mRNA happens at 24 h and only translates into a change in protein expression at 48 h.

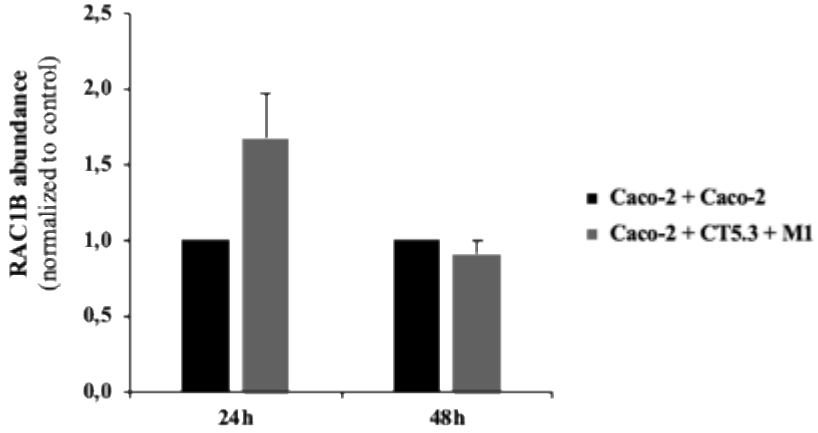

Figure 3.11 - **Effect of triple co-culture on RAC1B mRNA levels in Caco-2 cells.** Polarized Caco-2 cells were co-cultured with the indicated stromal cells and lysed after 24 h and 48 h of culture. Total RNA was extracted, cDNA synthetized and RAC1 and RAC1B were amplified by RT-PCR. Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM, of 5 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test.

3.5. Comparing co-culture conditions in a model of polarized T84 cells

As a second model of polarized colon cells, we used T84 cells, which also have the ability to form a layer of polarized cells when grown on filters and have been frequently used, similar to Caco-2, as a model for studying a tight and polarized enterocyte cell layer (Pereira *et al.* 2016). These cells are originally from a lung metastasis of a colon carcinoma, having a mutation in the *KRAS* and *PIK3CA* oncogenes, so with different genetic characteristics from Caco-2 cells (Donato *et al.* 2011; Pereira *et al.* 2016; Devriese *et al.* 2017).

In order to validate the T84 cell model, the cells were seeded on filter inserts for monolayer formation and cell polarization was monitored by measurement of TEER during 12 days.

As shown in Figure 3.12, the TEER of T84 cells was increasing over the days in culture, and between 6 and 12 days of culture the resistance reached the reference value

for T84 (400 to 600 $\Omega.cm^2$). Thus, the polarization of T84 cells is faster than that of Caco-2.

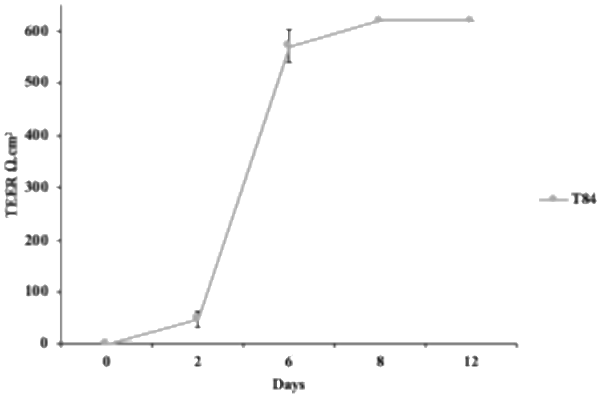

Figure 3.12 - **Polarization of T84 cells.** T84 cells were seeded on filter inserts for monolayer formation and cell polarization was monitored for 12 days by TEER measurement with a chopstick electrode STX2. Compilation of data from five independent experiments.

Using fully polarized T84 cells we compared the effect on RAC1B of the four most significant co-culture conditions identified with Caco-2 cells: T84 with T84 (control), T84 with CT5.3, T84 with CT5.3 plus M1, and T84 with CT5.3 plus M2. As shown in Figure 3.13, there is a slight increase in RAC1B protein in T84 cells in all three co-culture conditions after 48 h of co-culture, when compared to the control condition, reaching statistical significance in both triple co-cultures of stromal cells (T84 + CT5.3 + M1, or + M2).

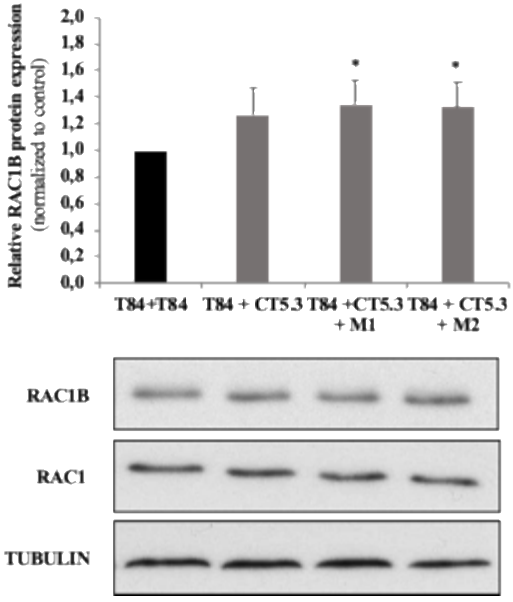

Figure 3.13 - **Effect of co-culture on RAC1B protein levels in T84 cells.** Polarized T84 cells were incubated with stromal cells and lysed after 48 h of culture. Proteins were analyzed by SDS-PAGE and WB and the indicated proteins

detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of T84 with T84 and the tubulin protein served as a loading control. Corresponding quantification of RAC1B levels in T84 obtained from at least three independent biological replicate experiments. Band intensities were measured, then normalized to tubulin levels, followed by normalization to total RAC1 levels. Then the RAC1B/RAC1 ratio was calculated (as both are derived from the same pre-mRNA transcript). Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM, of 5 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. * significantly different from the corresponding control (T84 + T84) with $P < 0.05$.

Unlike Caco-2 cells, and consistent with a less pronounced increase in RAC1B levels, polarized T84 cells maintained their TEER values unchanged, regardless of the type of co-culture to which they were subjected. This was also observable by confocal microscopy after immunofluorescent staining of epithelial markers. Figure 3.14 shows that although T84 monolayers are less organized than those established by Caco-2 cells, no loss in ZO-1 signal between the different co-culture conditions was apparent, and ZO-1 localized at the apical-basolateral interface, supporting epithelial cell polarity.

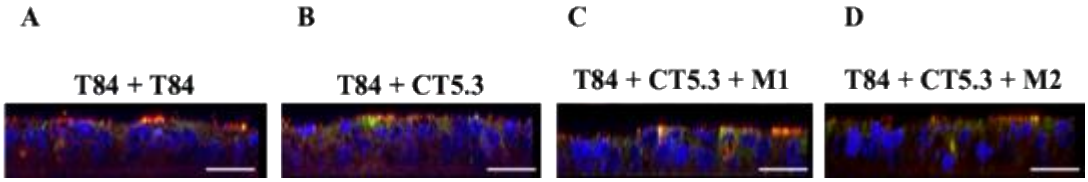

Figure 3.14 - **Polarization of T84 cells after co-culture with stromal cells.** Cell polarization was analyzed by confocal immunofluorescence microscopy, after 48 h of co-culture. Representative images resulted from merge of the colored overlay of three confocal immunofluorescence XZ images, which detected cell nuclei in blue (DAPI), actin in red (polyclonal anti-β-actin antibody) and tight junctions in green (polyclonal anti-ZO-1 antibody), followed by anti-mouse Alexa546 anti-rabbit Alexa488. **(A)** Co-culture of T84 + T84 (control), **(B)** T84 + CT5.3, **(C)** T84 + CT5.3 + M1 and **(D)** T84 + CT5.3 + M2.

Although Caco-2 and T84 cells have different origins and characteristics, the increase in RAC1B occurred in both cell lines under the same co-culture conditions.

3.6. Comparison with other non-polarized colorectal cell models

To confirm the importance of polarized cell models in the study of microenvironmental contribution of stromal cells, we decided to study other colorectal cell line models available in the laboratory, namely, DLD-1, NCM460 and HT29.

In this case, the cells were grown on filter inserts with the same methodology of co-cultures, however, DLD-1, NCM460 and HT29 do not have the capacity to polarize.

Thus, to complement the analysis, we also included non-polarized Caco-2 and T84 cell monolayers in the assay.

As shown in Figure 3.15, all three co-culture conditions increased the RAC1B protein in non-polarized Caco-2 and NCM460 cells, in particular, the co-culture with CT5.3 fibroblasts that reached statistical significance. DLD-1 cells responded only to the co-culture with CT5.3 fibroblasts, while HT29 and non-polarized T84 cells shown no significant variation in RAC1B expression in the three conditions.

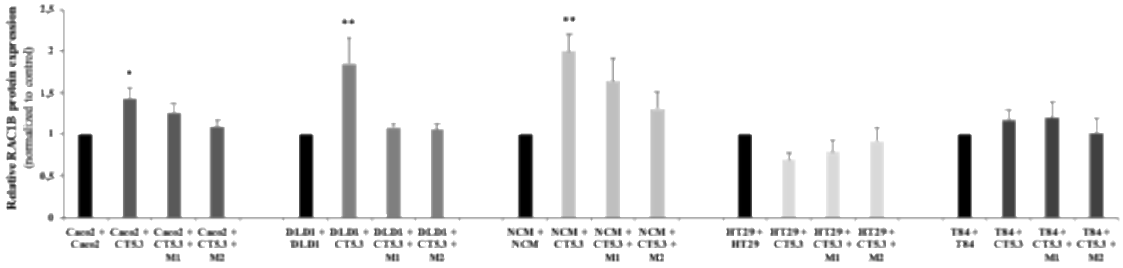

Figure 3.15 - **Effect of co-culture on RAC1B protein levels in non-polarized colorectal cells.** Colorectal cells were co-cultured with different stromal cells and lysed after 48 h of culture. Proteins were analyzed by SDS-PAGE and WB of the whole-cell lysates. The control of the assay corresponds to the co-culture of colorectal cells with each type of colorectal cells and the tubulin protein served as a loading control. Band intensities were measured, then normalized to tubulin levels, followed by normalization to total RAC1 levels. Then the RAC1B/RAC1 ratio was calculated (as both are derived from the same pre-mRNA transcript). Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM of 3 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. * significantly different from the corresponding control, respectively, with $P < 0.05$ or ** with $P < 0.01$.

Interestingly, the results with the non-polarized Caco-2 and T84 cells are, for the most part, different from those obtained when these cells were polarized. This indicates that the acquisition of the structural organization similar to that of an intact epithelium, changes the response of cancer cells to microenvironmental cues, highlighting the importance of using polarized cell models in these studies.

3.7. Implementation of culture conditions to form 3D Caco-2 spheroids

3D cultures are frequently used for studying the biological response of epithelial tissues, since they better represent the 3D organization and functional properties of the intestinal barrier (Shamir and Ewald 2014; Kawai *et al.* 2020).

Caco-2 cells have the ability to form spheroids when grown under the right conditions in the presence of Matrigel, a solubilized basement membrane preparation, rich in ECM proteins, secreted by mouse sarcoma cells. These conditions were tested in terms of the percentage of Matrigel and the time of spheroid formation.

Figure 3.16 shows the formation of Caco-2 spheroids with the development of an internal and central lumen, surrounded by polarized cells with an apical surface. Adherens junctions are basolateral and tight junctions are apical. The RAC1B staining showed preferential basolateral localization. In addition, in the internal lumen, there are cells in some spheroids that are probably in the apoptotic/necrotic process, although no marker has been used to confirm this state.

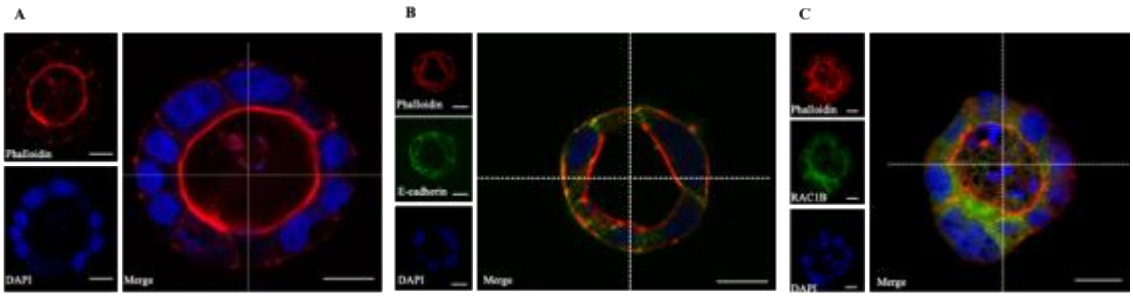

Figure 3.16 - **Caco-2 cells spheroid formation and morphological characterization.** Caco-2 cells were grown in Matrigel for 5 to 7 days**. (A-C)** Cells stained with phalloidin-TRITC (red) to detect actin and with DAPI to label the nuclei of cells (blue). **(B)** Cells additionally stained with mouse-anti-E-cadherin, followed by anti-mouse Alexa488, to detect cell junctions (green). **(C)** Cells additionally stained with rabbit-anti-RAC1B, followed by anti-rabbit Alexa488 (green). Merge is the colored overlay of the shown individual confocal immunofluorescence images.

The implementation of this type of cultures in the laboratory also allows to design co-culture experiments with stromal cells and will be a useful model for future studies, however, these Matrigel-based cultures were not further pursued because the transcript and protein contents of the different cell types cannot be analyzed separately.

3.8. Identification of secreted cytokines by co-cultured cells

In order to identify which cytokines were released by stromal cells and responsible for the RAC1B increase in Caco-2 cells, we pooled the conditioned basolateral media from co-cultures of Caco-2 with either CAFs CT5.3, M1 macrophages, or the triple co-culture between Caco-2, CT5.3 and macrophages M1. These media were incubated with human inflammation antibody arrays (AAH-INF-3-8; RayBiotech Inc.) consisting of 40

antibodies against human inflammatory factors, as cytokines/chemokines, spotted in duplicate onto a blotting membrane (Lin *et al.* 2003; Mahlknecht *et al.* 2012; Drolia *et al.* 2019).

The detected cytokines/chemokines spots using these arrays are shown in Figure 3.17.

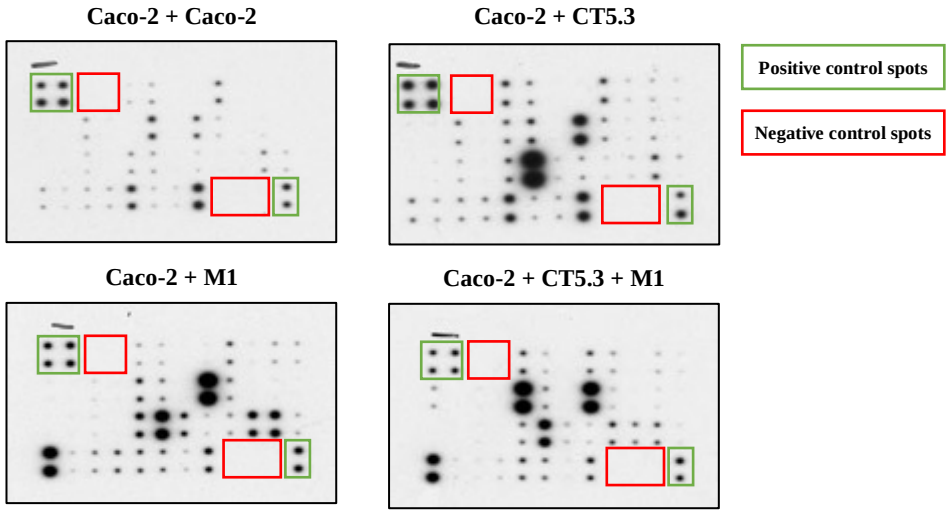

Figure 3.17 - **Identification of cytokines in the media recovered after co-culture conditions.** Representative picture of human cytokine antibody array (AAH-INF-3-8, Raybiotech Inc.) showing the reactivity of pooled basolateral media samples from co-cultures of polarized Caco-2 with the indicated stromal cells. Each antibody is present in duplicate. Membranes were digitalized, and images analyzed with ImageJ software (NIH). Green boxes: positive control spots (upper left corner and lower right corner). Red boxes: negative or blank control spots (upper left and lower right corner).

Subsequently, signal intensities of the array spots were analyzed on digital images using ImageJ (NIH). First, values were corrected against non-specific background and then equalized using as reference the positive spot intensities on each array, so that all arrays from one experiment can be compared. Results were displayed as a “heatmap” of arbitrary intensity units proportional to the abundance of each cytokine in the medium of each co-culture condition (Figure 3.18).

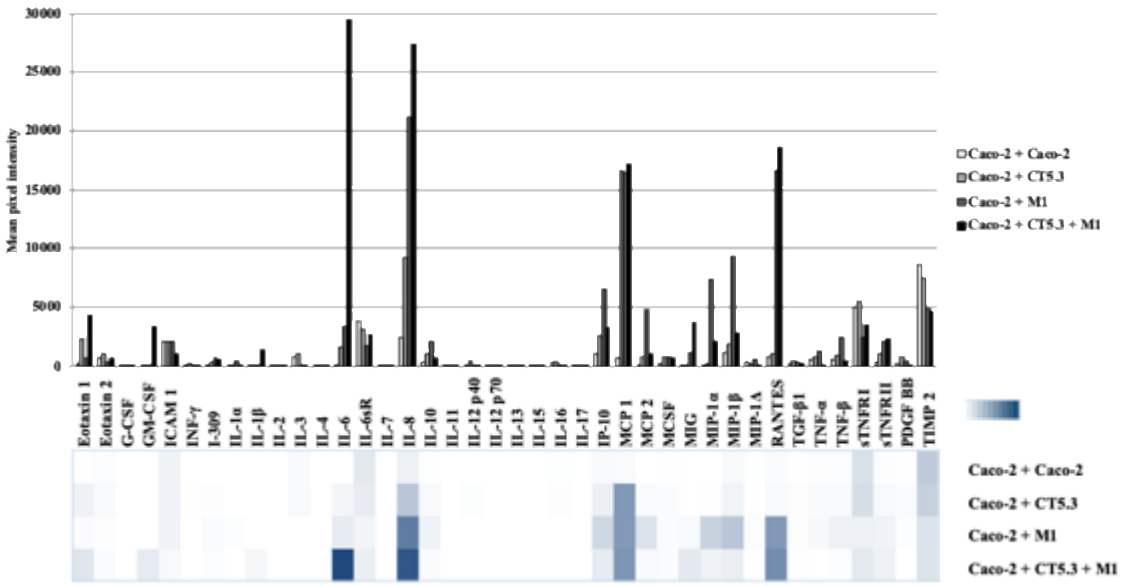

Figure 3.18 - **Differential cytokine levels in media from co-culture conditions.** The intensity of each spot, corresponding to each of the 40 cytokines in the array was analyzed with ImageJ (NIH), equalized and compared between the four Caco-2 co-cultures (Caco-2 + Caco-2 (control), Caco-2 + CT5.3, Caco-2 + M1 and Caco-2 + CT5.3 + M1). The determined values (arbitrary units) are shown as a graph and as a "heatmap", with signal intensity displayed in a scale from white (low) to dark blue (high) color.

Subsequently, the ratio between the signal intensity of each spot and that observed under control condition (Caco-2 + Caco-2) was determined. The results are summarized in Figure 3.19.

Analysis of Figure 3.19 shows cytokine variations that correlate with RAC1B expression among the various co-culture conditions and highlighted three candidates: GM-CSF, IL-1β and IL-6. We considered IL-8 as a forth candidate because, although its value in Caco-2 control was sufficiently high to lose significance after normalization, Figure 3.18 had revealed a pattern of increment with co-culture complexity similar to that of IL-6.

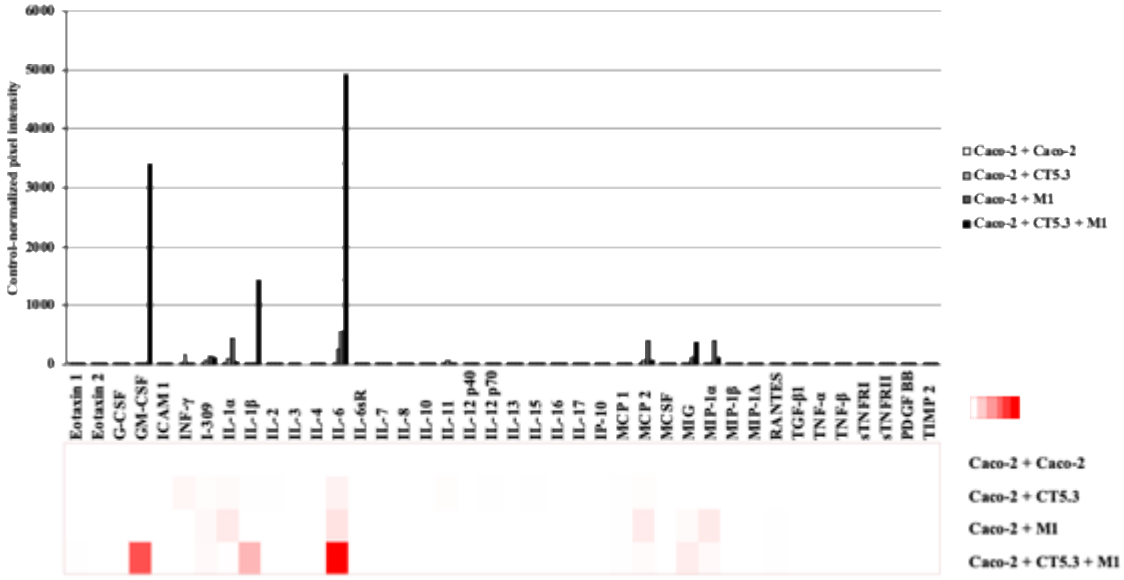

Figure 3.19 - **Relative difference in cytokine levels between control and co-culture conditions.** The values shown in Figure 3.18 for each cytokine in each condition (Caco-2 + CT5.3, Caco-2 + M1 and Caco-2 + CT5.3 + M1) were normalized to its corresponding value in control (Caco-2 + Caco-2) co-culture. The normalized values (fold change relative to control) are shown in graphical and "heatmap" forms, the latter with signal intensity displayed in a scale from white (low) to dark red (high) color.

On the other hand, and despite not being represented in the array, we decided to study the cytokine MIP-2α, which according to Kotelevets *et al* was increased and related to the accumulation of RAC1B in transgenic mouse models (Kotelevets *et al.* 2018). IL-11 was chosen as a control cytokine, since it was not detected in any of the co-culture conditions.

To validate the effect of each of the candidate cytokines, they were individually added as purified proteins at 1 ng/mL, 10 ng/mL or 100 ng/mL to the basolateral medium of polarized Caco-2 cells grown on filters during 10 days. RAC1B protein levels were evaluated by WB after 48 h of treatment (Figure 3.20). These experiments confirmed that IL-1β (Figure 3.20A), IL-6 (Figure 3.20B), IL-8 (Fig. 3.20D) and MIP-2α (Fig. 3.20E) increased the expression of RAC1B protein in a dose-dependent manner. Interestingly, GM-CSF, as well as the IL-11 control, caused no significant effect at any concentration used (Figures 3.20C and F, respectively).

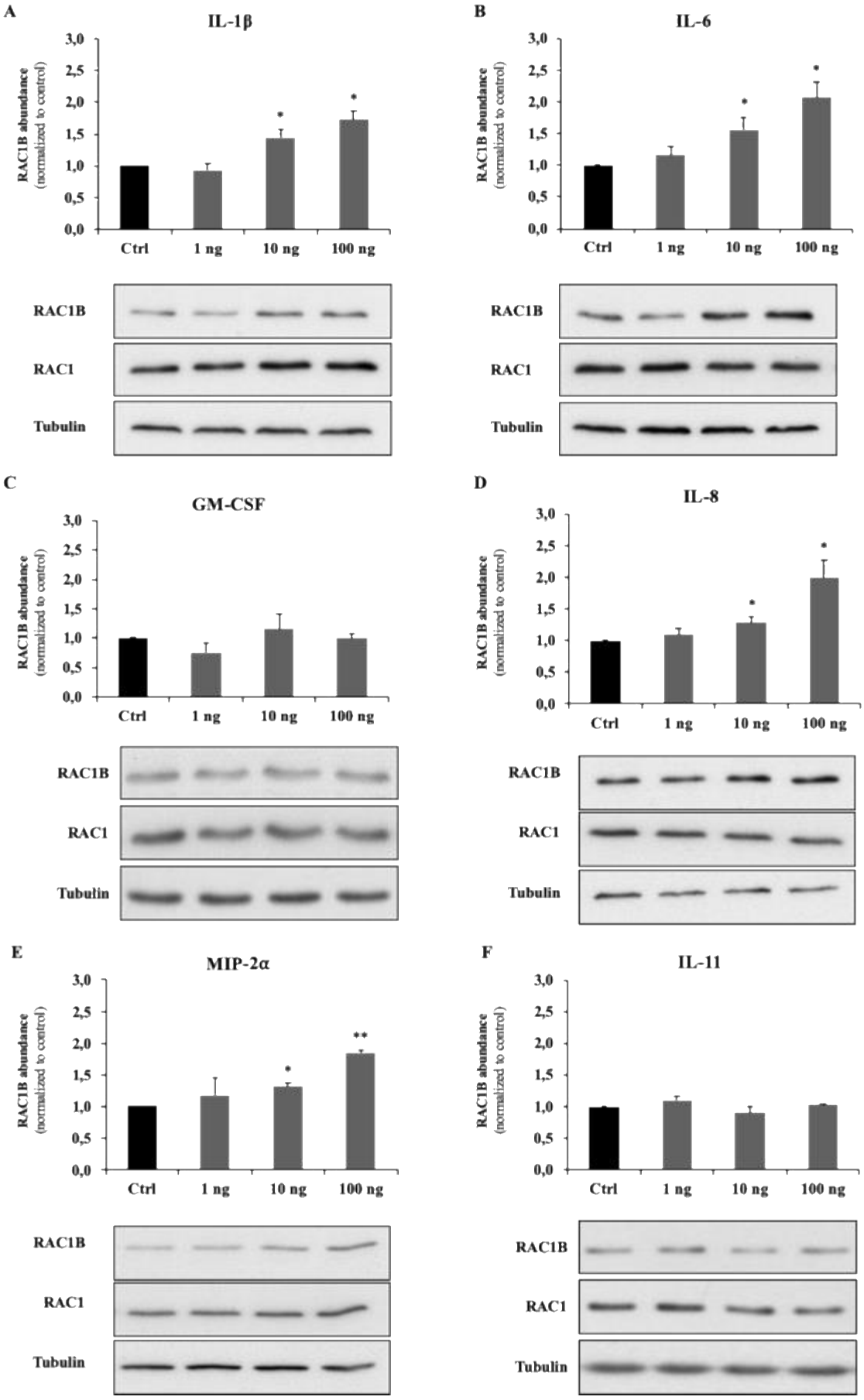
A
IL-1β
RAC1B abundance (normalized to control)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Ctrl
1 ng
10 ng
100 ng
RAC1B
RAC1
Tubulin
B
IL-6
C
GM-CSF
D
IL-8
E
MIP-2α
F
IL-11

Figure 3.20 - **Effect of purified cytokines on RAC1B protein levels in Caco-2 cells.** Polarized Caco-2 cells were grown for 10 days, then incubated with recombinant cytokines **(A)** IL-1β, **(B)** IL-6, **(C)** GM-CSF, **(D)** IL-8, **(E)** MIP-2α or **(F)** IL-11. After 48 h of culture, cells were lysed and proteins analyzed by SDS-PAGE and WB with the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the addition of the cytokines' solvent (PBS + 0.01% BSA). Detection of the tubulin protein served as a loading control. Data are shown as fold change of RAC1B/RAC1 ratio (as both are derived from the same pre-mRNA transcript) relative to control and represent means ± SEM of 3 independent experiments. Statistical analysis was carried out with a Welsh test. * significantly different from the corresponding control with $P < 0.05$ or ** with $P < 0.01$.

In order to understand if a combination of cytokines could have an additive or synergistic effect on the RAC1B protein levels in Caco-2, we used several combinations of cytokines, each at 10 ng/mL, the intermediate effective concentration observed in the above dose-response experiments. As shown in Figure 3.21, combining two different cytokines, as well as the combination of the three cytokines IL-1β, IL-6 and IL-8, did not seem to influence the expression of RAC1B, at least after 48 h of culture.

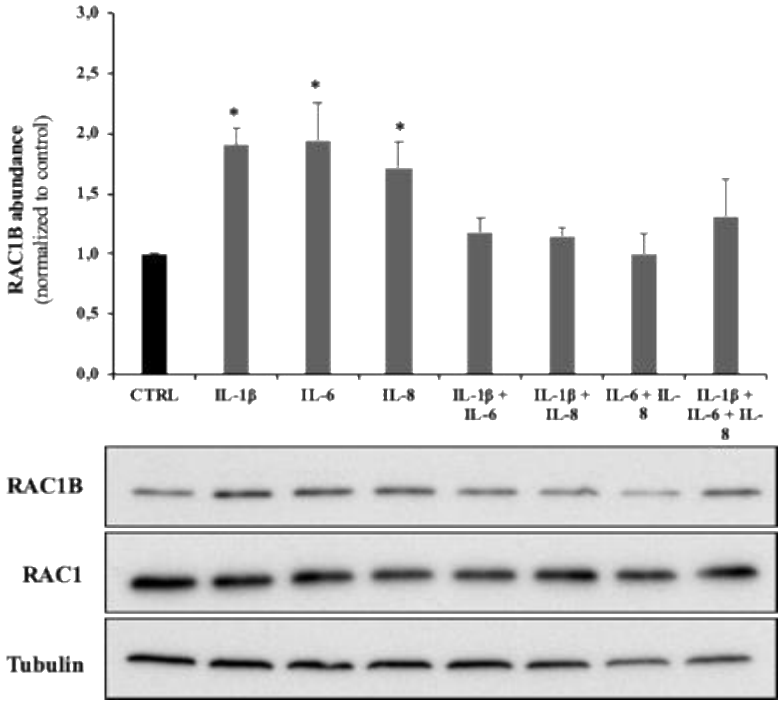

Figure 3.21 - **Effect of cytokine combinations on RAC1B protein levels in Caco-2 cells.** Polarized Caco-2 cells were incubated with combinations of purified cytokines, each at 10 ng/mL. After 48 h of culture, cells were lysed and proteins analyzed by SDS-PAGE and WB, with the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the addition of the cytokines' solvent (PBS + 0.01% BSA). Detection of the tubulin protein served as a loading control. Data are shown as fold change of RAC1B/RAC1 ratio (as both are derived from the same pre-mRNA transcript) relative to control and represent means ± SEM of 3 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. * significantly different from the corresponding control with $P < 0.05$.

As a further validation step for the identified cytokines, neutralizing antibodies were used to block their stimulating effect on RAC1B expression. For this, a neutralizing anti-human IL-6 antibody was added to either the basolateral medium of polarized Caco-2 containing recombinant IL-6, or to the basolateral medium of co-culture with CAFs and M1 macrophages. We detected that the increase in RAC1B protein levels in Caco-2 cells, that was observed both in triple co-culture and using purified IL-6, was completely blocked by the addition of 500 or 1000 ng/mL of the anti-human IL-6 antibody (Figure 3.22).

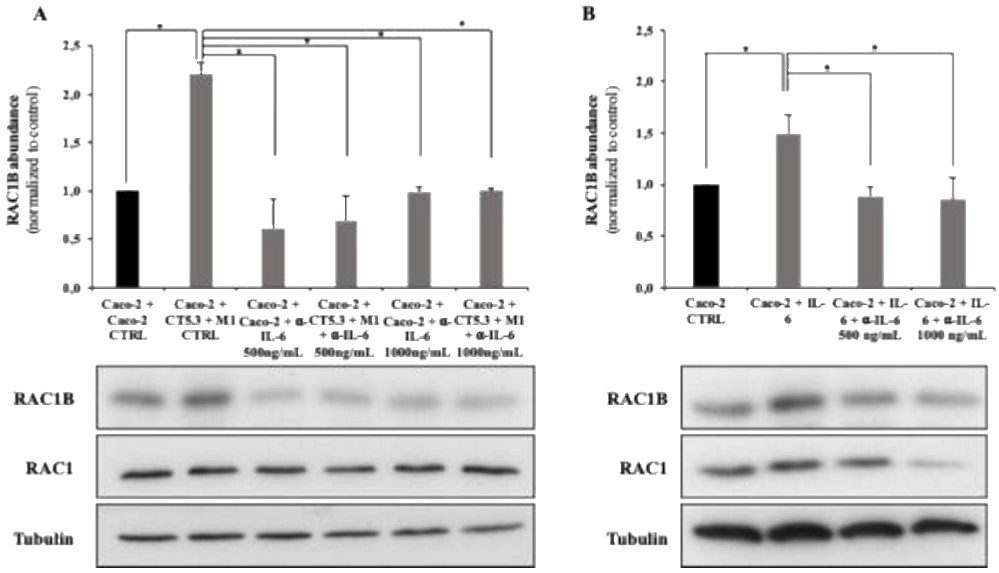

Figure 3.22 - **Effect of IL-6 neutralization on RAC1B protein in Caco-2 cells. (A)** Polarized Caco-2 cells were co-cultured with the indicated control or CT5.3 and M1 stromal cells, and the indicated concentration of anti-human-IL-6 antibody. Cells were lysed after 48 h of culture, proteins analyzed by SDS-PAGE and WB with the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of Caco-2 with Caco-2 with antibody solvent (PBS) and the tubulin protein served as a loading control. **(B)** Polarized Caco-2 cells were incubated with purified cytokine IL-6, at 10 ng/mL, and with the indicated concentration of anti-human-IL-6 antibody and lysed after 48 h of culture. Proteins were analyzed by SDS-PAGE and WB, and the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the addition of the cytokine solvent (PBS + 0.01% BSA) and antibody solvent (PBS). Detection of the tubulin protein served as a loading control. Data are shown as fold change of RAC1B/RAC1 ratio (as both are derived from the same pre-mRNA transcript) relative to control and represent means ± SEM, of 3 independent experiments. Statistical analysis was carried out with a Welsh test. * significantly different from the corresponding control with $P < 0.05$.

Although the corresponding neutralization experiments for the other cytokines could not yet be concluded, the results show clearly the causal relationship between the array-identified cytokine IL-6 and the increase in RAC1B.

3.9. Co-culture-mediated signaling events involved in the expression of RAC1B in Caco-2 cells

In order to identify which signaling events could be mediating the variation in RAC1B levels induced in the Caco-2 cells by the different co-culture conditions, changes in the phosphorylation profile of some key protein kinases were monitored with a phospho-kinase antibody array.

The proteome profiler human phospho-kinase antibody array (ARY003B; R&D Systems, Bio-techne) detects 45 proteins, namely the phosphorylation of 43 human kinases and total amount of 2 other signaling proteins simultaneously, and specific antibodies are spotted in duplicate on nitrocellulose membranes (Neelakantan *et al.* 2019; Ferraro *et al.* 2019).

We used the total Caco-2 cell lysates of control (co-culture Caco-2 + Caco-2) *versus* TME condition (co-culture Caco-2 + CT5.3 + M1). Cells were lysed both after 24 h of co-culture (to identify early protein phosphorylation events that regulate the splicing event) and after 48 h of co-culture (to distinguish signaling events that may be caused by the increase in the RAC1B protein, or that depend on extended co-culture time).

Polarized Caco-2 cell lysates were incubated with the phospho-kinase antibody array membranes and the resulting spot intensities are shown in Figure 3.23A for 24 h of co-culture and in Figure 3.24A for 48 h of co-culture. Subsequently, pixel intensities of each spot were analyzed on digital images of the different arrays using ImageJ (NIH). The arbitrary values were corrected for non-specific background noise and normalized to the intensity of positive spots present on each array. Results were displayed as “heatmaps” comparing two proteome profile events: Caco-2 + Caco-2 (control) and co-culture Caco-2 + CT5.3 + M1, each at 24 h (Figure 3.23B) and 48 h (Figure 3.24B).

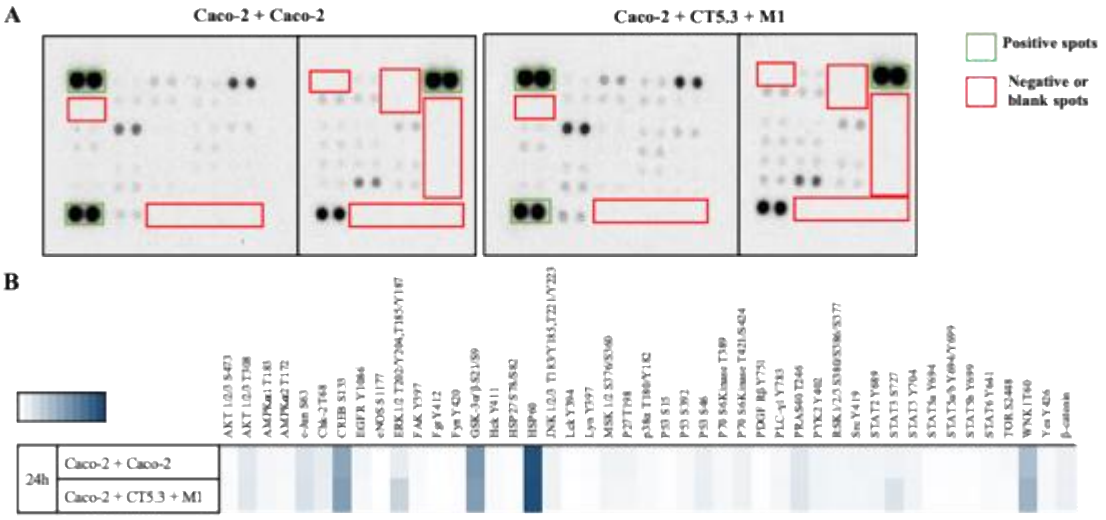

Figure 3.23 - **Protein phosphorylation differences in Caco-2 cells following 24 h of co-culture conditions**. **(A)** Representative image of the proteome profiler human phospho-kinase antibody array (ARY003B; R&D Systems, Biotechne), showing the reactivity of pooled Caco-2 lysate samples from Caco-2 + Caco-2 (control) and Caco-2 + CT5.3 + M1 (TME co-culture condition) co-cultures, at 24 h. Each antibody was present in duplicate. Images were digitalized and analyzed with ImageJ software (NIH). Green boxes: positive control spots (upper left corner, upper right corner and lower right corner). Red boxes: negative or blank control spots. **(B)** Signal intensity of the spots corresponding to the each of the 45 proteins after analysis by ImageJ (NIH), normalization to positive control signals, and comparison between the indicated co-cultures conditions. The relative signal intensities are shown as color "heatmap" with signal intensity represented in a scale of white (low) to dark blue (high) color.

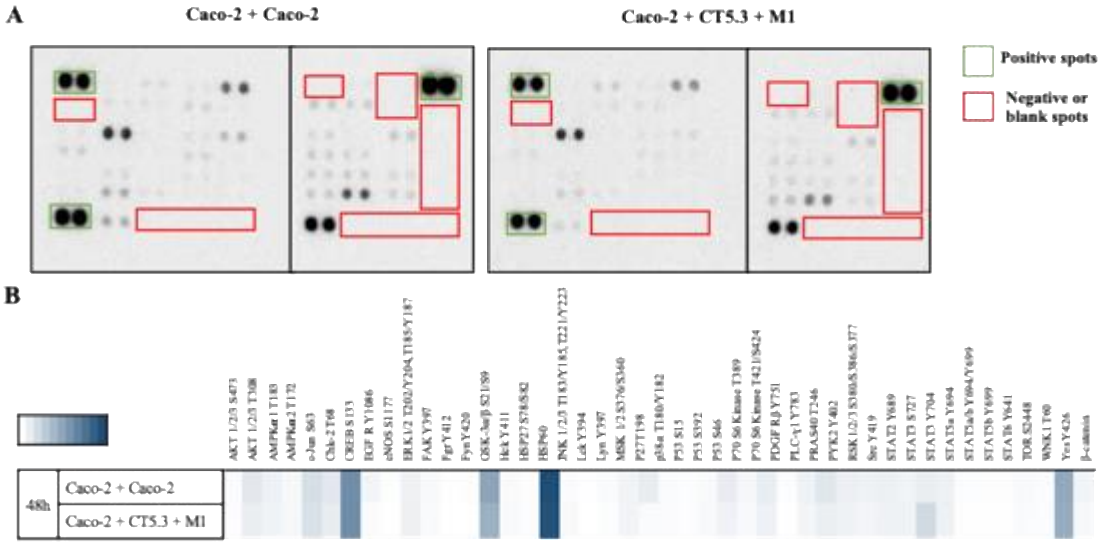

Figure 3.24 **Protein phosphorylation differences in Caco-2 cells following 48 h of co-culture conditions. (A)** Representative image of the proteome profiler human phospho-kinase antibody array (ARY003B; R&D Systems, Biotechne), showing the reactivity of pooled Caco-2 lysate samples from Caco-2 + Caco-2 (control) and Caco-2 + CT5.3 + M1 (TME co-culture condition) co-cultures, at 48 h. Each antibody was present in duplicate. Images were digitalized and analyzed with ImageJ software (NIH). Green boxes: positive control spots (upper left corner, upper right corner and lower right corner). Red boxes: negative or blank control spots. **(B)** Signal intensity of the spots corresponding to the each of the 45 proteins after analysis by ImageJ (NIH), normalization to positive control signals, and comparison

between the indicated co-cultures conditions. The relative signal intensities are shown as color "heatmap" with signal intensity represented in a scale of white (low) to dark blue (high) color.

Subsequently, the ratio between the signals from each spot of Caco-2 + CT5.3 + M1 and that measured under control condition (Caco-2 + Caco-2) was calculated, for each co-culture period, to identify which kinases were altered. Results are summarized in Figure 3.25, which allows visualizing the phosphorylation events that are altered in Caco-2 + CT5.3 + M1 co-cultures, both after 24 h (Figure 3.25A) and 48 h of co-culture (Figure 3.25B).

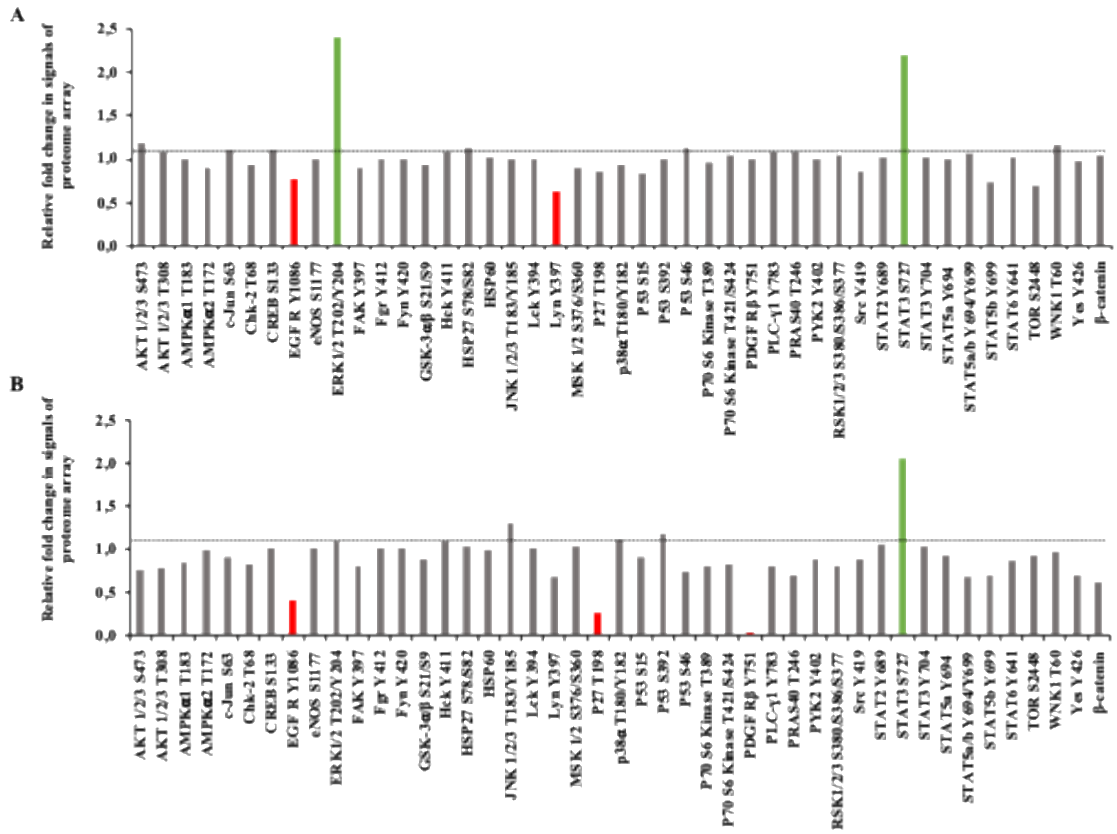

Figure 3.25 - **Differential protein phosphorylation or expression levels in Caco-2 cells following their co-culture with CT5.3 and M1 stromal cells**. Plotted are the fold change ratios between Caco-2 + CT5.3 + M1 and Caco-2 + Caco-2 cultures after, respectively, **(A)** 24 h and **(B)** 48 h of co-culture. The bars in green correspond to increased and red bars to reduced phosphorylation levels between co-culture and control conditions.

According to the above analysis, after 24 h of co-culture, we observed an increase in the phosphorylation events of ERK1/2 T202/Y204,T185/Y187 and STAT3 S727, while that of EGFR Y1086 and Lyn Y397 decreased.

On the other hand, after 48 h of co-culture, we mostly observed a decrease in kinase phosphorylations, with the exception of a sustained STAT3 S727 phosphorylation

increment. In addition to these, we identified a decrease in the phosphorylation of EGFR Y1086, beyond that verified at 24 h, of p27 T198 and PDGF-Rβ Y751.

3.10. Validation of candidate phosphorylation events in Caco-2 polarized cells

To proceed with the validation of putative signaling differences indicated by array analysis in response to the co-culture conditions, we prepared a pool of Caco-2 whole-cell lysates after co-cultures and used specific antibodies to detect the candidate proteins by WB (Figure 3.26).

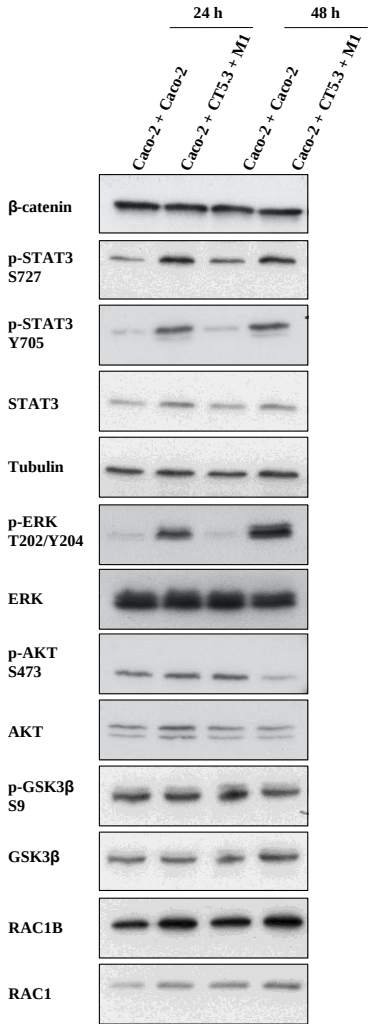

Figure 3.26 - **Validation of selected kinases activated in Caco-2 cells by co-culture.** Polarized Caco-2 cells were incubated with the indicated co-culture condition Caco-2 + Caco-2 or Caco-2 + CT5.3 + M1. After 24 h or 48 h of culture, cells were lysed, and proteins analyzed by SDS-PAGE and WB, with the indicated phosphorylated (p-) and total proteins detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of Caco-2 with Caco-2 at each time-point and the tubulin protein served as a loading control.

In addition to specific anti-phospho antibodies, we also detected total amounts of proteins and other proteins such as β-catenin, tubulin (for loading control), RAC1 and RAC1B. In agreement with the data presented in this thesis, the amount of RAC1 did not change under any condition or over time, whereas RAC1B increased in the condition Caco-2 + CT5.3 + M1 in relation to the control. According to the results of the array, the amount of β-catenin would decrease slightly after 48 h; however, using the specific antibody, it was not possible to verify this decrease.

Furthermore, we tested the phosphorylation of ERK with a phospho-specific antibody. While the total ERK protein levels did not fluctuate in the studied conditions, the phosphorylation of ERK1/2 T202/Y204, T185/Y187 increased significantly at 24 h and 48 h of triple co-culture. For 24 h the results are in agreement with the array data; however, for 48 h the results do not fully comply.

Concerning the phosphorylation of STAT3 at S727, we observed an increase in this phosphorylation in triple co-culture condition, both after 24 h and 48 h, confirming the array result. With regard to the total amount of STAT3, a slight increase was observed in the co-culture of Caco-2 with CT5.3 and M1, although to a lesser extent than for the specific phosphorylation. Curiously, the use of antibody specific for the phosphorylation of STAT3 at Y705 also demonstrated an increase in Caco-2 in triple co-culture, both at 24 h and 48 h, which is not in agreement with the results of the array, where no changes in this phosphorylation site were detected.

In addition, we also validated the AKT pathway with the use of specific antibodies for S473 AKT phosphorylation and S9 of GSK3β. According to the array, there are no significant changes in the amount of phosphorylation of AKT, however, in the WB there is a slight decrease in phosphorylation in the phosphorylation in TME co-culture condition, at 48 h, whereas the total AKT protein is not affected. Regarding the AKT-mediated inhibitory phosphorylation of GSK3β no alteration was observed in the validation WB, which corresponds to the results obtained with the array.

From this analysis, phosphorylation of ERK is one of the key differences observed in Caco-2 cells in co-culture with stromal cells. In order to determine whether this event is causally related to the observed increase in RAC1B, the co-culture experiment was repeated in the presence of a specific MEK1/2 kinase inhibitor, called AZD6244 or Selumetinib, which blocks the observed ERK phosphorylation As shown in Figure 3.27, a dramatic decrease in the phosphorylation of ERK1/2 T202/Y204 was observed after 24

h of triple co-culture, accompanied by a significant decrease in RAC1B protein levels, to values close to the control.

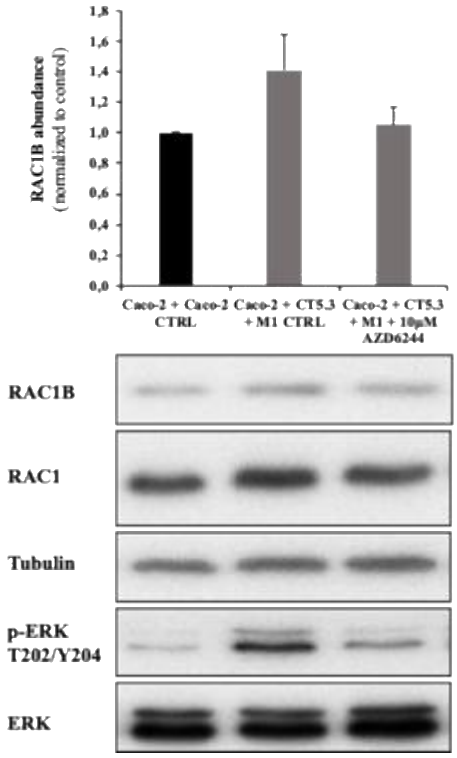

Figure 3.27 - **Effect of Selumetinib on RAC1B protein levels in Caco-2 cells.** Polarized Caco-2 cells were co-cultured with the indicated control or CT5.3 and M1 stromal cells, and with or without 10 μM of Selumetinib. Cells were lysed after 24 h of culture, proteins analyzed by SDS-PAGE and WB, and the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of Caco-2 with Caco-2 with addition of drug solvent (DMSO) and the tubulin protein served as a loading control. Data are shown as fold change of RAC1B/RAC1 ratio (as both are derived from the same pre-mRNA transcript) relative to control and represent means ± SEM 4 independent experiments. Statistical analysis was carried out with Welsh test.

Besides the above validation of the ERK pathway under the described co-culture conditions, we were able to validate the phosphorylation of STAT3 at Y705, using the neutralizing anti-IL-6 antibody. In addition to reducing the RAC1B protein levels previously shown in the Figure 3.22, the neutralizing antibody also reduced STAT3 Y705 phosphorylation in the Caco-2 cells, which demonstrates that this IL-6-induced pathway closely correlates with the observed changes in alternative spliced RAC1B protein levels, as can be observed in Figure 3.28.

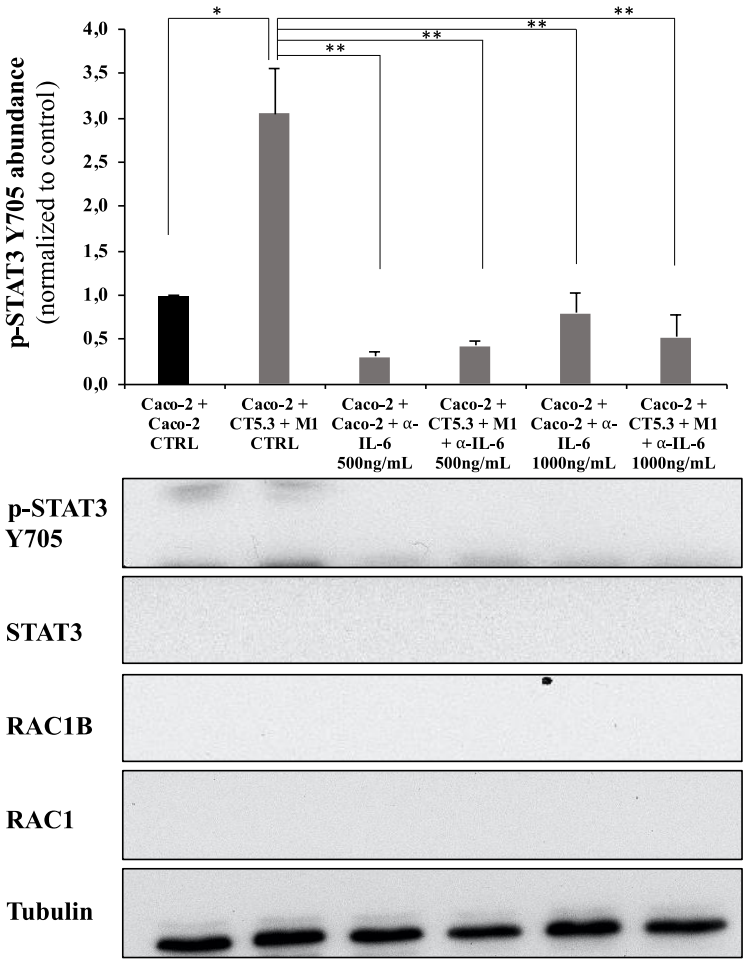

Figure 3.28 - **Effect of neutralizing IL-6 on STAT3 Y705 phosphorylation in Caco-2 cells.** Polarized Caco-2 cells were co-cultured with stromal cells and with the indicated concentration of anti-human-IL-6 antibody and lysed after 48 h of culture. Proteins were analyzed by SDS-PAGE and WB, and the indicated proteins detected in the whole-cell lysates. The control of the assay corresponds to the co-culture of Caco-2 with Caco-2 with antibody solvent (PBS) and the tubulin protein served as a loading control. Data are shown as fold change of RAC1B/RAC1 ratio (as both are derived from the same pre-mRNA transcript) relative to control and represent means ± SEM of 3 independent experiments. Statistical analysis was carried out with Welsh test. * or ** significantly different from the corresponding control, respectively, with $P < 0.05$ or $P < 0.01$.

Together, these data indicate that the pro-inflammatory co-culture conditions stimulate two specific signaling pathways in polarized Caco-2 cells, ERK and STAT3, and that these changes are related to increased expression of RAC1B.

3.11. RAC1B levels in the colon mucosa of IBD patients

IBD is a group of autoimmune diseases that are characterized by inflammation of both the small and large intestine, in which elements of the digestive system are attacked

by the body's own immune system (Fakhoury *et al.* 2014). This inflammatory condition encompasses two major forms, known as Crohn's disease and ulcerative colitis with increasing prevalence in the Portuguese population (Azevedo *et al.* 2010). Disease etiology involves a sequence of events in which changes in TJ permeability and actin cytoskeleton organization lead to a disturbed barrier function of the intestinal epithelial cell layer and consequently allow access of bacteria into the mucosa, generating sustained inflammation and tissue damage. With the use of Caco-2 polarized cells as an intestinal barrier model, we observed that fibroblasts-stimulated increase in RAC1B showed signs of epithelial barrier dedifferentiation (Figure 3.6). Besides that, previous work of our laboratory described that colon inflammation can generate increased RAC1B expression in acute colitis mouse models and also in surgical samples from patients with IBD (Matos *et al.* 2013).

So, in order to evaluate in clinical samples from patients with IBD, whether disease correlated with increased expression of RAC1B, we analyzed the levels of the two RAC1 transcripts in 32 FFPE samples, using qRT-PCR technique.

The samples were categorized on a scale of 0 to 5, according to the stage of development and characteristics of the disease. We had 6 control samples, that correspond to 19%, 7 samples of stage 2 that make up 22%, 9 samples of stage 4 (28%) and 4 samples of stage 5, that correspond to 12%. 19% of the samples were not categorized, due to the fact that they are poorly characterized or badly identified (Figure 3.29). Samples, clinical data and respective stages are summarized in Table 3.1.

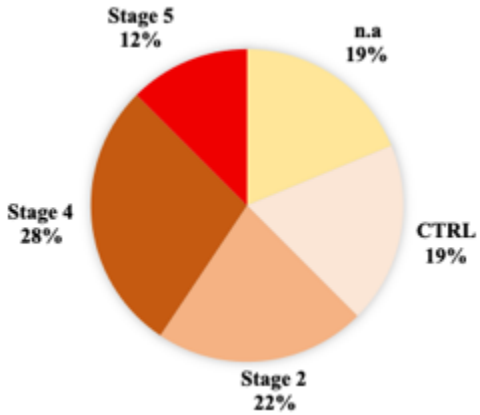

Figure 3.29 - **Distribution of FFPE samples of IBD patients.** Samples were categorized on a scale of 0 (without disease - control) to 5 (severe IBD) and n.a. correspond to samples without possibility of categorization.

Table 3.1 - **Clinical data of FFPE samples and stage of the diseases**. Samples were categorized on a scale of 0 (without disease - control) to 5 (severe IBD), taking into account the clinical aspects of the samples. n.a. corresponds to samples without possibility of categorization.

Sample	Clinical Data	Stage (0 to 5)
H17/5567 B1	Fragment of colon without appreciable changes	0
H17/6303	Fragment of large intestine mucosa without appreciable changes	0
H17/880	Normal colon mucosa	0
H17/4633	Colorectal mucosa without appreciable changes	0
H17/7148	Colorectal mucosa without appreciable changes	0
H17/1099	Granulation tissue from ulcerated lesion	n.a.
H17/1104	Fragments of colon mucosa, with slight inflammatory infiltrate.	2
H17/6075	Colorectal mucosa, which presents edema and inflammatory infiltrate, with epithelial erosion – IBD: ulcerative colitis	4
H17/27	Colon mucosa with slight distortion of the architecture and inflammatory infiltrate with superficial erosion. Clinical diagnosis of ulcerative colitis	4
H17/1501	Ulcerative colitis	5
E17/1822	Colon mucosa with slight distortion of the architecture and inflammatory infiltrate	4
E17/2084	Colorectal mucosa with inflammatory lesions, with ulceration and formation of cryptic micro-abscesses	4
H17/196	Chronic ileo-colitis lesions with ulceration - compatible with inflammatory disease, likely Crohn's disease	4
H17/197	Chronic ileo-colitis lesions with ulceration - compatible with inflammatory disease, likely Crohn's disease	4
H17/3226	Rectal mucosa with inflammatory bowel disease	2
H16/30481	Mucosa with deformation of villous architecture and marked inflammatory process with erosion / ulceration	5
H17/4394	Fragment of large intestine mucosa without appreciable changes	0
H17/6399	Fragment of colon mucosa with abundant inflammatory infiltrate	2
H17/6400	Fragment of colon mucosa with abundant inflammatory infiltrate	2
H17/1633	Without clinical data	n.a.
H98/5236	Ileal mucosa with trans-mural inflammatory infiltrate (particularly exuberant in the sub-mucosa and sub-serosa)	2
H99/10152	Colon sample, with predominantly lymphoplasmacytic inflammatory infiltrate, with extensive areas of ulceration and fistulation	4
H17/8226	Without clinical data	n.a.
H97/2655	Without clinical data	n.a.
H89/4601	Mucosa with transmural mononucleated inflammatory infiltrate - morphological aspects that in the appropriate clinical and laboratory context are compatible with Crohn's disease	5
H00/6249	Mucosa with chronic transmural inflammatory lesions and granulation tissue containing multinucleated giant foreign body cells	2
H04/5324	Mucosa with mononuclear inflammatory infiltrate	2
H03/12465	Mucosa with transmural inflammatory infiltrate, constituting lymphoid aggregates	4
H17/5236	Without clinical data	n.a.
H96/2032	Without clinical data	n.a.
H01/9697	Mucosa with polymorphic inflammatory infiltrate that reaches up to the muscle layer	5
H01/3381	Mucosa with chronic transmural inflammatory infiltrate, with lymphoid aggregates	4

The results of qRT-PCR are summarized in the graph of Figure 3.30 and show an increase in RAC1B expression with advanced disease stages. All control samples presented a normal RAC1B level, whereas the majority of the IBD samples had increased RAC1B levels. Only one of the four samples from stage 5 of the disease showed the normal RAC1B level, while the others showed a level at least 2 times increased. Samples from stage 2 showed an expression similar to the control group and stage 4 showed a slight increase, albeit not statistically significant. When we group all the IBD samples, we also see that there is a tendency for increased RAC1B expression.

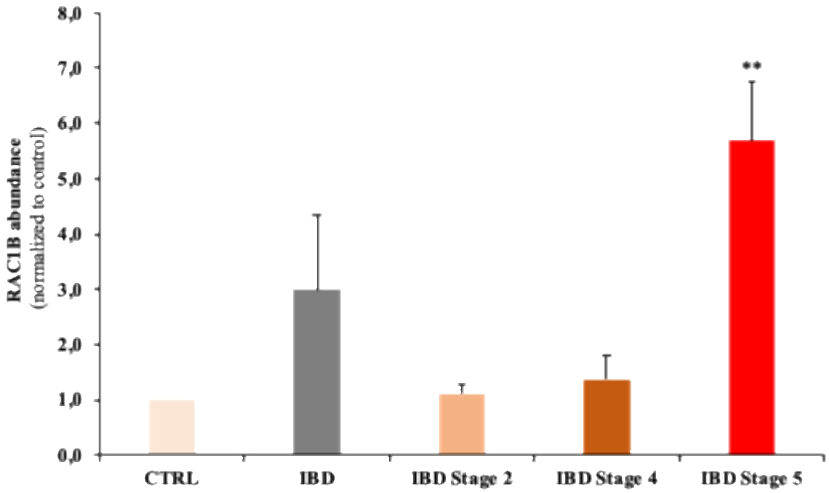

Figure 3.30 - **RAC1B mRNA levels of IBD patient samples.** Total RNA from paraffin-preserved samples was extracted, cDNA synthetized and RAC1 and RAC1B were amplified by qRT-PCR. The samples were grouped into categories according to the stage of the disease and subsequently the level of RAC1B was compared with the level of the control samples (mucosa without changes). IBD corresponds to all occurrences and IBD stage to those corresponding to each category. Data are shown as fold-change of the RAC1B/RAC1 ratio relative to control and represent means ± SEM, of 3 independent experiments. Statistical analysis was carried out with a one-way ANOVA test followed by a Dunnett post-test. ** significantly different from the corresponding control $P < 0.01$.

In short, the presence of increased RAC1B appears to correlate with the progression of inflammatory bowel disease and its severity. However, the small number of samples did not allow to draw solid conclusions and in the future, it would be fruitful to increase the number of patient samples.

Chapter 4 – General Discussion and Conclusion

The mucosa of our gastrointestinal tract forms a barrier that separates the gut lumen, containing ingested food, microorganisms and environmental toxins, from the surrounding tissues (Zhang *et al.* 2015). The barrier function relies on a close dialogue between epithelial and stromal immune cells. Thus, perturbation of the barrier function in the colon underlies inflammatory bowel diseases, which constitute a risk factor for colon cancer development (Baumgart and Carding 2007; Ullman and Itzkowitz 2011). In addition, the requirement for adult stem cells to constantly renew the colon mucosa epithelium, presents a proliferative microenvironment in the colon crypts that results in increased vulnerability of the mucosa to the growth of malignant cells (Barker *et al.* 2009; Clevers 2011, 2013; Gehart and Clevers 2019).

Previously, our lab found that inflammatory conditions in the colon mucosa led to increased expression of an AS variant of the small GTPase RAC1, designated RAC1B (Matos *et al.* 2013). Variant RAC1B is also overexpressed in a subtype of colorectal tumors, characterized by the presence of mutant *BRAF* and corresponding to up to 15% of all sporadic CRCs (Matos *et al.* 2008, 2016). The studies further suggested that the overexpression of RAC1B is a subsequent genetic event in tumor-initiated colorectal cells, allowing them to escape from oncogene-induced senescence (Henriques *et al.* 2015) and continue the malignant progression. However, mutations in the *RAC1* gene, that could explain a shift from RAC1 to RAC1B transcripts, have not been found, so the mechanism involved in triggering RAC1B overexpression remained to be determined.

The process of tumor formation and progression is influenced by two factors, namely genetic/epigenetic changes in the tumor cells and their mutual and dynamic interaction with the TME (Arneth 2019; Baghban *et al.* 2020). Solid tumors have been recognized as structures that consist of not only cancer cells but also a variety of morphologically distinct cell types. Although tumor formation is initiated by an oncogenic mutational event in a cell, further tumor-promoting stimuli are required for malignant progression to proceed. A pro-inflammatory microenvironment has been identified as one such stimulus (Coussens and Werb 2002; Bissell and Hines 2011).

4.1. A model to study the response of colorectal cells to a pro-inflammatory environment

The main objective of this work was to test the hypothesis that microenvironmental stimuli could be a trigger for tumor cells to change AS, namely RAC1B. For this, we grew Caco-2 cells on a microporous filter membrane into a polarized epithelial cell layer, as a model to implement co-cultures of colorectal and stromal cell types and determine the conditions that lead to increased RAC1B expression in colorectal cells, so to be able to respond to the first objective of this thesis.

Caco-2 cells were derived from an intermediate-stage human colorectal adenocarcinoma (Sambuy *et al.* 2005), show both colonocyte and enterocyte characteristics, and have a microsatellite-stable phenotype, mutations in the tumor-suppressor proteins APC, p53, and SMAD4 (Djelloul *et al.* 1997; Ilyas *et al.* 1997; De Bosscher *et al.* 2004), but not in the proliferation-driving oncogenes *KRAS*, *BRAF*, or *PIK3CA* (Forbes *et al.* 2010). Besides being a model for earlier stages of cell transformation, Caco-2 cells have the ability to form a layer of polarized cells, when grown on microporous membrane inserts, critical for these studies.

First, polarization has a profound impact on the wiring of the cell's signal transduction pathways. As described in a review by Weaver and colleagues (2014), the intracellular localization of many signaling molecules differs between cells grown as a flat monolayer on a stiff plastic matrix and cells differentiated into a polarized cell layer (Rubashkin *et al.* 2014). In part, the type of cell-cell or cell-matrix adhesion complexes assembled by the cell determines the connection to the intracellular cytoskeletal and signaling machinery, approaching such polarized cell layers more to the physiological situation of cells within an epithelium.

Second, it allowed co-culturing Caco-2 cells with stromal cell types without them being in direct physical contact, due to the separating membrane. This way, a pure Caco-2 extract could be obtained for analysis of RNA and protein, since the cells share only soluble factors released into the medium.

Based on co-culture experiments of stromal cells and Caco-2, the results showed that fibroblasts or CAFs and macrophages increased RAC1B protein expression, at 48 h. The most significant increase was observed with the triple cultures containing CAFs and M1-like macrophages. Besides RAC1B, these experimental conditions led to epithelial disorganization and, consequently, to cellular depolarization, but whether this is caused

by RAC1B signaling remains to be determined. Loss of apical-basal polarity is one of the hallmarks of epithelial cancers and it can occur both, in early stages of tumor progression, for example, in some breast tumor types (Hinck and Näthke 2014), or in later stages with the appearance of invasive migratory cells, as in CRC (Fleming *et al.* 2012).

Further support for the importance of using polarized Caco-2 cells was obtained from the use of other colorectal cell lines grown without polarization, including Caco-2, where a different response to co-culture with stromal cells was observed. As an alternative model of polarized colon cells, we used T84 cells, which also have the ability to form a layer of polarized cells (Donato *et al.* 2011; Pereira *et al.* 2016; Devriese *et al.* 2017). With these cells, co-culture with CAFs and macrophages also led to an increase in RAC1B, as verified with Caco-2 cells. The fact that the results obtained with other cell lines and with non-polarized Caco-2 and T84 differed from those obtained with polarized cells, underline the importance of using polarized cells as models closer to physiological conditions.

The importance and growing value of 3D cell models led us to develop and optimize Caco-2 spheroids, in which they self-organize around an internal and central lumen, surrounded by polarized cells with apical surface. However, the manipulation of Matrigel-based cultures have an associated degree of difficulty, since temperature variations make the Matrigel more liquid, leading to the rupture of the spheroids. On the other hand, Matrigel interferes with techniques such as WB, so it is necessary to use products that allow it to be removed first from the culture. Although these models will certainly be useful for future studies of the TME, the fact that cell types cannot be lysed separately precluded their further use in our co-cultures assays.

Altogether, these results allowed us to conclude that the study of polarized Caco-2 cells permitted identifying the effect of a pro-inflammatory stromal environment on the expression of alternative spliced RAC1B. This is in agreement with the growing understanding that a complex interplay exists between the developing tumor and the TME. In particular, an inflammatory microenvironment triggers adaptive responses in the tumor cells, which rely on the plasticity of gene expression, including the modulation of AS. It has now been established that alternatively spliced isoforms can have oncogenic potential and contribute to several cancer hallmarks, such as the cancer cell's ability to sustain proliferation, avoid apoptosis, invade and metastasize, as well as to manipulate cellular energy metabolism and evade the immune system (Hanahan and Weinberg 2011; Fouad and Aanei 2017; Silva *et al.* 2020). Several recent reviews have highlighted that

such oncogenic AS variants, including RAC1B, may become important new biomarkers for tumor progression or prognosis, or represent a novel type of therapeutic target for pharmacological intervention (Lee and Abdel-Wahab 2016; Urbanski *et al.* 2018; Desterro *et al.* 2020; Rahman *et al.* 2020; Bessa *et al.* 2020).

4.2. Molecular composition of the RAC1B-inducing pro-inflammatory environment

The intestinal epithelium can be injured by inflammation and then undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area (Iizuka 2011; Xue and Falcon 2019). These processes share similarities with cancer; however, at the level of regulation, cancer cells lost control of multiple cellular, molecular and biochemical processes that characterize each step of wound healing (Byun and Gardner 2013).

The molecular connection between cancer and inflammation resides in cytokines and chemokines, soluble factors released by immune cells to regulate their response, but which also stimulate proliferation of most human neoplastic cell types, including colorectal cells (Clevers 2004; Karin and Greten 2005; Terzić *et al.* 2010; DiDonato *et al.* 2012). These were primarily studied here, although we cannot exclude that additional communication of Caco-2 cells with the co-cultured cells exist, such as the release of exosomes or microvesicles which have a size range between 0.1 and 1.0 μm and could pass the microporous membrane (Gyorgy *et al.* 2011).

Through the use of a human inflammation antibody array, we were able to identify 4 main cytokines associated with increased RAC1B protein expression in Caco-2 cells after co-culture with CAFs and M1-like macrophages: IL-1β, IL-6, GM-CSF and IL-8, all of which are typical pro-inflammatory cytokines.

IL-1β levels in the medium were slightly increased by CAFs alone, but then increased over 50-fold in the triple co-culture. IL-1β has been identified as a key mediator of inflammation-induced pathological changes, including IBD (Mahida *et al.* 1989; Reimund *et al.* 1996; McAlindon *et al.* 1998; Reinecker *et al.* 2008; Voronov and Apte 2015). In Caco-2 cells, IL-1β promoted tight junction permeability (Al-Sadi *et al.* 2011, 2013) and in primary colon cancer cells or the HCT-116 colorectal cancer cell line, IL-

1β induced hallmarks of CSCs, such as an up-regulation of stemness factor genes, EMT and increased drug resistance (Li *et al.* 2012).

IL-1β has been strongly implicated in the inflammatory response of immune cells (Bent *et al.* 2018), for example by promoting the differentiation of monocytes to conventional dendritic cells (Yoshimura 2003) and to M1-like macrophages (Schenk *et al.* 2014). So, IL-1β is crucial to initiate acute inflammations. However, high levels of innate cytokines, as apparent in chronic inflammation, may promote tumor development by driving sustained NF-κB activation and MAPK activity. IL-1β is also a driver of auto-inflammatory diseases (Kim and Choi 2015; Bent *et al.* 2018).

The key pro-tumorigenic role of IL-1β is related to its ability to induce the production of other cytokines (Baker et al., 2019), including IL-6 production in breast cancer cells, as well as additional cytokines and growth factors such as TGF-β, TNF-α, and EGF. Indeed, these cytokines and growth factors seem to potentiate the effect of IL-1β on IL-6 expression (Oh *et al.* 2016). IL-1β further promotes tumor development by influencing angiogenesis, by stimulating secretion of VEGF by malignant cells and blood vessel density in tumors (Saijo *et al.* 2002; Voronov *et al.* 2003; Nakao *et al.* 2005).

In experimental studies, nucleotide-binding and oligomerization domain-like receptor 3 (NLRP3) and IL-1β expression levels were correlated with the tumor size and the metastatic status of the sentinel lymph node, in human oral squamous cell carcinoma (Wang *et al.* 2018). Clinically, a correlation between high levels of IL-1β and bad prognosis in cancer patients was observed. Therefore, IL-1β inhibition seems to target key components of tumor development and in most cases results in reduced tumor development (Bent *et al.* 2018).

On the other hand, divergent and conflicting data on the role of the IL-1β exist (Baker *et al.* 2019). Allen and collaborators (2010), ascribed tumor-inhibiting effects to IL-1β in a murine model of colitis-associated cancer, with the increased tumor burden correlating with attenuated levels of IL-1β and IL-18 at the tumor site, through inhibition of the NLRP3 inflammasome (Allen *et al.* 2010).

Although conflicting effects in terms of tumor development have been observed, IL-1β within the TME generally promotes carcinogenesis, tumor growth, and metastasis by different key mechanisms, such as driving chronic non-resolved inflammation, endothelial cell activation, tumor angiogenesis, and the induction of immune-suppressive cells. IL-1β blockers have already shown great efficacy and safety in the treatment of several auto-immune diseases; however, the pleotropic nature of IL-1β and its role under

homeostatic conditions, together with its crucial role in defense against infections, make it difficult to predict the systemic and long-term effects of its blocking in cancer treatment.

The highest signal intensity on the human inflammation array was seen for IL-6, which increased in single co-cultures of Caco-2 with either CAFs or M1-like macrophages alone, but increased at least 10-fold more in the triple co-culture containing both cell types. This mirrors our observation that RAC1B levels in Caco-2 cells were slightly increased in the presence of CAFs or M1-like macrophages alone, but induced to highest levels in the triple co-culture. Activated stromal fibroblasts can enhance IL-6 production by colon cancer cells and anti-IL-6 receptor antibody showed greater anti-tumor and anti-angiogenic activity *in vivo* (Nagasaki *et al.* 2014). So, the relationship between IL-6 and stromal fibroblasts offers new approaches to cancer therapy.

IL-6 levels were significantly increased in patients with CRC and correlated with tumor size and disease status (Chung and Chang 2003), and with metastasis and tumor progression (Ueda *et al.* 1994; Komoda *et al.* 1998; Kinoshita *et al.* 1999). In addition, an increase in serum IL-6 has been reported to be associated with poor prognosis in the patients with gastric cancer (Wu *et al.* 1996; To *et al.* 2004), renal cell carcinoma (Blay *et al.* 1992), prostate cancer (Nakashima *et al.* 2000), ovarian cancer (Plante *et al.* 1994), and breast cancer (Bachelot *et al.* 2003). Furthermore, IL-6 plays a crucial pathogenic role in IBD and its levels in serum and intestinal mucosa of patients with IBD are distinctly elevated and positively correlated with the severity of inflammation (Atreya and Neurath 2005).

As described above, IL-6 production can be stimulated by the presence of IL-1β in breast cancer cells, and their EMT and stem-cell-like phenotypes were attenuated by either anti-IL-6 or anti-IL-1β antibody treatment (Oh et al., 2016). The use of an IL-6 neutralizing antibody, showed that there is a decrease in the expression of RAC1B, showing that it could be a good target for study.

Another specific change in the array concerned GM-CSF, which occurred specifically in the triple co-culture condition but not with CAFs and M1 alone. GM-CSF is known to be involved in regulating macrophage polarization and may be a serum prognostic factor for colorectal cancer patients (Demirci *et al.* 2009; Nebiker *et al.* 2014). Constitutive GM-CSF secretion has been found in a variety of tumor models, including small-cell lung carcinomas, skin carcinoma, gliomas and head and neck squamous cell carcinomas (Hong 2016). Consistently, gene expression arrays show that ~70% of human

and murine colorectal cancers exhibit a consistent production and secretion of GM-CSF (Urdinguio *et al.* 2013). However, upon validation with the addition of purified protein to Caco-2 cells, GM-CSF had no effect on RAC1B levels.

Contrarily to what was referred above, GM-CSF production by tumor cells is at the same time an independent favorable prognostic factor in CRC, inhibiting colorectal cancer cell proliferation (Nebiker *et al.* 2014). Therefore, this double face of the GM-CSF may explain the fact that, despite being identified in our co-culture medium, it does not directly influence the expression of RAC1B.

We further considered IL-8 as a fourth candidate because it showed a pattern of increment from simple to triple co-cultures similar to IL-6, although its relative increase was low, due to a significant background secretion by Caco-2 cells in the control condition. Of particular note, signaling by the IL-8 has been shown to promote malignant progression of tumors (Waugh and Wilson 2008) and has been associated with inflammatory pathways and gastric cancer (Lurje *et al.* 2010). It is widely known that IL-8 acts as an autocrine growth factor for some colon carcinoma cell lines (Brew *et al.* 2000). Furthermore, a meta-analysis in CRC cases identified serum IL-8 as a promising biomarker, which may become a clinically useful tool to identify high-risk patients (Jin *et al.* 2014). In addition, data from population-based studies of colon cancer incidence identified the association of single nucleotide polymorphisms (SNPs) in IL-8 with colon cancer risk (Bondurant *et al.* 2013).

Besides these 4 cytokines that were identified through the cytokine array, we also studied the effect of MIP-2α, because it was found increased in a transgenic RAC1B-knock-in mice model (Kotelevets *et al.* 2018). Indeed, our results are in line with what was seen in this animal model, since the addition of purified MIP-2α led to an increase in RAC1B in Caco-2 cells. Although the activity of this chemokine has mainly been examined for immune cell recruitment, MIP-2α may also exhibit pro-tumorigenic activity (Phinney *et al.* 2018). MIP-2α may induce colorectal tumor cell proliferation in a C-X-C motif chemokine receptor 2 (CXCR2)-dependent manner (Kollmar *et al.* 2007) and also promote melanoma growth and metastasis (Kwon *et al.* 2015).

A limitation of our approach is that all cells in our co-culture system share the same surrounding medium, so that the observed changes in IL-6, IL-8, GM-CSF, MIP-2α and IL-1β cannot discriminate formally, which cell type was responsible for their

secretion, including Caco-2 cells. This may also explain why the combined addition of 2 or more purified cytokine did not show any additive effects on RAC1B expression levels.

4.3. Relation of the pro-inflammatory effects perceived in co-culture with those observed in patients with IBD

During the last years, the contribution of the immune system and inflammation to cancer development, progression, and therapy has regained enormous interest. It is now well known that inflammation predisposes to the development of cancer and promotes all stages of tumorigenesis (Greten and Grivennikov 2019).

Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract (Sanchez-Muñoz *et al.* 2008; Fakhoury *et al.* 2014). Currently, the pathogenesis of IBD is not completely understood, but it is known that cytokines are key signals in the intestinal immune system, and are known to participate in the disruption of the so-called normal state of controlled inflammation (Sanchez-Muñoz *et al.* 2008). The presence of certain chemokines, cytokines, and myeloid cell subsets correlate with poor prognosis in CRC (Tosolini *et al.* 2011; Mlecnik *et al.* 2016).

In fixed tissue sections obtained from patients with IBD, we observed a correlation with poor prognosis/stage of disease and the presence of overexpressed RAC1B. Nevertheless, the number of samples was too small to draw statistically meaningful conclusions.

T-helper (Th) cells are a subclass of lymphocytes that play a key role in IBD. Th1 cells mediate the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-2, which are part of cell-mediated immunity, whereas Th2 cells lead to production of anti-inflammatory cytokines. There is a mutual interaction between Th1 and Th2 lymphocytes. The administration of anti-inflammatory drugs reduce the extent of bowel inflammation disease by attenuating the Th1 response and enhancing Th2-mediated activity (Rutgeerts and Geboes 2001; Fichtner-Feigl 2005).

TNF-α is a pro-inflammatory cytokine that plays a major role in the inflammation caused by IBD, since clinical studies have demonstrated that serum TNF-α levels are elevated in these patients. An increase in TNF-α induces cell proliferation and differentiation, and leads to upregulation of adhesion molecules on endothelial cells (Fakhoury *et al.* 2014). TNF-α also exerts its pro-inflammatory effect through cytokines

such as INF-γ, IL-1β, and IL-6 (Florholmen and Fries 2011). So, TNF-α is the most promising candidate predictor of this disease (Florholmen and Fries 2011; Velikova *et al.* 2016).

In the results of the human inflammation array, TNF-α does not appear to be increased in the conditioned medium of our study model. This may be due to the fact that TNF-α is mainly secreted by lymphocytes, which are not present in our co-cultures. However, in the future, it would be necessary to assess its role in RAC1B production by Caco-2 cells, either in purified form or by adding lymphocytes to the triple co-culture condition.

Beyond this cytokine, IL-8 mRNA expression in the inflamed mucosa is shown to be significantly higher than the level in non-inflamed mucosa of IBD patients or in the normal mucosa of non-IBD patients (Matsuda *et al.* 2009) and there is also an upregulation of IL-6 levels in IBD patients. Mucosal IL-6 levels were correlated with the degree of clinical activity in Crohn's disease and ulcerative colitis (Florholmen and Fries 2011).

Altogether, the cytokines that were identified in the RAC1B-inducing pro-inflammatory co-culture system have already been largely identified in the context of inflammatory disease, further supporting the value of the developed Caco-2 co-culture model system to study the connection between inflammation of the intestine, the increase in RAC1B and the progression to cancerous conditions.

4.4. Signaling response of colorectal cells to a pro-inflammatory environment

Our data correlated the presence of 4 cytokines with the induction of RAC1B in Caco-2 cells and thus, we hypothesized that these were recognized by Caco-2 cell receptors and transformed into a signaling response. In order to identify signaling events that could be mediating the variation in RAC1B levels induced by the CAF with M1 co-culture, we analyzed Caco-2 cells for changes in the phosphorylation profile of some key protein kinases and signaling molecules, after 24 h and 48 h of co-culture.

According to the proteome profiler human phospho-kinase antibody array analysis, we identified several specific changes. First, STAT3 S727 phosphorylation increased after 24 h and 48 h of co-culture. This constitutive activation of STAT3 pathway has already been described in other cancers, such as melanoma (Sakaguchi *et al.*

2012) and chronic lymphocytic leukemia (Hazan-Halevy *et al.* 2010), where pS727 was detected in almost all the cases while very few of the samples presented pY705 STAT3 (Dimri *et al.* 2017). In our study model, we observed both phosphorylations of STAT3.

STAT3 is a transcription factor and a crucial mediator of tumor cell progression and tumor-associated immunosuppression (Wang and Sun 2014; Kitamura *et al.* 2017). Hyperactivation of STAT3 has been reported in several types of tumors, including head- and neck, brain, breast, liver, lung, kidney, pancreas, prostate, ovary cancer, and multiple myeloma, as well as acute myeloid leukemia. Expression levels of activated STAT3 are positively correlated with poor prognosis in these cancers (Lee *et al.* 2019).

In IBD, activation of STAT3 is mediated by the interaction of IL-6/soluble IL-6 receptor (sIL-6R) complexes and glycoprotein 130 (gp130) and enhances the expression of anti-apoptotic factors such as BCL-2 and BCL-xL, which cause apoptotic resistance and contribute to the perpetuation of chronic intestinal inflammation (Atreya and Neurath 2005; Luo and Zhang 2017). Collectively, it is suggested that IL-6 is a critical tumor promoter in colitis-associated cancer and STAT3 is essential for the transduction of tumor-promoting signals from IL-6 (Mager *et al.* 2016; Luo and Zhang 2017).

Targeting the IL-6/STAT3 signaling axis has already been shown to be beneficial in the treatment of certain cancers in human patients, therefore, many protein inhibitors of this pathway have been developed and tested as well as immunotherapy (Wang and Sun 2014; Wang *et al.* 2016; Johnson *et al.* 2018; Lee *et al.* 2019). Grivennikov and collaborators (2009) established the role of IL-6 and STAT3 signaling in pre-malignant intestinal epithelial cells, since the signaling protects their apoptosis, during inflammation-associated colon carcinogenesis. This created a rationale for the use of IL-6 blockers and STAT3 inhibitors in the treatment and prevention of colitis-associated cancer (Grivennikov *et al.* 2009).

In our results, the addition of anti-IL-6 antibody to the triple co-culture medium neutralized the effect of IL-6 and led to a decrease of both, STAT3 Y705 phosphorylation and RAC1B protein expression. Although further experiments are required to determine whether STAT3 directly affects RAC1B levels, it is possible that a transcriptional effect on, for example, an exon 3B-binding splicing factor such as SRSF1 could be involved.

Second, we observed an increase in ERK1/2 T202/Y204, T185/Y187, at 24 h of co-culture. RAF/MEK/ERK signaling is a key pathway in the occurrence and development of most human solid tumors (Guo *et al.* 2020). The RAF/MEK/ERK pathway has attracted much attention in the search for new chemotherapeutic agents, due

to both the high frequency of *KRAS* (40%) and *BRAF* (10%) mutations identified in colon tumors (Downward 2003; Arrington *et al.* 2012; Yaeger and Saltz 2012). It is a major pathway promoting proliferation by regulating cell cycle progression and apoptosis of human colon cancer cells (Zhou *et al.* 2018; Ducreux *et al.* 2019). Moreover, constitutive activation of ERK1/2 is frequently, though not invariably, observed in CRC cell lines and primary human tumors derived from colon (Hoshino *et al.* 1999).

Selumetinib (AZD6244, ARRY-142886) is an oral, highly specific, allosteric inhibitor of MEK1/2 that is currently undergoing clinical trials (Kirkwood *et al.* 2012; Catalanotti *et al.* 2013; Cheng and Tian 2017) as an anti-tumor agent (Grasso *et al.* 2014). It has shown to exert anti-proliferative and pro-apoptotic effects in various tumor cell lines grown in culture or as xenografts (Davies *et al.* 2007). Binding of Selumetinib to the inhibitor binding pocket of MEK1/2 prevents downstream phosphorylation of ERK1/2 and, thus, inhibits the RAF/MEK/ERK signaling pathway.

In our results, the use of Selumetinib not only decreased the phosphorylation of ERK1/2 but also prevented the co-culture induced increase in RAC1B, demonstrating that the activation of the MEK/ERK pathway has an important and direct role in the cytokine-mediated increase of AS variant RAC1B.

A BRAF-V600E mutation is frequently observed in melanoma and CRC and is associated with a very poor prognosis. This mutation leads to constitutive BRAF kinase phosphorylation of MEK and ERK kinases and sustained MAPK pathway signaling (Davies *et al.* 2002). This pathway is one of the most attractive targets for the design of small-molecule drugs, and currently many different BRAF inhibitors are on the market. Because these inhibitors alone have led to tumor relapse due to the selection of resistant cells, double and triple combinatorial therapeutic strategies that block several pathways in parallel have made remarkable progress in this area (Pan *et al.* 2018; Ducreux *et al.* 2019). Previous studies have suggested that BRAF-V600E alone is not sufficient to drive an increase in RAC1B levels in NCM460 cells (Henriques *et al.* 2015), however, in future studies, the treatment of the described co-culture model with RAF or EGFR inhibitors could help to further clarify the relation between ERK activation and RAC1B expression.

Besides being a transcriptional stimulator, the activation of ERK was also shown to affect AS through direct phosphorylation of splicing factors. For example, the inclusion of exon v5 into the cell surface tumor marker CD44 enhances malignancy and invasiveness of some tumors, and is regulated by KH domain containing, RNA binding, signal transduction associated 1 (SAM68). ERK phosphorylates SAM68 in T-lymphoma

(Weg-Remers 2001; Matter *et al.* 2002) and colorectal cancer cells, and leads to binding of p-SAM68 to the 3' UTR of the SRSF1 transcript (Valacca *et al.* 2010). Thus, ERK activation results in an increased level of SRSF1 protein, which has been shown to promote a switch in the splicing profile, for example, in case of the macrophage-stimulating protein receptor (RON) and RAC1B (Gonçalves *et al.* 2017). This presumable increase in SRSF1 levels could also be studied in future studies using the described co-culture model.

It has already been described that protein kinases SRSF protein kinase 1 (SRPK1) and GSK3β act upstream of SRSF1, and are required for inclusion of RAC1 exon 3B (Gonçalves *et al.* 2009, 2014). GSK3β is part of the canonical WNT signaling pathway but can also be regulated through an inhibitory phosphorylation at S9 by AKT. We observed in our co-cultures a decrease of phosphorylated active AKT and no change in the phosphorylation of S9 from GSK3β. Therefore, it seems unlikely that changes in GSK3β activity can explain the increase in alternative splicing variant RAC1B. Similarly to these alterations, the changes observed in P27, LYN, PDGFR and EGFR phosphorylation could not be validated in the context of this thesis and require further studies.

Surprisingly, changes in AS were described not only in tumor tissues but were also found in cells from the TME (Brosseau *et al.* 2014). This has stimulated research into antisense or decoy oligonucleotide technology to treat cancers with upregulated or hyperactive splicing factors. However, one challenge behind this technology is that several splicing factors with binding affinities for similar motifs might be sequestered; therefore, a broader range of their downstream splicing targets could be affected and result in compromised therapeutic efficiency (Rahman *et al.* 2020). Regarding the development of AS-targeting drugs, existing small-molecule compounds do mostly interfere with early spliceosome assembly or post-translational modification of SR proteins, but lack specific and efficient antitumor activity. Thus, it will first be necessary to establish in more detail how these drugs are connected with altered genotypes or signaling pathways that characterize tumor phenotypes. A more specific therapeutic approach would be to directly target the overexpression of tumor-related splice variants with antisense oligonucleotide technology.

Overall, the results obtained in this thesis are summarized in Figure 4.1. They suggest that a pro-inflammatory environment can lead to an increase in RAC1B expression in colon epithelial cells, through the cytokine-mediated activation of signaling

pathways, such as IL-6/STAT3 and MEK/ERK. Nevertheless, this does not exclude other pathways to be affected in this microenvironment. Since RAC1B was shown to sustain tumor cell survival and promote escape form OIS, the data further strengthen the causal connection between inflammatory conditions and the development of CRC. They are in line with recent progress in interpreting the crosstalk between signaling pathways in CRC (Koveitypour *et al.* 2019). Therefore, perceiving the connections between these pathways may trigger development of novel therapeutic or preventive approaches against CRC.

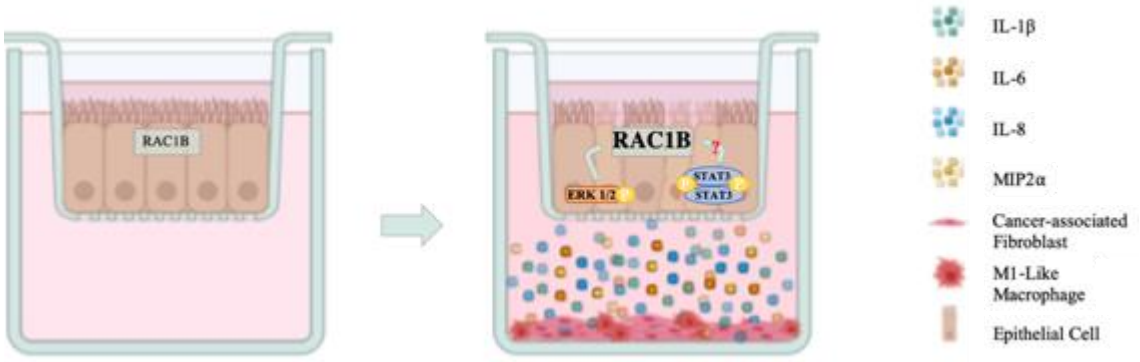

Figure 4.1 - **Scheme summarizing the results obtained in this work.** The polarized Caco-2 cells have a low RAC1B protein expression and form a polarized epithelial cell layer. In the presence of co-cultured pro-inflammatory stromal cells, mainly cancer-associated fibroblasts and M1-like macrophages, these release cytokines IL-1β, IL-6, IL-8 and MIP-2α, to the conditioned co-culture medium. and induce the phosphorylation of ERK 1/2 and STAT3 in the Caco-2 cells. This is associated with an increase in the expression of the alternative spliced RAC1B variant and perturbation of epithelial cell layer organization.

4.5. Future perspectives

The results obtained and explored in this thesis allowed for the first time the establishment of a molecular connection between inflammation and the increase in RAC1B. Following the identification of cytokines with a fundamental role in the increase of this splicing variant expression, and also of the signaling pathways directly involved in this context, new avenues for research have been opened to understand how the alteration of the splicing pattern is connected to early stages of tumorigenesis. However, it will be necessary to study the underlying signaling pathways in more detail, including other changes in kinases not futher validated in this study. This knowledge can lead to novel targeted therapy solutions for IBD and CRC patients.

Another future goal would be to establish a cause-effect relationship between RAC1B expression and IBD by perceiving whether an experimental manipulation of

RAC1B levels in polarized Caco-2 cells might be sufficient to dissolve the integrity of the intestinal barrier function, with implications for the understanding of IBD.

It has become increasingly clear that AS is tightly associated with human health and disease, such as cancer, cardiovascular disease, diabetes or neurological diseases (Kim *et al.* 2018). The emergence of advanced sequencing technologies at a genome level has made it possible to identify aberrant splicing signatures in cancer, allowing the establishment of AS patterns. Remarkably, these studies have contributed in a decisive way to develop strategies towards cancer precision medicine (Bessa *et al.* 2020). Therefore, future studies should apply this model of Caco-2 cells to determine whether the pro-inflammatory condition affects genome-wide changes in the splicing profile, using the RNA-sequencing technique. The results could take us to the discovery of new biomarkers for early tumorigenic stages or provide novel therapeutic targets of CRC, since it will permit to move our understanding from individual AS variants to the overall pattern of splicing changes in tumors. Thus, AS signature profiles or patterns may represent more meaningful biomarkers to understand the complexity of CRC.

Chapter 5 – Bibliography

Abbott Chalew T. E., and Schwab K. J. (2013). Toxicity of commercially available engineered nanoparticles to Caco-2 and SW480 human intestinal epithelial cells. *Cell Biol. Toxicol.* **29**, 101–116. doi:10.1007/s10565-013-9241-6

Achilli T.-M., Meyer J., and Morgan J. R. (2012). Advances in the formation, use and understanding of multi-cellular spheroids. *Expert Opin. Biol. Ther.* **12**, 1347–1360. doi:10.1517/14712598.2012.707181

Adjei A. A., and Hidalgo M. (2005). Intracellular Signal Transduction Pathway Proteins As Targets for Cancer Therapy. *J. Clin. Oncol.* **23**, 5386–5403. doi:10.1200/JCO.2005.23.648

Aggarwal B. B., and Gehlot P. (2009). Inflammation and cancer: how friendly is the relationship for cancer patients? *Curr. Opin. Pharmacol.* **9**, 351–369. doi:10.1016/j.coph.2009.06.020

Ahmed D., Eide P. W., Eilertsen I. A., Danielsen S. A., Eknæs M., Hektoen M., Lind G. E., and Lothe R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* **2**, e71–e71. doi:10.1038/oncsis.2013.35

Akagi Y., Liu W., Xie K., Zebrowski B., Shaheen R. M., and Ellis L. M. (1999). Regulation of vascular endothelial growth factor expression in human colon cancer by interleukin-1β. *Br. J. Cancer* **80**, 1506–1511. doi:10.1038/sj.bjc.6690553

Akinleye A., and Rasool Z. (2019). Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *J Hematol Oncol* **12**, 92. doi:10.1186/s13045-019-0779-5

Allen I. C., TeKippe E. M., Woodford R.-M. T., Uronis J. M., Holl E. K., Rogers A. B., Herfarth H. H., Jobin C., and Ting J. P.-Y. (2010). The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J. Exp. Med.* **207**, 1045–1056. doi:10.1084/jem.20100050

Almendro V., Marusyk A., and Polyak K. (2013). Cellular Heterogeneity and Molecular Evolution in Cancer. *Annu. Rev. Pathol. Mech. Dis.* **8**, 277–302. doi:10.1146/annurev-pathol-020712-163923

Alonso-Espinaco V., Cuatrecasas M., Alonso V., Escudero P., Marmol M., Horndler C., Ortego J., Gallego R., Codony-Servat J., Garcia-Albeniz X., Jares P., Castells A., Lozano J. J., Rosell R., and Maurel J. (2014). RAC1b overexpression correlates with poor prognosis in KRAS/BRAF WT metastatic colorectal cancer patients

treated with first-line FOLFOX/XELOX chemotherapy. *Eur. J. Cancer* **50**, 1973–1981. doi:10.1016/j.ejca.2014.04.019

Al-Sadi R., Guo S., Ye D., Dokladny K., Alhmoud T., Ereifej L., Said H. M., and Ma T. Y. (2013). Mechanism of IL-1β Modulation of Intestinal Epithelial Barrier Involves p38 Kinase and Activating Transcription Factor-2 Activation. *J. Immunol.* **190**, 6596–6606. doi:10.4049/jimmunol.1201876

Al-Sadi R., Ye D., Said H. M., and Ma T. Y. (2011). Cellular and molecular mechanism of interleukin-1β modulation of Caco-2 intestinal epithelial tight junction barrier. *J. Cell. Mol. Med.* **15**, 970–982. doi:10.1111/j.1582-4934.2010.01065.x

Alt F. W., Bothwell A. L. M., Knapp M., Siden E., Mather E., Koshiand M., and Baltimore D. (1980). Synthesis of Secreted and Membrane-Bound lmmunoglobulin Mu Heavy Chains Is Directed by mRNAs That Differ at Their 3' Ends. *Cell* **20**, 293–301. doi:10.1016/0092-8674(80)90615-7

Andreu P., Johansson M., Affara N. I., Pucci F., Tan T., Junankar S., Korets L., Lam J., Tawfik D., DeNardo D. G., Naldini L., de Visser K. E., De Palma M., and Coussens L. M. (2010). FcRγ Activation Regulates Inflammation-Associated Squamous Carcinogenesis. *Cancer Cell* **17**, 121–134. doi:10.1016/j.ccr.2009.12.019

Annunziata C. M., Davis R. E., Demchenko Y., Bellamy W., Gabrea A., Zhan F., Lenz G., Hanamura I., Wright G., Xiao W., Dave S., Hurt E. M., Tan B., Zhao H., Stephens O., Santra M., Williams D. R., Dang L., Barlogie B., Shaughnessy J. D., Kuehl W. M., and Staudt L. M. (2007). Frequent Engagement of the Classical and Alternative NF-κB Pathways by Diverse Genetic Abnormalities in Multiple Myeloma. *Cancer Cell* **12**, 115–130. doi:10.1016/j.ccr.2007.07.004

Araldi R. P., Sant'Ana T. A., Módolo D. G., de Melo T. C., Spadacci-Morena D. D., de Cassia Stocco R., Cerutti J. M., and de Souza E. B. (2018). The human papillomavirus (HPV)-related cancer biology: An overview. *Biomed. Pharmacother.* **106**, 1537–1556. doi:10.1016/j.biopha.2018.06.149

Arneth B. (2019). Tumor Microenvironment. *Medicina (Mex.)* **56**, 15. doi:10.3390/medicina56010015

Arrington A. K., Heinrich E. L., Lee W., Duldulao M., Patel S., Sanchez J., Garcia-Aguilar J., and Kim J. (2012). Prognostic and Predictive Roles of KRAS Mutation in Colorectal Cancer. *Int. J. Mol. Sci.* **13**, 12153–12168. doi:10.3390/ijms131012153

Atreya R., and Neurath M. F. (2005). Involvement of IL-6 in the Pathogenesis of Inflammatory Bowel Disease and Colon Cancer. *Clin. Rev. Allergy Immunol.* **28**, 187–196. doi:10.1385/CRIAI:28:3:187

Aykut B., Pushalkar S., Chen R., Li Q., Abengozar R., Kim J. I., Shadaloey S. A., Wu D., Preiss P., Verma N., Guo Y., Saxena A., Vardhan M., Diskin B., Wang W., Leinwand J., Kurz E., Kochen Rossi J. A., Hundeyin M., Zambrinis C., Li X., Saxena D., and Miller G. (2019). The fungal mycobiome promotes pancreatic oncogenesis via activation of MBL. *Nature* **574**, 264–267. doi:10.1038/s41586-019-1608-2

Azevedo L. F., Magro F., Portela F., Lago P., Deus J., Cotter J., Cremers I., Vieira A., Peixe P., Caldeira P., Lopes H., Gonçalves R., Reis J., Cravo M., Barros L., Ministro P., Lurdes M., Duarte A., Campos M., Carvalho L., and Costa-Pereira A. (2010). Estimating the prevalence of inflammatory bowel disease in Portugal using a pharmaco-epidemiological approach: Prevalence of IBD in Portugal - a pharmaco-epidemiological approach. *Pharmacoepidemiol. Drug Saf.* **19**, 499–510. doi:10.1002/pds.1930

Bachelot T., Ray-Coquard I., Menetrier-Caux C., Rastkha M., Duc A., and Blay J.-Y. (2003). Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br. J. Cancer* **88**, 1721–1726. doi:10.1038/sj.bjc.6600956

Baghban R., Roshangar L., Jahanban-Esfahlan R., Seidi K., Ebrahimi-Kalan A., Jaymand M., Kolahian S., Javaheri T., and Zare P. (2020). Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Commun. Signal.* **18**, 59. doi:10.1186/s12964-020-0530-4

Baier P. K., Wimmenauer S., Hirsch T., v. Specht B.-U., v. Kleist S., Keller H., and Farthmann E. H. (1998). Analysis of the T Cell Receptor Variability of Tumor-Infiltrating Lymphocytes in Colorectal Carcinomas. *Tumor Biol.* **19**, 205–212. doi:10.1159/000030008

Baker K. J., Houston A., and Brint E. (2019). IL-1 Family Members in Cancer; Two Sides to Every Story. *Front. Immunol.* **10**, 1197. doi:10.3389/fimmu.2019.01197

Balkwill F. R., Capasso M., and Hagemann T. (2012). The tumor microenvironment at a glance. *J. Cell Sci.* **125**, 5591–5596. doi:10.1242/jcs.116392

Barille S., and Harousseau J.-L. (1997). Metalloproteinases in Multiple Myeloma: Production of Matrix Metalloproteinase-9 (MMP-9), Activation of proMMP-2, and Induction of MMP-1 by Myeloma Cells. *Blood* **90**, 1649–1655. doi:10.1182/blood.v90.4.1649

Barker N., Ridgway R. A., van Es J. H., van de Wetering M., Begthel H., van den Born M., Danenberg E., Clarke A. R., Sansom O. J., and Clevers H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457**, 608–611. doi:10.1038/nature07602

Bates G. J., Fox S. B., Han C., Leek R. D., Garcia J. F., Harris A. L., and Banham A. H. (2006). Quantification of Regulatory T Cells Enables the Identification of High-Risk Breast Cancer Patients and Those at Risk of Late Relapse. *J. Clin. Oncol.* **24**, 5373–5380. doi:10.1200/JCO.2006.05.9584

Bats A. S., Zafrani Y., Pautier P., Duvillard P., and Morice P. (2008). Malignant transformation of abdominal wall endometriosis to clear cell carcinoma: case report and review of the literature. *Fertil. Steril.* **90**, 1197.e13-1197.e16. doi:10.1016/j.fertnstert.2007.08.080

Baumgart D. C., and Carding S. R. (2007). Inflammatory bowel disease: cause and immunobiology. *The Lancet* **369**, 1627–1640. doi:10.1016/S0140-6736(07)60750-8

Beck B., and Blanpain C. (2013). Unravelling cancer stem cell potential. *Nat. Rev. Cancer* **13**, 727–738. doi:10.1038/nrc3597

Béduneau A., Tempesta C., Fimbel S., Pellequer Y., Jannin V., Demarne F., and Lamprecht A. (2014). A tunable Caco-2/HT29-MTX co-culture model mimicking variable permeabilities of the human intestine obtained by an original seeding procedure. *Eur. J. Pharm. Biopharm.* **87**, 290–298. doi:10.1016/j.ejpb.2014.03.017

Bemmo A., Dias C., Rose A. A. N., Russo C., Siegel P., and Majewski J. (2010). Exon-Level Transcriptome Profiling in Murine Breast Cancer Reveals Splicing Changes Specific to Tumors with Different Metastatic Abilities (C Creighton, Ed.). *PLoS ONE* **5**, e11981. doi:10.1371/journal.pone.0011981

Bent R., Moll L., Grabbe S., and Bros M. (2018). Interleukin-1 Beta—A Friend or Foe in Malignancies? *Int. J. Mol. Sci.* **19**, 2155. doi:10.3390/ijms19082155

Bertram J. S. (2001). The molecular biology of cancer. *Mol. Aspects Med.* **21**, 167–223. doi:10.1016/s0098-2997(00)00007-8

Bessa C., Matos P., Jordan P., and Gonçalves V. (2020). Alternative Splicing: Expanding the Landscape of Cancer Biomarkers and Therapeutics. *Int. J. Mol. Sci.* **21**, 9032. doi:10.3390/ijms21239032

Bettington M., Walker N., Clouston A., Brown I., Leggett B., and Whitehall V. (2013). The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology* **62**, 367–386. doi:10.1111/his.12055

Bhowmick N. A. (2004). TGF- Signaling in Fibroblasts Modulates the Oncogenic Potential of Adjacent Epithelia. *Science* **303**, 848–851. doi:10.1126/science.1090922

Biamonti G., Bonomi S., Gallo S., and Ghigna C. (2012). Making alternative splicing decisions during epithelial-to-mesenchymal transition (EMT). *Cell. Mol. Life Sci.* **69**, 2515–2526. doi:10.1007/s00018-012-0931-7

Bingle L., Brown N. J., and Lewis C. E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J. Pathol.* **196**, 254–265. doi:10.1002/path.1027

Birbrair A. (Ed) (2020). 'Tumor Microenvironment: Non-Hematopoietic Cells.' (Springer International Publishing: Cham) doi:10.1007/978-3-030-37184-5

Bissell M. J., and Hines W. C. (2011). Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat. Med.* **17**, 320–329. doi:10.1038/nm.2328

Black D. L. (2003). Mechanisms of Alternative Pre-Messenger RNA Splicing. *Annu. Rev. Biochem.* **72**, 291–336. doi:10.1146/annurev.biochem.72.121801.161720

Blay J.-Y., Nã S., Mercatello A., Ravault A., Tourani J.-M., and Favrot M. (1992). Serum Level of Interleukin 6 as a Prognosis Factor in Metastatic Renal Cell Carcinoma. *Cancer Res* **52**, 3317–22.

Bollrath J., Phesse T. J., von Burstin V. A., Putoczki T., Bennecke M., Bateman T., Nebelsiek T., Lundgren-May T., Canli Ö., Schwitalla S., Matthews V., Schmid R. M., Kirchner T., Arkan M. C., Ernst M., and Greten F. R. (2009). gp130-Mediated Stat3 Activation in Enterocytes Regulates Cell Survival and Cell-Cycle Progression during Colitis-Associated Tumorigenesis. *Cancer Cell* **15**, 91–102. doi:10.1016/j.ccr.2009.01.002

Bondurant K. L., Lundgreen A., Herrick J. S., Kadlubar S., Wolff R. K., and Slattery M. L. (2013). Interleukin genes and associations with colon and rectal cancer risk and overall survival. *Int. J. Cancer* **132**, 905–915. doi:10.1002/ijc.27660

Bonnans C., Chou J., and Werb Z. (2014). Remodelling the extracellular matrix in development and disease. *Nat. Rev. Mol. Cell Biol.* **15**, 786–801. doi:10.1038/nrm3904

Bosco E. E., Mulloy J. C., and Zheng Y. (2009). Rac1 GTPase: A "Rac" of All Trades. *Cell. Mol. Life Sci.* **66**, 370–374. doi:10.1007/s00018-008-8552-x

Bosscher K. D., and Hill C. S. (2004). Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells. *Biochem. J.* **379**, 209–216. doi:10.1042/BJ20031886

Boureux A., Vignal E., Faure S., and Fort P. (2007). Evolution of the Rho Family of Ras-Like GTPases in Eukaryotes. *Mol. Biol. Evol.* **24**, 203–216. doi:10.1093/molbev/msl145

Brew R., Erikson J. S., West D. C., Kinsella A. R., Slavin J., and Christmas S. E. (2000). Interleukin-8 as an autocrine growth factor for human colon carcinoma cells in vitro. *Cytokine* **12**, 78–85. doi:10.1006/cyto.1999.0518

Brosseau J.-P., Lucier J.-F., Nwilati H., Thibault P., Garneau D., Gendron D., Durand M., Couture S., Lapointe E., Prinos P., Klinck R., Perreault J.-P., Chabot B., and Abou-Elela S. (2014). Tumor microenvironment-associated modifications of alternative splicing. *RNA* **20**, 189–201. doi:10.1261/rna.042168.113

Buhrmann C., Shayan P., Kraehe P., Popper B., Goel A., and Shakibaei M. (2015). Resveratrol induces chemosensitization to 5-fluorouracil through up-regulation of intercellular junctions, Epithelial-to-mesenchymal transition and apoptosis in colorectal cancer. *Biochem. Pharmacol.* **98**, 51–68. doi:10.1016/j.bcp.2015.08.105

Burdett E., Kasper F. K., Mikos A. G., and Ludwig J. A. (2010). Engineering Tumors: A Tissue Engineering Perspective in Cancer Biology. *Tissue Eng. Part B Rev.* **16**, 351–359. doi:10.1089/ten.teb.2009.0676

Burns J., and Manda G. (2017). Metabolic Pathways of the Warburg Effect in Health and Disease: Perspectives of Choice, Chain or Chance. *Int. J. Mol. Sci.* **18**, 2755. doi:10.3390/ijms18122755

Bustelo X. R., Sauzeau V., and Berenjeno I. M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *BioEssays* **29**, 356–370. doi:10.1002/bies.20558

Byun J. S., and Gardner K. (2013). Wounds That Will Not Heal. *Am. J. Pathol.* **182**, 1055–1064. doi:10.1016/j.ajpath.2013.01.009

Calon A., Lonardo E., Berenguer-Llergo A., Espinet E., Hernando-Momblona X., Iglesias M., Sevillano M., Palomo-Ponce S., Tauriello D. V. F., Byrom D., Cortina C., Morral C., Barceló C., Tosi S., Riera A., Attolini C. S.-O., Rossell D., Sancho E., and Batlle E. (2015). Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat. Genet.* **47**, 320–329. doi:10.1038/ng.3225

Camus M., Tosolini M., Mlecnik B., Pages F., Kirilovsky A., Berger A., Costes A., Bindea G., Charoentong P., Bruneval P., Trajanoski Z., Fridman W.-H., and Galon J. (2009). Coordination of Intratumoral Immune Reaction and Human Colorectal Cancer Recurrence. *Cancer Res.* **69**, 2685–2693. doi:10.1158/0008-5472.CAN-08-2654

Canavan C., Abrams K. R., and Mayberry J. (2006). Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment. Pharmacol. Ther.* **23**, 1097–1104. doi:10.1111/j.1365-2036.2006.02854.x

Cardama G. A., Alonso D. F., Gonzalez N., Maggio J., Gomez D. E., Rolfo C., and Menna P. L. (2018). Relevance of small GTPase Rac1 pathway in drug and radio-resistance mechanisms: Opportunities in cancer therapeutics. *Crit. Rev. Oncol. Hematol.* **124**, 29–36. doi:10.1016/j.critrevonc.2018.01.012

Carmeliet P., and Jain R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298–307. doi:10.1038/nature10144

Carragher L. A. S., Snell K. R., Giblett S. M., Aldridge V. S. S., Patel B., Cook S. J., Winton D. J., Marais R., and Pritchard C. A. (2010). V600E Braf induces gastrointestinal crypt senescence and promotes tumour progression through enhanced CpG methylation of p16 INK4a. *EMBO Mol. Med.* **2**, 458–471. doi:10.1002/emmm.201000099

Catalanotti F., Solit D. B., Pulitzer M. P., Berger M. F., Scott S. N., Iyriboz T., Lacouture M. E., Panageas K. S., Wolchok J. D., Carvajal R. D., Schwartz G. K., Rosen N., and Chapman P. B. (2013). Phase II Trial of MEK Inhibitor Selumetinib (AZD6244, ARRY-142886) in Patients with BRAFV600E/K-Mutated Melanoma. *Clin. Cancer Res.* **19**, 2257–2264. doi:10.1158/1078-0432.CCR-12-3476

Chanput W., Mes J. J., Savelkoul H. F. J., and Wichers H. J. (2013). Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. *Food Funct* **4**, 266–276. doi:10.1039/C2FO30156C

Chen X.-M., Elisia I., and Kitts D. D. (2010). Defining conditions for the co-culture of Caco-2 and HT29-MTX cells using Taguchi design. *J. Pharmacol. Toxicol. Methods* **61**, 334–342. doi:10.1016/j.vascn.2010.02.004

Chen Z., Malhotra P. S., Thomas G. R., Ondrey F. G., Duffey D. C., Smith C. W., Enamorado I., Yeh N. T., Kroog G. S., Rudy S., McCullagh L., Mousa S., Quezado M., Herscher L. L., and Waes C. V. (1999). Expression of Proinflammatory and Proangiogenic Cytokines in Patients with Head and Neck Cancer. *Clin. Cancer Res.* **5**, 1369–1379.

Chen J., and Weiss W. A. (2015). Alternative splicing in cancer: implications for biology and therapy. *Oncogene* **34**, 1–14. doi:10.1038/onc.2013.570

Chen F., Zhuang X., Lin L., Yu P., Wang Y., Shi Y., Hu G., and Sun Y. (2015). New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med.* **13**, 45. doi:10.1186/s12916-015-0278-7

Cheng G., Diebold B. A., Hughes Y., and Lambeth J. D. (2006). Nox1-dependent Reactive Oxygen Generation Is Regulated by Rac1. *J. Biol. Chem.* **281**, 17718–17726. doi:10.1074/jbc.M512751200

Cheng Y., and Tian H. (2017). Current Development Status of MEK Inhibitors. *Molecules* **22**, 1551. doi:10.3390/molecules22101551

Cherfils J., and Zeghouf M. (2013). Regulation of Small GTPases by GEFs, GAPs, and GDIs. *Physiol. Rev.* **93**, 269–309. doi:10.1152/physrev.00003.2012

Chronopoulos A., and Kalluri R. (2020). Emerging role of bacterial extracellular vesicles in cancer. *Oncogene* **39**, 6951–6960. doi:10.1038/s41388-020-01509-3

Chung Y.-C., and Chang Y.-F. (2003). Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J. Surg. Oncol.* **83**, 222–226. doi:10.1002/jso.10269

Clark W. H., Elder D. E., Guerry D., Braitman L. E., Trock B. J., Schultz D., Synnestvedt M., and Halpern A. C. (1989). Model Predicting Survival in Stage I Melanoma Based on Tumor Progression. *J. Natl. Cancer Inst.* **81**, 1893–1904. doi:10.1093/jnci/81.24.1893

Clemente C. G., Mihm M. C., Bufalino R., Zurrida S., Collini P., and Cascinelli N. (1996). Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77**, 8. doi:10.1002/(SICI)1097-0142

Clevers H. (2004). At the crossroads of inflammation and cancer. *Cell* **118**, 671–674. doi:10.1016/j.cell.2004.09.005

Clevers H. (2011). The cancer stem cell: premises, promises and challenges. *Nat. Med.* **17**, 313–319. doi:10.1038/nm.2304

Clevers H. (2013). The Intestinal Crypt, A Prototype Stem Cell Compartment. *Cell* **154**, 274–284. doi:10.1016/j.cell.2013.07.004

Cohen I. (2017). DNA damage talks to inflammation. *Cytokine Growth Factor Rev.* **33**, 35–39. doi:10.1016/j.cytogfr.2016.11.002

Collado M., Gil J., Efeyan A., Guerra C., Schuhmacher A. J., Barradas M., Benguría A., Zaballos A., Flores J. M., Barbacid M., Beach D., and Serrano M. (2005). Senescence in premalignant tumours. *Nature* **436**, 642–642. doi:10.1038/436642a

Colotta F., Allavena P., Sica A., Garlanda C., and Mantovani A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30**, 1073–1081. doi:10.1093/carcin/bgp127

Coronella J. A., Telleman P., Kingsbury G. A., Truong T. D., Hays S., and Junghans R. P. (2001). Evidence for an Antigen-driven Humoral Immune Response in Medullary Ductal Breast Cancer. *Cancer Res.* **61**, 7889–7899.

Coso O. A., Chiariello M., Yu J.-C., Teramoto H., Crespo P., Xu N., and Miki T. (1995). The Small GTP-Binding Proteins Racl, and Cdc42 Regulate the Activity of the JNK/SAPK Signaling Pathway. *Cell* **81**, 1137–1146. doi:10.1016/s0092-8674(05)80018-2

Coussens L. M., and Werb Z. (2002). Inflammation and cancer. *Nature* **420**, 860–867. doi:10.1038/nature01322

Cox T. R., and Erler J. T. (2011). Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Dis. Model. Mech.* **4**, 165–178. doi:10.1242/dmm.004077

Cui J., Shin T., Kawano T., Sato H., Kondo E., Toura I., Kaneko Y., Koseki H., Kanno M., and Taniguchi M. (1997). Requirement for Vα14 NKT Cells in IL-12-Mediated Rejection of Tumors. *Science* **278**, 1623–1626. doi:10.1126/science.278.5343.1623

Curiel T. J., Coukos G., Zou L., Alvarez X., Cheng P., Mottram P., Evdemon-Hogan M., Conejo-Garcia J. R., Zhang L., Burow M., Zhu Y., Wei S., Kryczek I., Daniel B., Gordon A., Myers L., Lackner A., Disis M. L., Knutson K. L., Chen L., and Zou W. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **10**, 942–949. doi:10.1038/nm1093

Czerwinski M., Shroyer N. F., and Spence J. R. (2018). WNT Signaling in the Intestine: Development, Homeostasis, Disease. 'Physiology of the Gastrointestinal Tract'. pp. 185–196. (Elsevier) doi:10.1016/B978-0-12-809954-4.00007-4

Dahlin A. M., Henriksson M. L., Van Guelpen B., Stenling R., Öberg Å., Rutegård J., and Palmqvist R. (2011). Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. *Mod. Pathol.* **24**, 671–682. doi:10.1038/modpathol.2010.234

Dai B., Zhang X., Shang R., Wang J., Yang X., Zhang H., Liu Q., Wang D., Wang L., and Dou K. (2018). Blockade of ARHGAP11A reverses malignant progress via

inactivating Rac1B in hepatocellular carcinoma. *Cell Commun. Signal.* **16**, 99. doi:10.1186/s12964-018-0312-4

Daigneault M., Preston J. A., Marriott H. M., Whyte M. K. B., and Dockrell D. H. (2010). The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages (TM Doherty, Ed.). *PLoS ONE* **5**, e8668. doi:10.1371/journal.pone.0008668

Dave S. S., Gascoyne R. D., Rimsza L. M., Greiner T. C., Armitage J. O., Delabie J., Lister T. A., Ott G., Jaffe E. S., and Goldschmidt N. (2004). Prediction of Survival in Follicular Lymphoma Based on Molecular Features of Tumor-Infiltrating Immune Cells. *N. Engl. J. Med.* **351**, 2159–2169. doi:10.1056/NEJMoa041869

David C. J., and Manley J. L. (2010). Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev.* **24**, 2343–2364. doi:10.1101/gad.1973010

Davies H., Bignell G. R., Cox C., Stephens P., Edkins S., Clegg S., Teague J., Woffendin H., Garnett M. J., Bottomley W., Davis N., Dicks E., Ewing R., Floyd Y., Gray K., Hall S., Hawes R., Hughes J., Kosmidou V., Menzies A., Mould C., Parker A., Stevens C., Watt S., Hooper S., Wilson R., Jayatilake H., Gusterson B. A., Cooper C., Shipley J., Hargrave D., Pritchard-Jones K., Maitland N., Chenevix-Trench G., Riggins G. J., Bigner D. D., Palmieri G., Cossu A., Flanagan A., Nicholson A., Ho J. W. C., Leung S. Y., Yuen S. T., Weber B. L., Seigler H. F., Darrow T. L., Paterson H., Marais R., Marshall C. J., Wooster R., Stratton M. R., and Futreal P. A. (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**, 949–954. doi:10.1038/nature00766

Davies B. R., Logie A., McKay J. S., Martin P., Steele S., Jenkins R., Cockerill M., Cartlidge S., and Smith P. D. (2007). AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. *Mol. Cancer Ther.* **6**, 2209–2219. doi:10.1158/1535-7163.MCT-07-0231

De Bosscher K., Hill C. S., and Nicolás F. J. (2004). Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells. *Biochem. J.* **379**, 209–216. doi:10.1042/BJ20031886

De Palma F., D'Argenio V., Pol J., Kroemer G., Maiuri M., and Salvatore F. (2019). The Molecular Hallmarks of the Serrated Pathway in Colorectal Cancer. *Cancers* **11**, 1017. doi:10.3390/cancers11071017

Dekker E., Tanis P. J., Vleugels J. L. A., Kasi P. M., and Wallace M. B. (2019). Colorectal cancer. *The Lancet* **394**, 1467–1480. doi:10.1016/S0140-6736(19)32319-0

Demirci U., Coskun U., Sancak B., Ozturk B., Bahar B., Benekli M., and Buyukberber S. (2009). Serum Granulocyte Macrophage-Colony Stimulating Factor: a Tumor Marker in Colorectal Carcinoma? *Asian Pac. J. Cancer Prev.* **10**, 1021–1024.

Desmouliere A., Darby I. A., Laverdet B., and Bonté F. (2014). Fibroblasts and myofibroblasts in wound healing. *Clin. Cosmet. Investig. Dermatol.* 301. doi:10.2147/CCID.S50046

Desterro J., Bak-Gordon P., and Carmo-Fonseca M. (2020). Targeting mRNA processing as an anticancer strategy. *Nat. Rev. Drug Discov.* **19**, 112–129. doi:10.1038/s41573-019-0042-3

Devriese S., Van den Bossche L., Van Welden S., Holvoet T., Pinheiro I., Hindryckx P., De Vos M., and Laukens D. (2017). T84 monolayers are superior to Caco-2 as a model system of colonocytes. *Histochem. Cell Biol.* **148**, 85–93. doi:10.1007/s00418-017-1539-7

Dhomen N., Reis-Filho J. S., da Rocha Dias S., Hayward R., Savage K., Delmas V., Larue L., Pritchard C., and Marais R. (2009). Oncogenic Braf Induces Melanocyte Senescence and Melanoma in Mice. *Cancer Cell* **15**, 294–303. doi:10.1016/j.ccr.2009.02.022

Di Modugno F., Iapicca P., Boudreau A., Mottolese M., Terrenato I., Perracchio L., Carstens R. P., Santoni A., Bissell M. J., and Nistico P. (2012). Splicing program of human MENA produces a previously undescribed isoform associated with invasive, mesenchymal-like breast tumors. *Proc. Natl. Acad. Sci.* **109**, 19280–19285. doi:10.1073/pnas.1214394109

DiDonato J. A., Mercurio F., and Karin M. (2012). NF-κB and the link between inflammation and cancer. *Immunol. Rev.* **246**, 379–400. doi:10.1111/j.1600-065X.2012.01099.x

Dieu-Nosjean M.-C., Antoine M., Danel C., Heudes D., Wislez M., Poulot V., Rabbe N., Laurans L., Tartour E., de Chaisemartin L., Lebecque S., Fridman W.-H., and Cadranel J. (2008). Long-Term Survival for Patients With Non–Small-Cell Lung Cancer With Intratumoral Lymphoid Structures. *J. Clin. Oncol.* **26**, 4410–4417. doi:10.1200/JCO.2007.15.0284

Dimri S., Sukanya, and De A. (2017). Approaching non-canonical STAT3 signaling to redefine cancer therapeutic strategy. *Integr. Mol. Med.* **4**, 1–10. doi:10.15761/IMM.1000268

Djelloul S., Forgue-Lafitte M.-E., Hermelin B., Mareel M., Bruyneel E., Baldi A., Giordano A., Chastre E., and Gespach C. (1997). Enterocyte differentiation is compatible with SV40 large T expression and loss of p53 function in human colonic Caco-2 cells: Status of the pRb1 and pRb2 tumor suppressor gene products. *FEBS Lett.* **406**, 234–242. doi:10.1016/S0014-5793(97)00208-1

Dolznig H., Rupp C., Puri C., Haslinger C., Schweifer N., Wieser E., Kerjaschki D., and Garin-Chesa P. (2011). Modeling Colon Adenocarcinomas in Vitro. *Am. J. Pathol.* **179**, 487–501. doi:10.1016/j.ajpath.2011.03.015

Döme B., Hendrix M. J. C., Paku S., Tóvári J., and Tímár J. (2007). Alternative Vascularization Mechanisms in Cancer. *Am. J. Pathol.* **170**, 1–15. doi:10.2353/ajpath.2007.060302

Donato R. P., El-Merhibi A., Gundsambuu B., Mak K. Y., Formosa E. R., Wang X., Abbott C. A., and Powell B. C. (2011). Studying Permeability in a Commonly Used Epithelial Cell Line: T84 Intestinal Epithelial Cells. 'Permeability Barrier'. (Ed K Turksen) Methods in Molecular Biology. pp. 115–137. (Humana Press: Totowa, NJ) doi:10.1007/978-1-61779-191-8_8

Downward J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **3**, 11–22. doi:10.1038/nrc969

Downward J. (2009). A tumour gene's fatal flaws. *Nature* **462**, 44–45. doi:10.1038/462044a

Drolia R., Tenguria S., Durkes A. C., Turner J. R., and Bhunia A. K. (2019). Listeria Adhesion Protein Induces Intestinal Epithelial Barrier Dysfunction for Bacterial Translocation. *Cell Host Microbe* **23**, 470–484. doi:10.1016/j.chom.2018.03.004

Ducreux M., Chamseddine A., Laurent-Puig P., Smolenschi C., Hollebecque A., Dartigues P., Samallin E., Boige V., Malka D., and Gelli M. (2019). Molecular targeted therapy of BRAF-mutant colorectal cancer. *Ther. Adv. Med. Oncol.* **11**, 175883591985649. doi:10.1177/1758835919856494

Duronio R. J., and Xiong Y. (2013). Signaling Pathways that Control Cell Proliferation. *Cold Spring Harb. Perspect. Biol.* **5**, a008904–a008904. doi:10.1101/cshperspect.a008904

Eaden J. A., Abrams K. R., and Mayberry J. F. (2001). The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* **48**, 526–535. doi:10.1136/gut.48.4.526

Early P., Rogers J., Davis M., Calame K., Bond M., Wall R., and Hood L. (1980). Two mRNAs Can Be Produced from a Single lmmunoglobulin p Gene by Alternative RNA Processing Pathways. *Cell* **20**, 313–319. doi:10.1016/0092-8674(80)90617-0

Eden S., Rohatgi R., Podtelejnikov A. V., Mann M., and Kirschner M. W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* **418**, 790–793. doi:10.1038/nature00859

Edwards J. P., Zhang X., Frauwirth K. A., and Mosser D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. *J. Leukoc. Biol.* **80**, 1298–1307. doi:10.1189/jlb.0406249

Egeblad M., and Werb Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2**, 161–174. doi:10.1038/nrc745

Elaraj D. M. (2006). The Role of Interleukin 1 in Growth and Metastasis of Human Cancer Xenografts. *Clin. Cancer Res.* **12**, 1088–1096. doi:10.1158/1078-0432.CCR-05-1603

Elimrani I., Dionne S., Saragosti D., Qureshi I., Levy E., Delvin E., and Seidman E. G. (2015). Acetylcarnitine potentiates the anticarcinogenic effects of butyrate on SW480 colon cancer cells. *Int. J. Oncol.* **47**, 755–763. doi:10.3892/ijo.2015.3029

El-Serag H. B. (2012). Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterology* **142**, 1264-1273.e1. doi:10.1053/j.gastro.2011.12.061

Esufali S., Charames G. S., Pethe V. V., Buongiorno P., and Bapat B. (2007). Activation of Tumor-Specific Splice Variant Rac1b by Dishevelled Promotes Canonical Wnt Signaling and Decreased Adhesion of Colorectal Cancer Cells. *Cancer Res.* **67**, 2469–2479. doi:10.1158/0008-5472.CAN-06-2843

Etemadmoghadam D., Weir B. A., Au-Yeung G., Alsop K., Mitchell G., George J., Australian Ovarian Cancer Study Group, Davis S., D'Andrea A. D., Simpson K., Hahn W. C., and Bowtell D. D. L. (2013). Synthetic lethality between CCNE1 amplification and loss of BRCA1. *Proc. Natl. Acad. Sci.* **110**, 19489–19494. doi:10.1073/pnas.1314302110

Etienne-Manneville S., and Hall A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629–635. doi:10.1038/nature01148

Fakhoury M., Al-Salami H., Negrulj R., and Mooranian A. (2014). Inflammatory bowel disease: clinical aspects and treatments. *J. Inflamm. Res.* **7**, 113–120. doi:10.2147/JIR.S65979

Faria M., Capinha L., Simões-Pereira J., Bugalho M. J., and Silva A. L. (2016). Extending the Impact of RAC1b Overexpression to Follicular Thyroid Carcinomas. *Int. J. Endocrinol.* **2016**, 1–6. doi:10.1155/2016/1972367

Faria M., Matos P., Pereira T., Cabrera R., Cardoso B. A., Bugalho M. J., and Silva A. L. (2017). RAC1b overexpression stimulates proliferation and NF-kB-mediated anti-apoptotic signaling in thyroid cancer cells. *PLOS ONE* **12**, e0172689. doi:10.1371/journal.pone.0172689

Feagins L. A., Souza R. F., and Spechler S. J. (2009). Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. *Nat. Rev. Gastroenterol. Hepatol.* **6**, 297–305. doi:10.1038/nrgastro.2009.44

Fearon E. R., and Vogelstein B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**, 759–767. doi:10.1016/0092-8674(90)90186-I

Ferlay J., Colombet M., Soerjomataram I., Mathers C., Parkin D. M., Piñeros M., Znaor A., and Bray F. (2019). Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int. J. Cancer* **144**, 1941–1953. doi:10.1002/ijc.31937

Ferraro D. A., Patella F., Zanivan S., Donato C., Aceto N., Giannotta M., Dejana E., Diepenbruck M., Christofori G., and Buess M. (2019). Endothelial cell-derived nidogen-1 inhibits migration of SK-BR-3 breast cancer cells. *BMC Cancer* **19**, 312. doi:10.1186/s12885-019-5521-8

Fichtner-Feigl S. (2005). Treatment of murine Th1- and Th2-mediated inflammatory bowel disease with NF- B decoy oligonucleotides. *J. Clin. Invest.* **115**, 3057–3071. doi:10.1172/JCI24792

Fiegen D., Haeusler L.-C., Blumenstein L., Herbrand U., Dvorsky R., Vetter I. R., and Ahmadian M. R. (2004). Alternative Splicing of Rac1 Generates Rac1b, a Self-activating GTPase. *J. Biol. Chem.* **279**, 4743–4749. doi:10.1074/jbc.M310281200

Flanagan D., Austin C., Vincan E., and Phesse T. (2018). Wnt Signalling in Gastrointestinal Epithelial Stem Cells. *Genes* **9**, 178. doi:10.3390/genes9040178

Fleming M., Ravula S., Tatishchev S. F., and Wang H. L. (2012). Colorectal carcinoma: Pathologic aspects. *J. Gastrointest. Oncol.* **3**, 153–173. doi:10.3978/j.issn.2078-6891.2012.030

Florholmen J., and Fries W. (2011). Candidate mucosal and surrogate biomarkers of inflammatory bowel disease in the era of new technology. *Scand. J. Gastroenterol.* **46**, 1407–1417. doi:10.3109/00365521.2011.627449

Forbes S. A., Bindal N., Bamford S., Cole C., Kok C. Y., Beare D., Jia M., Shepherd R., Leung K., Menzies A., Teague J. W., Campbell P. J., Stratton M. R., and Futreal P. A. (2011). COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res.* **39**, D945–D950. doi:10.1093/nar/gkq929

Forrester M. A., Wassall H. J., Hall L. S., Cao H., Wilson H. M., Barker R. N., and Vickers M. A. (2018). Similarities and differences in surface receptor expression by THP-1 monocytes and differentiated macrophages polarized using seven different conditioning regimens. *Cell. Immunol.* **332**, 58–76. doi:10.1016/j.cellimm.2018.07.008

Fouad Y. A., and Aanei C. (2017). Revisiting the hallmarks of cancer. *Am J Cancer Res* **7**, 1016–1036. doi:ajcr0053932

Fox J. G., Dewhirst F. E., Shen Z., Feng Y., Taylor N. S., Paster B. J., Ericson R. L., Lau C. N., Correa P., Araya J. C., and Roa I. (1998). Hepatic Helicobacter species identified in bile and gallbladder tissue from chileans with chronic cholecystitis. *Gastroenterology* **114**, 755–763. doi:10.1016/S0016-5085(98)70589-X

Franco-Barraza J., Francescone R., Luong T., Shah N., Madhani R., Cukierman G., Dulaimi E., Devarajan K., Egleston B. L., Nicolas E., Katherine Alpaugh R., Malik R., Uzzo R. G., Hoffman J. P., Golemis E. A., and Cukierman E. (2017). Matrix-regulated integrin αvβ5 maintains α5β1-dependent desmoplastic traits prognostic of neoplastic recurrence. *eLife* **6**, e20600. doi:10.7554/eLife.20600

Frankiw L., Baltimore D., and Li G. (2019). Alternative mRNA splicing in cancer immunotherapy. *Nat. Rev. Immunol.* **19**, 675–687. doi:10.1038/s41577-019-0195-7

Freeman T. J., Smith J. J., Chen X., Washington M. K., Roland J. T., Means A. L., Eschrich S. A., Yeatman T. J., Deane N. G., and Beauchamp R. D. (2012). Smad4-Mediated Signaling Inhibits Intestinal Neoplasia by Inhibiting Expression of β-Catenin. *Gastroenterology* **142**, 562-571.e2. doi:10.1053/j.gastro.2011.11.026

Fridman W. H., Pagès F., Sautès-Fridman C., and Galon J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev. Cancer* **12**, 298–306. doi:10.1038/nrc3245

Galon J., Costes A., Sanchez-Cabo F., and Franck P. (2006). Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science* **313**, 1960–1964. doi:10.1126/science.1129139

Garraway L. A., and Lander E. S. (2013). Lessons from the Cancer Genome. *Cell* **153**, 17–37. doi:10.1016/j.cell.2013.03.002

Gehart H., and Clevers H. (2019). Tales from the crypt: new insights into intestinal stem cells. *Nat. Rev. Gastroenterol. Hepatol.* **16**, 19–34. doi:10.1038/s41575-018-0081-y

Gehler S., Baldassarre M., Lad Y., Leight J. L., Wozniak M. A., Riching K. M., Eliceiri K. W., Weaver V. M., Calderwood D. A., and Keely P. J. (2009). Filamin A–alpha1 Integrin Complex Tunes Epithelial Cell Response to Matrix Tension. *Mol. Biol. Cell* **20**, 3224–3238. doi:10.1091/mbc.e08-12-1186

Gemma A., Takenaka K., Hosoya Y., Matuda K., Seike M., Kurimoto F., Ono Y., Uematsu K., Takeda Y., Hibino S., Yoshimura A., Shibuya M., and Kudoh S. (2001). Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line. *Eur. J. Cancer* **37**, 1554–1561. doi:10.1016/S0959-8049(01)00154-X

Genin M., Clement F., Fattaccioli A., Raes M., and Michiels C. (2015). M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer* **15**, 577. doi:10.1186/s12885-015-1546-9

Ghigna C., Valacca C., and Biamonti G. (2008). Alternative Splicing and Tumor Progression. *Curr. Genomics* **9**, 556–570. doi:10.2174/138920208786847971

Ginhoux F., and Jung S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* **14**, 392–404. doi:10.1038/nri3671

Giraldo N. A., Sanchez-Salas R., Peske J. D., Vano Y., Becht E., Petitprez F., Validire P., Ingels A., Cathelineau X., Fridman W. H., and Sautès-Fridman C. (2019). The clinical role of the TME in solid cancer. *Br. J. Cancer* **120**, 45–53. doi:10.1038/s41416-018-0327-z

Gonçalves V., Henriques A., Pereira J., Neves Costa A., Moyer M. P., Moita L. F., Gama-Carvalho M., Matos P., and Jordan P. (2014). Phosphorylation of SRSF1 by SRPK1 regulates alternative splicing of tumor-related Rac1b in colorectal cells. *RNA* **20**, 474–482. doi:10.1261/rna.041376.113

Gonçalves V., Matos P., and Jordan P. (2009). Antagonistic SR proteins regulate alternative splicing of tumor-related Rac1b downstream of the PI3-kinase and Wnt pathways. *Hum. Mol. Genet.* **18**, 3696–3707. doi:10.1093/hmg/ddp317

Gonçalves V., Pereira J. F., and Jordan P. (2017). Signaling Pathways Driving Aberrant Splicing in Cancer Cells. *Genes* **9**, 9. doi:10.3390/genes9010009

Gopalakrishnan V., Spencer C. N., Nezi L., Reuben A., Andrews M. C., Karpinets T. V., Prieto P. A., Vicente D., Hoffman K., Wei S. C., Cogdill A. P., Zhao L., Hudgens C. W., Hutchinson D. S., Manzo T., Petaccia de Macedo M., Cotechini T., Kumar T., Chen W. S., Reddy S. M., Szczepaniak Sloane R., Galloway-Pena J., Jiang H., Chen P. L., Shpall E. J., Rezvani K., Alousi A. M., Chemaly R. F., Shelburne S., Vence L. M., Okhuysen P. C., Jensen V. B., Swennes A. G., McAllister F., Marcelo Riquelme Sanchez E., Zhang Y., Le Chatelier E., Zitvogel L., Pons N., Austin-Breneman J. L., Haydu L. E., Burton E. M., Gardner J. M., Sirmans E., Hu J., Lazar A. J., Tsujikawa T., Diab A., Tawbi H., Glitza I. C., Hwu W. J., Patel S. P., Woodman S. E., Amaria R. N., Davies M. A., Gershenwald J. E., Hwu P., Lee J. E., Zhang J., Coussens L. M., Cooper Z. A., Futreal P. A., Daniel C. R., Ajami N. J., Petrosino J. F., Tetzlaff M. T., Sharma P., Allison J. P., Jenq R. R., and Wargo J. A. (2018). Gut microbiome modulates response to anti–PD-1 immunotherapy in melanoma patients. *Science* **359**, 97–103. doi:10.1126/science.aan4236

Grasso S., Tristante E., Saceda M., Carbonell P., Mayor-López L., Carballo-Santana M., Carrasco-García E., Rocamora-Reverte L., García-Morales P., Carballo F., Ferragut J. A., and Martínez-Lacaci I. (2014). Resistance to Selumetinib (AZD6244) in Colorectal Cancer Cell Lines is Mediated by p70S6K and RPS6 Activation. *Neoplasia* **16**, 845–860. doi:10.1016/j.neo.2014.08.011

Greten F. R., Eckmann L., Greten T. F., Park J. M., Li Z.-W., Egan L. J., Kagnoff M. F., and Karin M. (2004). IKKβ Links Inflammation and Tumorigenesis in a Mouse Model of Colitis-Associated Cancer. *Cell* **118**, 285–296. doi:10.1016/j.cell.2004.07.013

Greten F. R., and Grivennikov S. I. (2019). Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* **51**, 27–41. doi:10.1016/j.immuni.2019.06.025

Grivennikov S. I., Greten F. R., and Karin M. (2010). Immunity, Inflammation, and Cancer. *Cell* **140**, 883–899. doi:10.1016/j.cell.2010.01.025

Grivennikov S., Karin E., Terzic J., Mucida D., Yu G.-Y., Vallabhapurapu S., Scheller J., Rose-John S., Cheroutre H., Eckmann L., and Karin M. (2009). IL-6 and Stat3

Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer. *Cancer Cell* **15**, 103–113. doi:10.1016/j.ccr.2009.01.001

Guinney J., Dienstmann R., Wang X., de Reyniès A., Schlicker A., Soneson C., Marisa L., Roepman P., Nyamundanda G., Angelino P., Bot B. M., Morris J. S., Simon I. M., Gerster S., Fessler E., De Sousa E Melo F., Missiaglia E., Ramay H., Barras D., Homicsko K., Maru D., Manyam G. C., Broom B., Boige V., Perez-Villamil B., Laderas T., Salazar R., Gray J. W., Hanahan D., Tabernero J., Bernards R., Friend S. H., Laurent-Puig P., Medema J. P., Sadanandam A., Wessels L., Delorenzi M., Kopetz S., Vermeulen L., and Tejpar S. (2015). The consensus molecular subtypes of colorectal cancer. *Nat. Med.* **21**, 1350–1356. doi:10.1038/nm.3967

Guo Y., Pan W., Liu S., Shen Z., Xu Y., and Hu L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Exp. Ther. Med.* **19**, 1997–2007. doi:10.3892/etm.2020.8454

Gupta R. B., Harpaz N., Itzkowitz S., Hossain S., Matula S., Bodian C., and Ullman T. (2008). Histologic Inflammation Is a Risk Factor for Progression to Colorectal Neoplasia in Ulcerative Colitis: A Cohort Study. *Gastroenterology* **133**, 1099–1341. doi:10.1053/j.gastro.2007.08.001

Guttridge D. C., Albanese C., Reuther J. Y., Pestell R. G., and Baldwin A. S. (1999). NF-kB Controls Cell Growth and Differentiation through Transcriptional Regulation of Cyclin D. *Mol. Cell. Biol.* **19**, 5785–5799. doi:10.1128/mcb.19.8.5785

Gyorgy B., Szabo T. G., Pasztoi M., Pal Z., Misjak P., Aradi B., Laszlo V., and Pap E. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell. Mol. Life Sci.* **68**, 2667–2688. doi:10.1007/s00018-011-0689-3

Hall A. (1998). Rho GTPases and the Actin Cytoskeleton. *Science* **279**, 509–514. doi:10.1126/science.279.5350.509

Hanahan D., and Weinberg R. A. (2000). The Hallmarks of Cancer. *Cell* **100**, 57–70. doi:10.1016/S0092-8674(00)81683-9

Hanahan D., and Weinberg R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674. doi:10.1016/j.cell.2011.02.013

Harvey A. J. (2019). Overview of Cell Signaling Pathways in Cancer. 'Predictive Biomarkers in Oncology'. (Eds S Badve and GL Kumar) pp. 167–182. (Springer International Publishing: Cham) doi:10.1007/978-3-319-95228-4_12

Harvey K. F., and Hariharan I. K. (2012). The Hippo Pathway. *Cold Spring Harb. Perspect. Biol.* **4**, a011288–a011288. doi:10.1101/cshperspect.a011288

Harvey K. F., Zhang X., and Thomas D. M. (2013). The Hippo pathway and human cancer. *Nat. Rev. Cancer* **13**, 246–257. doi:10.1038/nrc3458

Hassanpour S. H., and Dehghani M. (2017). Review of cancer from perspective of molecular. *J. Cancer Res. Pract.* **4**, 127–129. doi:10.1016/j.jcrpr.2017.07.001

Hay M., Thomas D. W., Craighead J. L., Economides C., and Rosenthal J. (2014). Clinical development success rates for investigational drugs. *Nat. Biotechnol.* **32**, 40–51. doi:10.1038/nbt.2786

Hazan-Halevy I., Harris D., Liu Z., Liu J., Li P., Chen X., Shanker S., Ferrajoli A., Keating M. J., and Estrov Z. (2010). STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. *Blood* **115**, 2852–2863. doi:10.1182/blood-2009-10-230060

Henriques A. F. A., Barros P., Moyer M. P., Matos P., and Jordan P. (2015). Expression of tumor-related Rac1b antagonizes B-Raf-induced senescence in colorectal cells. *Cancer Lett.* **369**, 368–375. doi:10.1016/j.canlet.2015.08.027

Hickman J. A., Graeser R., de Hoogt R., Vidic S., Brito C., Gutekunst M., van der Kuip H., and IMI PREDECT consortium (2014). Three-dimensional models of cancer for pharmacology and cancer cell biology: Capturing tumor complexity in vitro/ex vivo. *Biotechnol. J.* **9**, 1115–1128. doi:10.1002/biot.201300492

Hida K., Maishi N., Annan D., and Hida Y. (2018). Contribution of Tumor Endothelial Cells in Cancer Progression. *Int. J. Mol. Sci.* **19**, 1272. doi:10.3390/ijms19051272

Hinck L., and Näthke I. (2014). Changes in cell and tissue organization in cancer of the breast and colon. *Curr. Opin. Cell Biol.* **26**, 87–95. doi:10.1016/j.ceb.2013.11.003

Hinz M., Krappmann D., Eichten A., Heder A., Scheidereit C., and Strauss M. (1999). NF-kB Function in Growth Control: Regulation of Cyclin D1 Expression and G0/G1-to-S-Phase Transition. *Mol. Cell. Biol.* **19**, 9. doi:10.1128/mcb.19.4.2690

Hiraoka N., Onozato K., Kosuge T., and Hirohashi S. (2006). Prevalence of FOXP3+ Regulatory T Cells Increases During the Progression of Pancreatic Ductal Adenocarcinoma and Its Premalignant Lesions. *Clin. Cancer Res.* **12**, 5423–5434. doi:10.1158/1078-0432.CCR-06-0369

Hirsch D., Barker N., McNeil N., Hu Y., Camps J., McKinnon K., Clevers H., Ried T., and Gaiser T. (2014). LGR5 positivity defines stem-like cells in colorectal cancer. *Carcinogenesis* **35**, 849–858. doi:10.1093/carcin/bgt377

Hodge R. G., and Ridley A. J. (2016). Regulating Rho GTPases and their regulators. *Nat. Rev. Mol. Cell Biol.* **17**, 496–510. doi:10.1038/nrm.2016.67

Hodi F. S., O'Day S. J., McDermott D. F., Weber R. W., Sosman J. A., Haanen J. B., Gonzalez R., Robert C., Schadendorf D., Hassel J. C., Akerley W., van den Eertwegh A. J. M., Lutzky J., Lorigan P., Vaubel J. M., Linette G. P., Hogg D., Ottensmeier C. H., Lebbé C., Peschel C., Quirt I., Clark J. I., Wolchok J. D., Weber J. S., Tian J., Yellin M. J., Nichol G. M., Hoos A., and Urba W. J. (2010). Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N. Engl. J. Med.* **363**, 711–723. doi:10.1056/NEJMoa1003466

Hong I.-S. (2016). Stimulatory versus suppressive effects of GM-CSF on tumor progression in multiple cancer types. *Exp. Mol. Med.* **48**, e242–e242. doi:10.1038/emm.2016.64

Hoshino R., Chatani Y., Yamori T., Tsuruo T., Oka H., Yoshida O., Shimada Y., Ari-i S., Wada H., Fujimoto J., and Kohno M. (1999). Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18**, 813–822. doi:10.1038/sj.onc.1202367

Hurley B. P., Pirzai W., Eaton A. D., Harper M., Roper J., Zimmermann C., Ladics G. S., Layton R. J., and Delaney B. (2016). An experimental platform using human intestinal epithelial cell lines to differentiate between hazardous and non-hazardous proteins. *Food Chem. Toxicol.* **92**, 75–87. doi:10.1016/j.fct.2016.04.003

Hynes R. O. (2009). The Extracellular Matrix: Not Just Pretty Fibrils. *Science* **326**, 1216–1219. doi:10.1126/science.1176009

Iizuka M. (2011). Wound healing of intestinal epithelial cells. *World J. Gastroenterol.* **17**, 2161. doi:10.3748/wjg.v17.i17.2161

Ilan N., Elkin M., and Vlodavsky I. (2006). Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int. J. Biochem. Cell Biol.* **38**, 2018–2039. doi:10.1016/j.biocel.2006.06.004

Ilyas M., Tomlinson I. P. M., Rowan A., Pignatelli M., and Bodmer W. F. (1997). Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc. Natl. Acad. Sci.* **94**, 10330–10334. doi:10.1073/pnas.94.19.10330

Italiani P., and Boraschi D. (2014). From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front. Immunol.* **5**, 22. doi:10.3389/fimmu.2014.00514

Ito N., Suzuki Y., Taniguchi Y., Ishiguro K., Nakamura H., and Ohgi S. (2005). Prognostic Significance of T Helper 1 and 2 and T Cytotoxic 1 and 2 Cells in Patients with Non-small Cell Lung Cancer. *Anticancer Res.* **25**, 2027–2032.

Iyer P., Moslim M., Farma J. M., and Denlinger C. S. (2020). Diffuse gastric cancer: histologic, molecular, and genetic basis of disease. *Transl. Gastroenterol. Hepatol.* **5**, 52–52. doi:10.21037/tgh.2020.01.02

Jaffe A. B., and Hall A. (2005). RHO GTPASES: Biochemistry and Biology. *Annu. Rev. Cell Dev. Biol.* **21**, 247–269. doi:10.1146/annurev.cellbio.21.020604.150721

Jain R. K. (2005). Normalization of Tumor Vasculature: An Emerging Concept in Antiangiogenic Therapy. *Science* **307**, 58–62. doi:10.1126/science.1104819

Jass J. R. (1986). Lymphocytic infiltration and survival in rectal cancer. *J. Clin. Pathol.* **39**, 585–589. doi:10.1136/jcp.39.6.585

Jass J. R. (2007). Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* **50**, 113–130. doi:10.1111/j.1365-2559.2006.02549.x

Jess T., Loftus E. V., Velayos F. S., Harmsen W. S., Zinsmeister A. R., Smyrk T. C., Schleck C. D., Tremaine W. J., Melton L. J., Munkholm P., and Sandborn W. J. (2006). Risk of Intestinal Cancer in Inflammatory Bowel Disease: A Population-Based Study From Olmsted County, Minnesota. *Gastroenterology* **130**, 1039–1046. doi:10.1053/j.gastro.2005.12.037

Jiang B., and Liu L. (2009). Chapter 2 PI3K/PTEN Signaling in Angiogenesis and Tumorigenesis. 'Advances in Cancer Research'. pp. 19–65. (Elsevier) doi:10.1016/S0065-230X(09)02002-8

Jin W.-J., Xu J.-M., Xu W.-L., Gu D.-H., and Li P.-W. (2014). Diagnostic value of interleukin-8 in colorectal cancer: A case-control study and meta-analysis. *World J. Gastroenterol.* **20**, 16334. doi:10.3748/wjg.v20.i43.16334

Johnson D. E., O'Keefe R. A., and Grandis J. R. (2018). Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat. Rev. Clin. Oncol.* **15**, 234–248. doi:10.1038/nrclinonc.2018.8

Joneson T., and Bar-Sagi D. (1998). A Rac1 Effector Site Controlling Mitogenesis through Superoxide Production. *J. Biol. Chem.* **273**, 17991–17994. doi:10.1074/jbc.273.29.17991

Jordan P. (2018). Colorectal Cancer Subtypes – The Current Portrait. 'Targeted Therapy of Colorectal Cancer Subtypes'. (Ed P Jordan) pp. 1–6. (Springer International Publishing: Cham) doi:10.1007/978-3-030-02771-1_1

Jordan P., Brazão R., Boavida M. G., Gespach C., and Chastre E. (1999). Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. *Oncogene* **18**, 6835–6839. doi:10.1038/sj.onc.1203233

Joyce D., Bouzahzah B., Fu M., Albanese C., D'Amico M., Steer J., Klein J. U., Lee R. J., Segall J. E., Westwick J. K., Der C. J., and Pestell R. G. (1999). Integration of Rac-dependent Regulation of Cyclin D1 Transcription through a Nuclear Factor-kB-dependent Pathway. *J. Biol. Chem.* **274**, 6. doi:10.1074/jbc.274.36.25245

Junttila M. R., and de Sauvage F. J. (2013). Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* **501**, 346–354. doi:10.1038/nature12626

Kalluri R. (2016). The biology and function of fibroblasts in cancer. *Nat. Rev. Cancer* **16**, 582–598. doi:10.1038/nrc.2016.73

Kalluri R., and Zeisberg M. (2006). Fibroblasts in cancer. *Nat. Rev. Cancer* **6**, 392–401. doi:10.1038/nrc1877

Karin M., and Greten F. R. (2005). NF-κB: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* **5**, 749–759. doi:10.1038/nri1703

Kass L., Erler J. T., Dembo M., and Weaver V. M. (2007). Mammary epithelial cell: Influence of extracellular matrix composition and organization during development and tumorigenesis. *Int. J. Biochem. Cell Biol.* **39**, 1987–1994. doi:10.1016/j.biocel.2007.06.025

Kawai O., Ishii G., Kubota K., Murata Y., Naito Y., Mizuno T., Aokage K., Saijo N., Nishiwaki Y., Gemma A., Kudoh S., and Ochiai A. (2008). Predominant infiltration of macrophages and CD8 $^{+}$ T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. *Cancer* **113**, 1387–1395. doi:10.1002/cncr.23712

Kawai S., Yamazaki M., Shibuya K., Yamazaki M., Fujii E., Nakano K., and Suzuki M. (2020). Three-dimensional culture models mimic colon cancer heterogeneity induced by different microenvironments. *Sci. Rep.* **10**, 3156. doi:10.1038/s41598-020-60145-9

Kawanishi S., Hiraku Y., Pinlaor S., and Ma N. (2006). Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to

inflammation-related carcinogenesis. *Biol. Chem.* **387**, 365–372. doi:10.1515/BC.2006.049

Kawano T., Nakayama T., Kamada N., Kaneko Y., Harada M., Ogura N., Akutsu Y., Motohashi S., Iizasa T., Endo H., Fujisawa T., Shinkai H., and Taniguchi M. (1999). Antitumor Cytotoxicity Mediated by Ligand-activated Human Vα24 NKT Cells. *Cancer Res.* **59**, 5102–5105.

Keats J. J., Fonseca R., Chesi M., Schop R., Baker A., Chng W.-J., Wier S. V., Tiedemann R., Shi C.-X., Sebag M., Braggio E., Henry T., Zhu Y.-X., Fogle H., Price-Troska T., Ahmann G., Mancini C., Brents L. A., Kumar S., Greipp P., Dispenzieri A., Bryant B., Mulligan G., Bruhn L., Barrett M., Valdez R., Trent J., Stewart A. K., Carpten J., and Bergsagel P. L. (2008). Promiscuous Mutations Activate the Non-Canonical NF-kB Pathway in Multiple Myeloma. *Cancer Cell* **12**, 131–144. doi:10.1016/j.ccr.2007.07.003

Kessenbrock K., Plaks V., and Werb Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* **141**, 52–67. doi:10.1016/j.cell.2010.03.015

Kim E. K., and Choi E.-J. (2015). Compromised MAPK signaling in human diseases: an update. *Arch. Toxicol.* **89**, 867–882. doi:10.1007/s00204-015-1472-2

Kim H. J., and Ingber D. E. (2013). Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation. *Integr. Biol.* **5**, 1130. doi:10.1039/c3ib40126j

Kim H. K., Pham M. H. C., Ko K. S., Rhee B. D., and Han J. (2018). Alternative splicing isoforms in health and disease. *Pflüg. Arch. - Eur. J. Physiol.* **470**, 995–1016. doi:10.1007/s00424-018-2136-x

Kinoshita T., Ito H., and Miki C. (1999). Serum interleukin-6 level reflects the tumor proliferative activity in patients with colorectal carcinoma. *Am. Cancer Soc.* **85**, 2526–2531. doi:10.1002/(sici)1097-0142(19990615)85:12<2526::aid-cncr6>3.0.co;2-3

Kirkwood J. M., Bastholt L., Robert C., Sosman J., Larkin J., Hersey P., Middleton M., Cantarini M., Zazulina V., Kemsley K., and Dummer R. (2012). Phase II, Open-Label, Randomized Trial of the MEK1/2 Inhibitor Selumetinib as Monotherapy versus Temozolomide in Patients with Advanced Melanoma. *Clin. Cancer Res.* **18**, 555–567. doi:10.1158/1078-0432.CCR-11-1491

Kitamura H., Ohno Y., Toyoshima Y., Ohtake J., Homma S., Kawamura H., Takahashi N., and Taketomi A. (2017). Interleukin-6/STAT3 signaling as a promising

target to improve the efficacy of cancer immunotherapy. *Cancer Sci.* **108**, 1947–1952. doi:10.1111/cas.13332

Klein E. A., and Assoian R. K. (2008). Transcriptional regulation of the cyclin D1 gene at a glance. *J. Cell Sci.* **121**, 3853–3857. doi:10.1242/jcs.039131

Klimp A. H., de Vries E. G. E., Scherphof G. L., and Daemen T. (2002). A potential role of macrophage activation in the treatment of cancer. *Crit. Rev. Oncol. Hematol.* **44**, 143–161. doi:10.1016/S1040-8428(01)00203-7

Klinck R., Bramard A., Inkel L., Dufresne-Martin G., Gervais-Bird J., Madden R., Paquet E. R., Koh C., Venables J. P., Prinos P., Jilaveanu-Pelmus M., Wellinger R., Rancourt C., Chabot B., and Abou Elela S. (2008). Multiple Alternative Splicing Markers for Ovarian Cancer. *Cancer Res.* **68**, 657–663. doi:10.1158/0008-5472.CAN-07-2580

Knight E., and Przyborski S. (2015). Advances in 3D cell culture technologies enabling tissue-like structures to be created *in vitro*. *J. Anat.* **227**, 746–756. doi:10.1111/joa.12257

Kollmar O., Junker B., Rupertus K., Menger M. D., and Schilling M. K. (2007). Studies on MIP-2 and CXCR2 expression in a mouse model of extrahepatic colorectal metastasis. *Eur. J. Surg. Oncol. EJSO* **33**, 803–811. doi:10.1016/j.ejso.2007.01.012

Komoda H., Tanaka Y., Honda M., Matsuo Y., Hazama K., and Takao T. (1998). Interleukin-6 Levels in Colorectal Cancer Tissues. *World J. Surg.* **22**, 895–898. doi:10.1007/s002689900489

Konishi N., Miki C., Yoshida T., Tanaka K., Toiyama Y., and Kusunoki M. (2005). Interleukin-1 Receptor Antagonist Inhibits the Expression of Vascular Endothelial Growth Factor in Colorectal Carcinoma. *Oncology* **68**, 138–145. doi:10.1159/000086768

Koreishi A. F., Saenz A. J., Persky D. O., Cui H., Moskowitz A., Moskowitz C. H., and Teruya-Feldstein J. (2010). The Role of Cytotoxic and Regulatory T cells in Relapsed/Refractory Hodgkin Lymphoma: *Appl. Immunohistochem. Mol. Morphol.* **18**, 206–211. doi:10.1097/PAI.0b013e3181c7138b

Korgaonkar N., and Yadav K. S. (2019). Understanding the biology and advent of physics of cancer with perspicacity in current treatment therapy. *Life Sci.* **239**, 117060. doi:10.1016/j.lfs.2019.117060

Kotelevets L., Walker F., Mamadou G., Lehy T., Jordan P., and Chastre E. (2018). The Rac1 splice form Rac1b favors mouse colonic mucosa regeneration and contributes

to intestinal cancer progression. *Oncogene* **37**, 6054–6068. doi:10.1038/s41388-018-0389-7

Koukourakis M., Giatromanolaki A., Kakolyris S., O'Byrne K., Apostolikas N., Skarlatos J., Gatter K., and Harris A. (1998). Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. *Br. J. Cancer* **77**, 1696–1703. doi:10.1038/bjc.1998.280

Koveitypour Z., Panahi F., Vakilian M., Peymani M., Seyed Forootan F., Nasr Esfahani M. H., and Ghaedi K. (2019). Signaling pathways involved in colorectal cancer progression. *Cell Biosci.* **9**, 97. doi:10.1186/s13578-019-0361-4

Krieger J. N., Riley D. E., Vesella R. L., Miner D. C., Ross S. O., and Lange P. H. (2000). Bacterial dna sequences in prostate tissue from patients with prostate cancer and chronic prostatitis. *J. Urol.* **164**, 1221–1228. doi:0022-5347/00/1644-1221/0

Kriegl L., Neumann J., Vieth M., Greten F. R., Reu S., Jung A., and Kirchner T. (2011). Up and downregulation of p16Ink4a expression in BRAF-mutated polyps/adenomas indicates a senescence barrier in the serrated route to colon cancer. *Mod. Pathol.* **24**, 1015–1022. doi:10.1038/modpathol.2011.43

Kundu J., and Surh Y. (2008). Inflammation: Gearing the journey to cancer. *Mutat. Res. Mutat. Res.* **659**, 15–30. doi:10.1016/j.mrrev.2008.03.002

Kwon Y., Kim Y., Eom S., Kim M., Park D., Kim H., Noh K., Lee H., Lee Y. S., Choe J., Kim Y. M., and Jeoung D. (2015). MicroRNA-26a/-26b-COX-2-MIP-2 Loop Regulates Allergic Inflammation and Allergic Inflammation-promoted Enhanced Tumorigenic and Metastatic Potential of Cancer Cells. *J Biol Chem* **290**, 14245–14266. doi:10.1074/jbc.M115.645580

Lam B. D., and Hordijk P. L. (2013). The Rac1 hypervariable region in targeting and signaling: A tail of many stories. *Small GTPases* **4**, 78–89. doi:10.4161/sgtp.23310

Lamarche N., Tapon N., Stowers L., Burbelo P. D., Aspenström P., Bridges T., Chant J., and Hall A. (1996). Rac and Cdc42 Induce Actin Polymerization and G1 Cell Cycle Progression Independently of p65PAK and the JNK/SAPK MAP Kinase Cascade. *Cell* **87**, 519–529. doi:10.1016/S0092-8674(00)81371-9

Landskron G., De la Fuente M., Thuwajit P., Thuwajit C., and Hermoso M. A. (2014). Chronic Inflammation and Cytokines in the Tumor Microenvironment. *J. Immunol. Res.* **2014**, 1–19. doi:10.1155/2014/149185

Lee S. C.-W., and Abdel-Wahab O. (2016). Therapeutic targeting of splicing in cancer. *Nat. Med.* **22**, 976–986. doi:10.1038/nm.4165

Lee W. Y., Chin A. C., Voss S., and Parkos C. A. (2006). In Vitro Neutrophil Transepithelial Migration. 'Cell-Cell Interactions in Health and Disease'. pp. 205–216. (Humana Press: New Jersey) doi:10.1385/1-59745-113-4:205

Lee H., Jeong A. J., and Ye S.-K. (2019). Highlighted STAT3 as a potential drug target for cancer therapy. *BMB Rep.* **52**, 415–423. doi:10.5483/BMBRep.2019.52.7.152

Leek D., Lewis E., and Harris L. (1996). Association of Macrophage Infiltration with Angiogenesis and Prognosis in Invasive. *Cancer Res.* **56**, 4625–4629.

Lekakos L., Nikolaos P, Dimitrios Dimitroulis, and Nikolaos Nikiteas (2011). Barrett's esophagus with high-grade dysplasia: Focus on current treatment options. *World J. Gastroenterol.* **17**, 4174–4188. doi:10.3748/wjg.v17.i37.4174

Levental K. R., Yu H., Kass L., Lakins J. N., Egeblad M., Erler J. T., Fong S. F. T., Csiszar K., Giaccia A., Weninger W., Yamauchi M., Gasser D. L., and Weaver V. M. (2009). Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell* **139**, 891–906. doi:10.1016/j.cell.2009.10.027

Levin B. (1999). Gallbladder carcinoma. *Ann. Oncol.* **10**, S129–S130. doi:10.1093/annonc/10.suppl_4.S129

Lewis A. M., Varghese S., Xu H., and Alexander H. R. (2006). Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J. Transl. Med.* **4**, 48. doi:10.1186/1479-5876-4-48

Li H., Fan X., and Houghton J. (2007). Tumor microenvironment: The role of the tumor stroma in cancer. *J. Cell. Biochem.* **101**, 805–815. doi:10.1002/jcb.21159

Li Q., Lee J.-A., and Black D. L. (2007). Neuronal regulation of alternative pre-mRNA splicing. *Nat. Rev. Neurosci.* **8**, 819–831. doi:10.1038/nrn2237

Li H., Sun S., Lei Q., Lei P., Cai X., Wan C., and Shen G. (2018). M1-Polarized Macrophages Promote Self-Renewing Phenotype of Hepatic Progenitor Cells with Jagged1-Notch Signalling Involved: Relevance in Primary Sclerosing Cholangitis. *J. Immunol. Res.* **2018**, 1–12. doi:10.1155/2018/4807145

Li Y., Wang L., Pappan L., Galliher-Beckley A., and Shi J. (2012). IL-1β promotes stemness and invasiveness of colon cancer cells through Zeb1 activation. *Mol. Cancer* **11**, 87. doi:10.1186/1476-4598-11-87

Li L., and Xie T. (2005). Stem Cell Niche: Structure and Function. *Annu. Rev. Cell Dev. Biol.* **21**, 605–631. doi:10.1146/annurev.cellbio.21.012704.131525

Liberti M. V., and Locasale J. W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **41**, 211–218. doi:10.1016/j.tibs.2015.12.001

Lin Y., Huang R., Cao X., Wang S.-M., Shi Q., and Huang R.-P. (2003). Detection of Multiple Cytokines by Protein Arrays from Cell Lysate and Tissue Lysate. *Clin. Chem. Lab. Med.* **41**, 139–145. doi:10.1515/CCLM.2003.023

Lin E. Y., Nguyen A. V., Russell R. G., and Pollard J. W. (2001). Colony-Stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. *J. Exp. Med.* **193**, 727–740. doi:10.1084/jem.193.6.727

Lin K. C., Park H. W., and Guan K.-L. (2018). Deregulation and Therapeutic Potential of the Hippo Pathway in Cancer. *Annu. Rev. Cancer Biol.* **2**, 59–79. doi:10.1146/annurev-cancerbio-030617-050202

Litmanovich A., Khazim K., and Cohen I. (2018). The Role of Interleukin-1 in the Pathogenesis of Cancer and its Potential as a Therapeutic Target in Clinical Practice. *Oncol. Ther.* **6**, 109–127. doi:10.1007/s40487-018-0089-z

Littlefield M. J. (2014). Polarization of Human THP-1 Macrophages: Link between Adenosine Receptors, Inflammation and Lipid Accumulation. *Int. J. Immunol. Immunother.* **1**, 001. doi:10.23937/2378-3672/1410001

Lopez J. I., Mouw J. K., and Weaver V. M. (2008). Biomechanical regulation of cell orientation and fate. *Oncogene* **27**, 6981–6993. doi:10.1038/onc.2008.348

Lozoya-Agullo I., Araújo F., González-Álvarez I., Merino-Sanjuán M., González-Álvarez M., Bermejo M., and Sarmento B. (2017). Usefulness of Caco-2/HT29-MTX and Caco-2/HT29-MTX/Raji B Coculture Models To Predict Intestinal and Colonic Permeability Compared to Caco-2 Monoculture. *Mol. Pharm.* **14**, 1264–1270. doi:10.1021/acs.molpharmaceut.6b01165

Lu P., Weaver V. M., and Werb Z. (2012). The extracellular matrix: A dynamic niche in cancer progression. *J. Cell Biol.* **196**, 395–406. doi:10.1083/jcb.201102147

Luo C., and Zhang H. (2017). The Role of Proinflammatory Pathways in the Pathogenesis of Colitis-Associated Colorectal Cancer. *Mediators Inflamm.* **2017**, 1–8. doi:10.1155/2017/5126048

Lurje G., Husain H., Power D. G., Yang D., Groshen S., Pohl A., Zhang W., Ning Y., Manegold P. C., El-Khoueiry A., Iqbal S., Tang L. H., Shah M. A., and Lenz H.-J. (2010). Genetic variations in angiogenesis pathway genes associated with clinical outcome in localized gastric adenocarcinoma. *Ann. Oncol.* **21**, 78–86. doi:10.1093/annonc/mdp280

Mackensen A., Ferradini L., Carcelain G., Triebel F., Faure F., Viel S., and Hercend T. (1993). Evidence for in Situ Amplification of Cytotoxic T-Lymphocytes with Antitumor Activity in a Human Regressive Melanoma. *Cancer Res.* **53**, 3569–3573.

Maeda S., Kamata H., Luo J.-L., Leffert H., and Karin M. (2005). IKKβ Couples Hepatocyte Death to Cytokine-Driven Compensatory Proliferation that Promotes Chemical Hepatocarcinogenesis. *Cell* **121**, 977–990. doi:10.1016/j.cell.2005.04.014

Mager L. F., Wasmer M.-H., Rau T. T., and Krebs P. (2016). Cytokine-Induced Modulation of Colorectal Cancer. *Front. Oncol.* **6**, 96. doi:10.3389/fonc.2016.00096

Mahida Y. R., Wu K., and Jewell D. P. (1989). Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut* **30**, 835–838. doi:10.1136/gut.30.6.835

Mahlknecht P., Stemberger S., Sprenger F., Rainer J., Hametner E., Kirchmair R., Grabmer C., Scherfler C., Wenning G. K., Seppi K., Poewe W., and Reindl M. (2012). An antibody microarray analysis of serum cytokines in neurodegenerative Parkinsonian syndromes. *Proteome Sci.* **10**, 71. doi:10.1186/1477-5956-10-71

Mahmoud S. M. A., Paish E. C., Powe D. G., Macmillan R. D., Grainge M. J., Lee A. H. S., Ellis I. O., and Green A. R. (2011). Tumor-Infiltrating $CD8^+$ Lymphocytes Predict Clinical Outcome in Breast Cancer. *J. Clin. Oncol.* **29**, 1949–1955. doi:10.1200/JCO.2010.30.5037

Manser E., Leung T., Salihuddin H., Zhao Z., and Lim L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**, 40–46. doi:10.1038/367040a0

Mantovani A., Allavena P., Sica A., and Balkwill F. (2008). Cancer-related inflammation. *Nature* **454**, 436–444. doi:10.1038/nature07205

Mantovani A., Schioppa T., Porta C., Allavena P., and Sica A. (2006). Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev.* **25**, 315–322. doi:10.1007/s10555-006-9001-7

Mantovani A., Sica A., Sozzani S., Allavena P., Vecchi A., and Locati M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25**, 677–686. doi:10.1016/j.it.2004.09.015

Marjanovic N. D., Weinberg R. A., and Chaffer C. L. (2013). Cell Plasticity and Heterogeneity in Cancer. *Clin. Chem.* **59**, 168–179. doi:10.1373/clinchem.2012.184655

Marrogi A. J., Munshi A., Merogi A., and Freeman S. M. (1997). Study of tumor infiltrating lymphocytes and transforming growth factor-β as prognostic factors in breast

carcinoma. *Int J Cancer Pred Oncol* **74**, 492–501. doi:10.1002/(sici)1097-0215(19971021)74:5<492::aid-ijc3>3.0.co;2-z

Mathias A., Duc M., Favre L., Benyacoub J., Blum S., and Corthésy B. (2010). Potentiation of Polarized Intestinal Caco-2 Cell Responsiveness to Probiotics Complexed with Secretory IgA. *J. Biol. Chem.* **285**, 33906–33913. doi:10.1074/jbc.M110.135111

Matos P., Collard J. G., and Jordan P. (2003). Tumor-related Alternatively Spliced Rac1b Is Not Regulated by Rho-GDP Dissociation Inhibitors and Exhibits Selective Downstream Signaling. *J. Biol. Chem.* **278**, 50442–50448. doi:10.1074/jbc.M308215200

Matos P., Gonçalves V., and Jordan P. (2016). Targeting the serrated pathway of colorectal cancer with mutation in BRAF. *Biochim. Biophys. Acta BBA - Rev. Cancer* **1866**, 51–63. doi:10.1016/j.bbcan.2016.06.003

Matos P., and Jordan P. (2005). Expression of Rac1b stimulates NF-κB-mediated cell survival and G1/S progression. *Exp. Cell Res.* **305**, 292–299. doi:10.1016/j.yexcr.2004.12.029

Matos P., and Jordan P. (2006). Rac1, but Not Rac1B, Stimulates RelB-mediated Gene Transcription in Colorectal Cancer Cells. *J. Biol. Chem.* **281**, 13724–13732. doi:10.1074/jbc.M513243200

Matos P., and Jordan P. (2008). Increased Rac1b Expression Sustains Colorectal Tumor Cell Survival. *Mol. Cancer Res.* **6**, 1178–1184. doi:10.1158/1541-7786.MCR-08-0008

Matos P., and Jordan P. (2018). Targeting Colon Cancers with Mutated BRAF and Microsatellite Instability. 'Targeted Therapy of Colorectal Cancer Subtypes'. (Ed P Jordan) pp. 7–21. (Springer International Publishing: Cham) doi:10.1007/978-3-030-02771-1_2

Matos P., Kotelevets L., Jordan P., Gonçalves V., Henriques A., Zerbib P., Moyer M. P., and Chastre E. (2013). Ibuprofen Inhibits Colitis-Induced Overexpression of TumorRelated Rac1b. *Neoplasia* **15**, 102–111. doi:10.1593/neo.121890

Matos P., Oliveira C., Velho S., Gonçalves V., da Costa L. T., Moyer M. P., Seruca R., and Jordan P. (2008). B-RafV600E Cooperates With Alternative Spliced Rac1b to Sustain Colorectal Cancer Cell Survival. *Gastroenterology* **135**, 899–906. doi:10.1053/j.gastro.2008.05.052

Matos P., Skaug J., Marques B., Beck S., Veríssimo F., Gespach C., Boavida M. G., Scherer S. W., and Jordan P. (2000). Small GTPase Rac1: Structure, Localization,

and Expression of the Human Gene. *Biochem. Biophys. Res. Commun.* **277**, 741–751. doi:10.1006/bbrc.2000.3743

Matsuda R., Koide T., Tokoro C., and Umemura S. (2009). Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid naïve ulcerative colitis during active and quiescent disease. *Inflamm Bowel Dis* **15**, 328–334. doi:10.1002/ibd.20759

Matsuda Y., Miura K., Yamane J., Shima H., Fujibuchi W., Ishida K., Fujishima F., Ohnuma S., Sasaki H., Nagao M., Tanaka N., Satoh K., Naitoh T., and Unno M. (2016). SERPINI1 regulates epithelial-mesenchymal transition in an orthotopic implantation model of colorectal cancer. *Cancer Sci.* **107**, 619–628. doi:10.1111/cas.12909

Matter N., Herrlich P., and König H. (2002). Signal-dependent regulation of splicing via phosphorylation of Sam68. *Nature* **420**, 691–695. doi:10.1038/nature01153

McAlindon M. E., Hawkey C. J., and Mahida Y. R. (1998). Expression of interleukin 1 and interleukin 1 converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut* **42**, 214–219. doi:10.1136/gut.42.2.214

Mehner C., Miller E., Khauv D., Nassar A., Oberg A. L., Bamlet W. R., Zhang L., Waldmann J., Radisky E. S., Crawford H. C., and Radisky D. C. (2014). Tumor Cell–Derived MMP3 Orchestrates Rac1b and Tissue Alterations That Promote Pancreatic Adenocarcinoma. *Mol. Cancer Res.* **12**, 1430–1439. doi:10.1158/1541-7786.MCR-13-0557-T

Mehner C., Miller E., Nassar A., Bamlet W. R., Radisky E. S., and Radisky D. C. (2015). Tumor cell expression of MMP3 as a prognostic factor for poor survival in pancreatic, pulmonary, and mammary carcinoma. *Genes Cancer* **6**, 480–489. doi:10.18632/genesandcancer.90

Mellman I., and Nelson W. J. (2008). Coordinated protein sorting, targeting and distribution in polarized cells. *Nat. Rev. Mol. Cell Biol.* **9**, 833–845. doi:10.1038/nrm2525

Melo F. D. S. E., Vermeulen L., Fessler E., and Medema J. P. (2013). Cancer heterogeneity—a multifaceted view. *EMBO Rep.* **14**, 686–695. doi:10.1038/embor.2013.92

Melzer C., Hass R., Lehnert H., and Ungefroren H. (2019). RAC1B: A Rho GTPase with Versatile Functions in Malignant Transformation and Tumor Progression. *Cells* **8**, 21. doi:10.3390/cells8010021

Melzer C., von der Ohe J., Hass R., and Ungefroren H. (2017). TGF-β-Dependent Growth Arrest and Cell Migration in Benign and Malignant Breast Epithelial Cells Are Antagonistically Controlled by Rac1 and Rac1b. *Int. J. Mol. Sci.* **18**, 1574. doi:10.3390/ijms18071574

Menon R., and Omenn G. S. (2011). Identification of Alternatively Spliced Transcripts Using a Proteomic Informatics Approach. 'Data Mining in Proteomics'. (Eds M Hamacher, M Eisenacher and C Stephan) Methods in Molecular Biology. pp. 319–326. (Humana Press: Totowa, NJ) doi:10.1007/978-1-60761-987-1_20

Meredith M. M., Liu K., Darrasse-Jeze G., Kamphorst A. O., Schreiber H. A., Guermonprez P., Idoyaga J., Cheong C., Yao K.-H., Niec R. E., and Nussenzweig M. C. (2012). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J. Exp. Med.* **209**, 1153–1165. doi:10.1084/jem.20112675

Meylan E., Dooley A. L., Feldser D. M., Shen L., Turk E., Ouyang C., and Jacks T. (2009). Requirement for NF-κB signalling in a mouse model of lung adenocarcinoma. *Nature* **462**, 104–107. doi:10.1038/nature08462

Michaloglou C., Vredeveld L. C. W., Soengas M. S., Denoyelle C., Kuilman T., van der Horst C. M. A. M., Majoor D. M., Shay J. W., Mooi W. J., and Peeper D. S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720–724. doi:10.1038/nature03890

Mills C. D., Kincaid K., Alt J. M., Heilman M. J., and Hill A. M. (2000). M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J. Immunol.* **164**, 6166–6173. doi:10.4049/jimmunol.164.12.6166

Milne K., Köbel M., Kalloger S. E., Barnes R. O., Gao D., Gilks C. B., Watson P. H., and Nelson B. H. (2009). Systematic Analysis of Immune Infiltrates in High-Grade Serous Ovarian Cancer Reveals CD20, FoxP3 and TIA-1 as Positive Prognostic Factors (D Unutmaz, Ed.). *PLoS ONE* **4**, e6412. doi:10.1371/journal.pone.0006412

Mima K., Nishihara R., Qian Z. R., Cao Y., Sukawa Y., Nowak J. A., Yang J., Dou R., Masugi Y., Song M., Kostic A. D., Giannakis M., Bullman S., Milner D. A., Baba H., Giovannucci E. L., Garraway L. A., Freeman G. J., Dranoff G., Garrett W. S., Huttenhower C., Meyerson M., Meyerhardt J. A., Chan A. T., Fuchs C. S., and Ogino S. (2016). Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* **65**, 1973–1980. doi:10.1136/gutjnl-2015-310101

Mina M., Raynaud F., Tavernari D., Battistello E., Sungalee S., Saghafinia S., Laessle T., Sanchez-Vega F., Schultz N., Oricchio E., and Ciriello G. (2017). Conditional Selection of Genomic Alterations Dictates Cancer Evolution and Oncogenic Dependencies. *Cancer Cell* **32**, 155-168.e6. doi:10.1016/j.ccell.2017.06.010

Misquitta-Ali C. M., Cheng E., O'Hanlon D., Liu N., McGlade C. J., Tsao M. S., and Blencowe B. J. (2011). Global Profiling and Molecular Characterization of Alternative Splicing Events Misregulated in Lung Cancer. *Mol. Cell. Biol.* **31**, 138–150. doi:10.1128/MCB.00709-10

Mlecnik B., Bindea G., Angell H. K., Maby P., Angelova M., Tougeron D., Church S. E., Lafontaine L., Fischer M., Fredriksen T., Sasso M., Bilocq A. M., Kirilovsky A., Obenauf A. C., Hamieh M., Berger A., Bruneval P., Tuech J.-J., Sabourin J.-C., Le Pessot F., Mauillon J., Rafii A., Laurent-Puig P., Speicher M. R., Trajanoski Z., Michel P., Sesboüe R., Frebourg T., Pagès F., Valge-Archer V., Latouche J.-B., and Galon J. (2016). Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* **44**, 698–711. doi:10.1016/j.immuni.2016.02.025

Mlecnik B., Tosolini M., Kirilovsky A., Berger A., Bindea G., Meatchi T., Bruneval P., Trajanoski Z., Fridman W.-H., Pagès F., and Galon J. (2011). Histopathologic-Based Prognostic Factors of Colorectal Cancers Are Associated With the State of the Local Immune Reaction. *J. Clin. Oncol.* **29**, 610–618. doi:10.1200/JCO.2010.30.5425

Mosser D. M., and Edwards J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8**, 958–969. doi:10.1038/nri2448

Mouradov D., Sloggett C., Jorissen R. N., Love C. G., Li S., Burgess A. W., Arango D., Strausberg R. L., Buchanan D., Wormald S., O'Connor L., Wilding J. L., Bicknell D., Tomlinson I. P. M., Bodmer W. F., Mariadason J. M., and Sieber O. M. (2014). Colorectal Cancer Cell Lines Are Representative Models of the Main Molecular Subtypes of Primary Cancer. *Cancer Res.* **74**, 3238–3247. doi:10.1158/0008-5472.CAN-14-0013

Murata M., Thanan R., Ma N., and Kawanishi S. (2012). Role of Nitrative and Oxidative DNA Damage in Inflammation-Related Carcinogenesis. *J. Biomed. Biotechnol.* **2012**, 1–11. doi:10.1155/2012/623019

Nagasaki T., Hara M., Nakanishi H., Takahashi H., Sato M., and Takeyama H. (2014). Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour

angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour–stroma interaction. *Br. J. Cancer* **110**, 469–478. doi:10.1038/bjc.2013.748

Nakano O., Sato M., Naito Y., Suzuki K., Orikasa S., Aizawa M., Suzuki Y., Shintaku I., Nagura H., and Ohtani H. (2001). Proliferative Activity of Intratumoral CD8+ T-Lymphocytes As a Prognostic Factor in Human Renal Cell Carcinoma: Clinicopathologic Demonstration of Antitumor Immunity. *Cancer Res.* **61**, 5132–5136.

Nakao S., Kuwano T., Tsutsumi-Miyahara C., Ueda S., Kimura Y. N., Hamano S., Sonoda K., Saijo Y., Nukiwa T., Strieter R. M., Ishibashi T., Kuwano M., and Ono M. (2005). Infiltration of COX-2–expressing macrophages is a prerequisite for IL-1β–induced neovascularization and tumor growth. *J. Clin. Invest.* **115**, 2979–2991. doi:10.1172/JCI23298

Nakashima J., Tachibana M., Horiguchi Y., Oya M., Ohigashi T., Asakura H., and Murai M. (2000). Serum Interleukin 6 as a Prognostic Factor in Patients with Prostate Cancer. *Clin. Cancer Res.* **6**, 2702–2706.

Nassar D., and Blanpain C. (2016). Cancer Stem Cells: Basic Concepts and Therapeutic Implications. *Annu. Rev. Pathol. Mech. Dis.* **11**, 47–76. doi:10.1146/annurev-pathol-012615-044438

Naugler W. E., Sakurai T., Kim S., Maeda S., Kim K., Elsharkawy A. M., and Karin M. (2007). Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-Dependent IL-6 Production. *Science* **317**, 121–124. doi:10.1126/science.1140485

Nebiker C. A., Han J., Eppenberger-Castori S., Iezzi G., Hirt C., Amicarella F., Cremonesi E., Huber X., Padovan E., Angrisani B., Droeser R. A., Rosso R., Bolli M., Oertli D., von Holzen U., Adamina M., Muraro M. G., Mengus C., Zajac P., Sconocchia G., Zuber M., Tornillo L., Terracciano L., and Spagnoli G. C. (2014). GM-CSF Production by Tumor Cells Is Associated with Improved Survival in Colorectal Cancer. *Clin. Cancer Res.* **20**, 3094–3106. doi:10.1158/1078-0432.CCR-13-2774

Neelakantan D., Dogra S., Devapatla B., Jaiprasart P., Mukashyaka M. C., Janknecht R., Dwivedi S. K. D., Bhattacharya R., Husain S., Ding K., and Woo S. (2019). Multifunctional APJ Pathway Promotes Ovarian Cancer Progression and Metastasis. *Mol. Cancer Res.* **17**, 1378–1390. doi:10.1158/1541-7786.MCR-18-0989

Nelson W. J. (2003). Adaptation of core mechanisms to generate cell polarity. *Nature* **422**, 766–774. doi:10.1038/nature01602

Nilsen T. W., and Graveley B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature* **463**, 457–463. doi:10.1038/nature08909

Nissan M. H., Pratilas C. A., Jones A. M., Ramirez R., Won H., Liu C., Tiwari S., Kong L., Hanrahan A. J., Yao Z., Merghoub T., Ribas A., Chapman P. B., Yaeger R., Taylor B. S., Schultz N., Berger M. F., Rosen N., and Solit D. B. (2014). Loss of NF1 in Cutaneous Melanoma Is Associated with RAS Activation and MEK Dependence. *Cancer Res.* **74**, 2340–2350. doi:10.1158/0008-5472.CAN-13-2625

Noguchi T., and Inoue H. (1986). The M1- and M2-type isozymes of rat pyruvate kinase are produced from the same gene by alternative RNA splicing. *J. Biol. Chem.* **261**, 6.

Nusse R., and Clevers H. (2017). Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* **169**, 985–999. doi:10.1016/j.cell.2017.05.016

Oh K., Lee O.-Y., Park Y., Seo M. W., and Lee D.-S. (2016). IL-1β induces IL-6 production and increases invasiveness and estrogen-independent growth in a TG2-dependent manner in human breast cancer cells. *BMC Cancer* **16**, 724. doi:10.1186/s12885-016-2746-7

Orecchioni M., Ghosheh Y., Pramod A. B., and Ley K. (2019). Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages. *Front. Immunol.* **10**, 1084. doi:10.3389/fimmu.2019.01084

Orlichenko L., Geyer R., Yanagisawa M., Khauv D., Radisky E. S., Anastasiadis P. Z., and Radisky D. C. (2010). The 19-Amino Acid Insertion in the Tumor-associated Splice Isoform Rac1b Confers Specific Binding to p120 Catenin. *J. Biol. Chem.* **285**, 19153–19161. doi:10.1074/jbc.M109.099382

O'Connor P. M., Lapointe T. K., Beck P. L., and Buret A. G. (2010). Mechanisms by which inflammation may increase intestinal cancer risk in inflammatory bowel disease: *Inflamm. Bowel Dis.* **16**, 1411–1420. doi:10.1002/ibd.21217

Pagès F., Kirilovsky A., Mlecnik B., Asslaber M., Tosolini M., Bindea G., Lagorce C., Wind P., Marliot F., Bruneval P., Zatloukal K., Trajanoski Z., Berger A., Fridman W.-H., and Galon J. (2009). In Situ Cytotoxic and Memory T Cells Predict Outcome in Patients With Early-Stage Colorectal Cancer. *J. Clin. Oncol.* **27**, 5944–5951. doi:10.1200/JCO.2008.19.6147

Pagliarini V., Naro C., and Sette C. (2015). Splicing Regulation: A Molecular Device to Enhance Cancer Cell Adaptation. *BioMed Res. Int.* **2015**, 1–13. doi:10.1155/2015/543067

Pan Q., Shai O., Lee L. J., Frey B. J., and Blencowe B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40**, 1413–1415. doi:10.1038/ng.259

Pan J., Zhou H., Zhu S., Huang J., Zhao X., Ding H., and Pan Y. (2018). Development of small-molecule therapeutics and strategies for targeting RAF kinase in BRAF-mutant colorectal cancer. *Cancer Manag. Res.* **10**, 2289–2301. doi:10.2147/CMAR.S170105

Park H. S., Lee S. H., Park D., Lee J. S., Ryu S. H., Lee W. J., Rhee S. G., and Bae Y. S. (2004). Sequential Activation of Phosphatidylinositol 3-Kinase, Pix, Rac1, and Nox1 in Growth Factor-Induced Production of H2O2. *Mol Cell Biol* **24**, 4384–4394. doi:10.1128/mcb.24.10.4384-4394.2004

Paszek M. J., Zahir N., Johnson K. R., Lakins J. N., Rozenberg G. I., Gefen A., Reinhart-King C. A., Margulies S. S., Dembo M., Boettiger D., Hammer D. A., and Weaver V. M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell* **8**, 241–254. doi:10.1016/j.ccr.2005.08.010

Pattabiraman D. R., and Weinberg R. A. (2014). Tackling the cancer stem cells — what challenges do they pose? *Nat. Rev. Drug Discov.* **13**, 497–512. doi:10.1038/nrd4253

Peek R. M., and Blaser M. J. (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer* **2**, 28–37. doi:10.1038/nrc703

Pereira J. F. S., Awatade N. T., Loureiro C. A., Matos P., Amaral M. D., and Jordan P. (2016). The third dimension: new developments in cell culture models for colorectal research. *Cell. Mol. Life Sci.* **73**, 3971–3989. doi:10.1007/s00018-016-2258-2

Perona R., Montaner S., Saniger L., Sanchez-Perez I., Bravo R., and Lacal J. C. (1997). Activation of the nuclear factor-KB by Rho, CDC42, and Rac-1 proteins. *Genes Dev.* **11**, 463–475. doi:10.1101/gad.11.4.463

Phinney B. B., Ray A. L., Peretti A. S., Jerman S. J., Grim C., Pinchuk I. V., and Beswick E. J. (2018). MK2 Regulates Macrophage Chemokine Activity and Recruitment to Promote Colon Tumor Growth. *Front. Immunol.* **9**, 1857. doi:10.3389/fimmu.2018.01857

Plante M., Rubin S. C., Wong G. Y., Federici M. G., Finstad C. L., and Gastl G. A. (1994). Interleukin-6 level in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer. *Cancer* **73**, 1882–1888. doi:10.1002/1097-0142(19940401)73:7<1882::aid-cncr2820730718>3.0.co;2-r

Poore G. D., Kopylova E., Zhu Q., Carpenter C., Fraraccio S., Wandro S., Kosciolek T., Janssen S., Metcalf J., Song S. J., Kanbar J., Miller-Montgomery S., Heaton R., Mckay R., Patel S. P., Swafford A. D., and Knight R. (2020). Microbiome analyses of blood and tissues suggest cancer diagnostic approach. *Nature* **579**, 567–574. doi:10.1038/s41586-020-2095-1

Prenen H., and Mazzone M. (2019). Tumor-associated macrophages: a short compendium. *Cell. Mol. Life Sci.* **76**, 1447–1458. doi:10.1007/s00018-018-2997-3

Qian B.-Z., and Pollard J. W. (2010). Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell* **141**, 39–51. doi:10.1016/j.cell.2010.03.014

Qin Z., Richter G., Schuler T., Ibe S., Cao X., and Blankenstein T. (1998). B cells inhibit induction of T cell-dependent tumor immunity. *Nat. Med.* **4**, 627–630. doi:10.1038/nm0598-627

Radisky D. C., Levy D. D., Littlepage L. E., Liu H., Nelson C. M., Fata J. E., Leake D., Godden E. L., Albertson D. G., Angela Nieto M., Werb Z., and Bissell M. J. (2005). Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* **436**, 123–127. doi:10.1038/nature03688

Raftopoulou M., and Hall A. (2004). Cell migration: Rho GTPases lead the way. *Dev. Biol.* **265**, 23–32. doi:10.1016/j.ydbio.2003.06.003

Rahman M. A., Nasrin F., Bhattacharjee S., and Nandi S. (2020). Hallmarks of Splicing Defects in Cancer: Clinical Applications in the Era of Personalized Medicine. *Cancers* **12**, 1381. doi:10.3390/cancers12061381

Reimund J.-M., Wittersheim C., Dumont S., Muller C. D., Baumann R., Poindron P., and Duclos B. (1996). Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. *J. Clin. Immunol.* **16**, 144–150. doi:10.1007/BF01540912

Reinecker H.-C., Steffen M., Witthoeft T., Pflueger I., Schreiber S., MAcDERMOTT R. P., and Raedler A. (2008). Enhand secretion of tumour necrosis factor-alpha, IL-6, and IL-1β by isolated lamina ropria monouclear cells from patients with ulcretive cilitis and Crohn's disease. *Clin. Exp. Immunol.* **94**, 174–181. doi:10.1111/j.1365-2249.1993.tb05997.x

Riquelme E., Zhang Y., Zhang L., Montiel M., Zoltan M., Dong W., Quesada P., Sahin I., Chandra V., San Lucas A., Scheet P., Xu H., Hanash S. M., Feng L., Burks J. K., Do K.-A., Peterson C. B., Nejman D., Tzeng C.-W. D., Kim M. P., Sears C. L., Ajami N., Petrosino J., Wood L. D., Maitra A., Straussman R., Katz M., White J. R., Jenq R.,

Wargo J., and McAllister F. (2019). Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. *Cell* **178**, 795-806.e12. doi:10.1016/j.cell.2019.07.008

Rőszer T. (2015). Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm.* **2015**, 1–16. doi:10.1155/2015/816460

Roy B., M. Haupt L., and R. Griffiths L. (2013). Review: Alternative Splicing (AS) of Genes As An Approach for Generating Protein Complexity. *Curr. Genomics* **14**, 182–194. doi:10.2174/1389202911314030004

Rubashkin M. G., Ou G., and Weaver V. M. (2014). Deconstructing Signaling in Three Dimensions. *Biochemistry* **53**, 2078–2090. doi:10.1021/bi401710d

Rutgeerts P., and Geboes K. (2001). Understanding inflammatory bowel disease-the clinician's perspective. *Eur. J. Surg.* **167**, 66–72. doi:10.1080/110241501317076281

Rutter M., Saunders B., Wilkinson K., Rumbles S., Schofield G., Kamm M., Williams C., Price A., Talbot I., and Forbes A. (2004). Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* **126**, 451–459. doi:10.1053/j.gastro.2003.11.010

Saaf A. M., Halbleib J. M., Chen X., Yuen S. T., Leung S. Y., Nelson W. J., and Brown P. O. (2007). Parallels between Global Transcriptional Programs of Polarizing Caco-2 Intestinal Epithelial Cells In Vitro and Gene Expression Programs in Normal Colon and Colon Cancer. *Mol. Biol. Cell* **18**, 4245–4260. doi:10.1091/mbc.e07-04-0309

Sahai E., Astsaturov I., Cukierman E., DeNardo D. G., Egeblad M., Evans R. M., Fearon D., Greten F. R., Hingorani S. R., Hunter T., Hynes R. O., Jain R. K., Janowitz T., Jorgensen C., Kimmelman A. C., Kolonin M. G., Maki R. G., Powers R. S., Puré E., Ramirez D. C., Scherz-Shouval R., Sherman M. H., Stewart S., Tlsty T. D., Tuveson D. A., Watt F. M., Weaver V., Weeraratna A. T., and Werb Z. (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nat. Rev. Cancer* **20**, 174–186. doi:10.1038/s41568-019-0238-1

Sahu V., Gupta A., Kumar R., Gupta T., Mohan A., and Dey S. (2016). Quantification of Rac1 and Rac1b in serum of non small cell lung cancer by label free real time assay. *Clin. Chim. Acta* **460**, 231–235. doi:10.1016/j.cca.2016.07.009

Saijo Y., Tanaka M., Miki M., Usui K., Suzuki T., Maemondo M., Hong X., Tazawa R., Kikuchi T., Matsushima K., and Nukiwa T. (2002). Proinflammatory Cytokine IL-1β Promotes Tumor Growth of Lewis Lung Carcinoma by Induction of

Angiogenic Factors: In Vivo Analysis of Tumor-Stromal Interaction. *J. Immunol.* **169**, 469–475. doi:10.4049/jimmunol.169.1.469

Sakaguchi M., Oka M., Iwasaki T., Fukami Y., and Nishigori C. (2012). Role and Regulation of STAT3 Phosphorylation at Ser727 in Melanocytes and Melanoma Cells. *J. Invest. Dermatol.* **132**, 1877–1885. doi:10.1038/jid.2012.45

Sakharov D., Maltseva D., Knyazev E., Nikulin S., Poloznikov A., Shilin S., Baranova A., Tsypina I., and Tonevitsky A. (2019). Towards embedding Caco-2 model of gut interface in a microfluidic device to enable multi-organ models for systems biology. *BMC Syst. Biol.* **13**, 19. doi:10.1186/s12918-019-0686-y

Samadani A. A., Norollahi S. E., Rashidy-Pour A., Mansour-Ghanaei F., Nemati S., Joukar F., Afshar A. M., Ghazanfari S., Safizadeh M., Rostami P., and Gatei M. (2018). Cancer signaling pathways with a therapeutic approach: An overview in epigenetic regulations of cancer stem cells. *Biomed. Pharmacother.* **108**, 590–599. doi:10.1016/j.biopha.2018.09.048

Sambuy Y., Angelis I. D., Ranaldi G., Scarino M. L., Stammati A., and Zucco F. (2005). The Caco-2 cell line as a model of the intestinal barrier: in£uence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* **21**, 1–26. doi:10.1007/s10565-005-0085-6

Sanchez-Muñoz F., Dominguez-Lopez A., and Yamamoto-Furusho J. K. (2008). Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* **14**, 4280. doi:10.3748/wjg.14.4280

Sanchez-Vega F., Mina M., Armenia J., Chatila W. K., Luna A., La K. C., Dimitriadoy S., Liu D. L., and Schultz N. (2018). Oncogenic Signaling Pathways in The Cancer Genome Atlas. *Cell* **173**, 321-337.e10. doi:10.1016/j.cell.2018.03.035

Sandmeier D., Benhattar J., Martin P., and Bouzourene H. (2009). Serrated polyps of the large intestine: a molecular study comparing sessile serrated adenomas and hyperplastic polyps. *Histopathology* **55**, 206–213. doi:10.1111/j.1365-2559.2009.03356.x

Sangodkar J., DiFeo A., Feld L., Bromberg R., Schwartz R., Huang F., Terzo E. A., Choudhri A., and Narla G. (2009). Targeted reduction of KLF6-SV1 restores chemotherapy sensitivity in resistant lung adenocarcinoma. *Lung Cancer* **66**, 292–297. doi:10.1016/j.lungcan.2009.02.014

Sanz-Garcia E., Argiles G., Elez E., and Tabernero J. (2017). BRAF mutant colorectal cancer: prognosis, treatment, and new perspectives. *Ann. Oncol.* **28**, 2648–2657. doi:10.1093/annonc/mdx401

Schaub F. X., Dhankani V., Berger A. C., Trivedi M., Richardson A. B., Shaw R., Zhao W., Zhang X., Ventura A., Liu Y., Ayer D. F., Hurlin P. J., Cherniack A. D., Eisenman R. N., Bernard B., and Grandori C. (2018). Pan-cancer Alterations of the MYC Oncogene and Its Proximal Network across the Cancer Genome Atlas. *Cell Syst.* **6**, 282–300. doi:10.1016/j.cels.2018.03.003

Schenk M., Fabri M., Krutzik S. R., Lee D. J., Vu D. M., Sieling P. A., Montoya D., Liu P. T., and Modlin R. L. (2014). Interleukin-1β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells. *Immunology* **141**, 174–180. doi:10.1111/imm.12167

Schioppa T., Moore R., Thompson R. G., Rosser E. C., Kulbe H., Nedospasov S., Mauri C., Coussens L. M., and Balkwill F. R. (2011). B regulatory cells and the tumor-promoting actions of TNF- during squamous carcinogenesis. *Proc. Natl. Acad. Sci.* **108**, 10662–10667. doi:10.1073/pnas.1100994108

Schnelzer A., Prechtel D., Knaus U., Dehne K., Gerhard M., Graeff H., Harbeck N., Schmitt M., and Lengyel E. (2000). Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. *Oncogene* **19**, 3013–3020. doi:10.1038/sj.onc.1203621

Segev N. (2001). Ypt/Rab GTPases: Regulators of Protein Trafficking. *Sci STKE* **100**, 18. doi:10.1126/stke.2001.100.re11

Sette C., Ladomery M., and Ghigna C. (2013). Alternative Splicing: Role in Cancer Development and Progression. *Int. J. Cell Biol.* 1–2. doi:10.1155/2013/421606

Sever R., and Brugge J. S. (2015). Signal Transduction in Cancer. *Cold Spring Harb. Perspect. Med.* **5**, a006098–a006098. doi:10.1101/cshperspect.a006098

Shamir E. R., and Ewald A. J. (2014). Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nat. Rev. Mol. Cell Biol.* **15**, 647–664. doi:10.1038/nrm3873

Shultz J. C., Goehe R. W., Wijesinghe D. S., Murudkar C., Hawkins A. J., Shay J. W., Minna J. D., and Chalfant C. E. (2010). Alternative Splicing of Caspase 9 Is Modulated by the Phosphoinositide 3-Kinase/Akt Pathway via Phosphorylation of SRp30a. *Cancer Res.* **70**, 9185–9196. doi:10.1158/0008-5472.CAN-10-1545

Sica A., and Mantovani A. (2012). Macrophage plasticity and polarization: in vivo veritas. *J. Clin. Invest.* **122**, 787–795. doi:10.1172/JCI59643

Silva A. L., Carmo F., and Bugalho M. J. (2013). RAC1b overexpression in papillary thyroid carcinoma: a role to unravel. *Eur. J. Endocrinol.* **168**, 795–804. doi:10.1530/EJE-12-0960

Silva A. L., Faria M., Capinha L., and Bugalho M. J. (2016). RAC1b: A New Player in the Scenario of Thyroid Tumorigenesis? *Adv. Mol. Diasnostics* **1**, 5. doi:10.4172/2572-5073.1000103

Silva A. L., Faria M., and Matos P. (2020). Inflammatory Microenvironment Modulation of Alternative Splicing in Cancer: A Way to Adapt. *Adv Exp Med Biol* **1219**, 243–258. doi:10.1007/978-3-030-34025-4_13

Singh R. K., Gershenwald J. E., Gutman M., Reich R., and Bar-Eli M. (1995). Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8: 188. *Melanoma Res.* **55**, 3669-3674S54. doi:10.1097/00008390-199706001-00188

Singh A., Karnoub A. E., Palmby T. R., Lengyel E., Sondek J., and Der C. J. (2004). Rac1b, a tumor associated, constitutively active Rac1 splice variant, promotes cellular transformation. *Oncogene* **23**, 9369–9380. doi:10.1038/sj.onc.1208182

Smyth M. J., Thia K. Y. T., Street S. E. A., Cretney E., Trapani J. A., Taniguchi M., Kawano T., Pelikan S. B., Crowe N. Y., and Godfrey D. I. (2000). Differential Tumor Surveillance by Natural Killer (Nk) and Nkt Cells. *J. Exp. Med.* **191**, 661–668. doi:10.1084/jem.191.4.661

Snover D. C. (2011). Update on the serrated pathway to colorectal carcinoma. *Hum. Pathol.* **42**, 1–10. doi:10.1016/j.humpath.2010.06.002

Song S., Cong W., Zhou S., Shi Y., Dai W., Zhang H., Wang X., He B., and Zhang Q. (2019). Small GTPases: Structure, biological function and its interaction with nanoparticles. *Asian J. Pharm. Sci.* **14**, 30–39. doi:10.1016/j.ajps.2018.06.004

Srinivasan B., Kolli A. R., Esch M. B., Abaci H. E., Shuler M. L., and Hickman J. J. (2015). TEER Measurement Techniques for In Vitro Barrier Model Systems. *J. Lab. Autom.* **20**, 107–126. doi:10.1177/2211068214561025

Stallings-Mann M. L., Waldmann J., Zhang Y., Miller E., Gauthier M. L., Visscher D. W., Downey G. P., Radisky E. S., Fields A. P., and Radisky D. C. (2012). Matrix Metalloproteinase Induction of Rac1b, a Key Effector of Lung Cancer Progression. *Sci. Transl. Med.* **4**, 142ra95-142ra95. doi:10.1126/scitranslmed.3004062

Straussman R., Morikawa T., Shee K., Barzily-Rokni M., Qian Z. R., Du J., Davis A., Mongare M. M., Gould J., Frederick D. T., Cooper Z. A., Chapman P. B., Solit D. B., Ribas A., Lo R. S., Flaherty K. T., Ogino S., Wargo J. A., and Golub T. R. (2012). Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* **487**, 500–504. doi:10.1038/nature11183

Suhail Y., Cain M. P., Vanaja K., Kurywchak P. A., Levchenko A., Kalluri R., and Kshitiz (2019). Systems Biology of Cancer Metastasis. *Cell Syst.* **9**, 109–127. doi:10.1016/j.cels.2019.07.003

Sulciner D. J., Irani K., Yu Z.-X., Ferrans V. J., Goldschmidt-Clermont P., and Finkel T. (1996). Rac1 Regulates a Cytokine-Stimulated, Redox-Dependent Pathway Necessary for NF-kB Activation. *Mol Cell Biol* **16**, 7115–7121.

Sun H., Chow E. C., Liu S., Du Y., and Pang K. S. (2008). The Caco-2 cell monolayer: usefulness and limitations. *Expert Opin. Drug Metab. Toxicol.* **4**, 395–411. doi:10.1517/17425255.4.4.395

Suzuki T. (2013). Regulation of intestinal epithelial permeability by tight junctions. *Cell. Mol. Life Sci.* **70**, 631–659. doi:10.1007/s00018-012-1070-x

Swann J. B., Vesely M. D., Silva A., Sharkey J., Akira S., Schreiber R. D., and Smyth M. J. (2008). Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc. Natl. Acad. Sci.* **105**, 652–656. doi:10.1073/pnas.0708594105

Tachibana T. (2005). Increased Intratumor V 24-Positive Natural Killer T Cells: A Prognostic Factor for Primary Colorectal Carcinomas. *Clin. Cancer Res.* **11**, 7322–7327. doi:10.1158/1078-0432.CCR-05-0877

Tarin D., and Croft C. B. (1970). Ultrastructural studies of wound healing in mouse skin. *J Anat* **106**, 79–91. doi:5413644

Tauriello D. V. F., Palomo-Ponce S., Stork D., Berenguer-Llergo A., Badia-Ramentol J., Iglesias M., Sevillano M., Ibiza S., Cañellas A., Hernando-Momblona X., Byrom D., Matarin J. A., Calon A., Rivas E. I., Nebreda A. R., Riera A., Attolini C. S.-O., and Batlle E. (2018). TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature* **554**, 538–543. doi:10.1038/nature25492

Terabe M., Matsui S., Noben-Trauth N., Chen H., Watson C., Donaldson D. D., Carbone D. P., Paul W. E., and Berzofsky J. A. (2000). NKT cell–mediated repression of tumor immunosurveillance by IL-13 and the IL-4R–STAT6 pathway. *Nat. Immunol.* **1**, 515–520. doi:10.1038/82771

Terzić J., Grivennikov S., Karin E., and Karin M. (2010). Inflammation and Colon Cancer. *Gastroenterology* **138**, 2101-2114.e5. doi:10.1053/j.gastro.2010.01.058

Teschendorff A. E., Gomez S., Arenas A., El-Ashry D., Schmidt M., Gehrmann M., and Caldas C. (2010). Improved prognostic classification of breast cancer defined by antagonistic activation patterns of immune response pathway modules. *BMC Cancer* **10**, 604. doi:10.1186/1471-2407-10-604

To K. F., Chan M. W. Y., Leung W. K., Ng E. K. W., Yu J., Bai A. H. C., Lo A. W. I., Chu S. H., Tong J. H. M., Lo K. W., Sung J. J. Y., and Chan F. K. L. (2004). Constitutional activation of IL-6-mediated JAK/STAT pathway through hypermethylation of SOCS-1 in human gastric cancer cell line. *Br. J. Cancer* **91**, 1335–1341. doi:10.1038/sj.bjc.6602133

Torras N., García-Díaz M., Fernández-Majada V., and Martínez E. (2018). Mimicking Epithelial Tissues in Three-Dimensional Cell Culture Models. *Front. Bioeng. Biotechnol.* **6**, 197. doi:10.3389/fbioe.2018.00197

Tosolini M., Kirilovsky A., Mlecnik B., Fredriksen T., Mauger S., Bindea G., Berger A., Bruneval P., Fridman W.-H., Pagès F., and Galon J. (2011). Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer. *Cancer Res.* **71**, 1263–1271. doi:10.1158/0008-5472.CAN-10-2907

Tzankov A., Meier C., Hirschmann P., Went P., Pileri S. A., and Dirnhofer S. (2008). Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. *Haematologica* **93**, 193–200. doi:10.3324/haematol.11702

Ueda T., Shimada E., and Urakawa T. (1994). Serum levels of cytokines in patients with colorectal cancer: Possible involvement of interleukin-6 and interleukin-8 in hematogenous metastasis. *J. Gastroenterol.* **29**, 423–429. doi:10.1007/BF02361238

Ullman T. A., and Itzkowitz S. H. (2011). Intestinal Inflammation and Cancer. *Gastroenterology* **140**, 1807-1816.e1. doi:10.1053/j.gastro.2011.01.057

Ungefroren H., Witte D., and Lehnert H. (2018). The role of small GTPases of the Rho/Rac family in TGF-β-induced EMT and cell motility in cancer: Role of Small GTPases in Cancer. *Dev. Dyn.* **247**, 451–461. doi:10.1002/dvdy.24505

Urbanski L. M., Leclair N., and Anczuków O. (2018). Alternative-splicing defects in cancer: Splicing regulators and their downstream targets, guiding the way to novel cancer therapeutics. *Wiley Interdiscip. Rev. RNA* **9**, e1476. doi:10.1002/wrna.1476

Urdinguio R. G., Fernandez A. F., Moncada-Pazos A., Huidobro C., Rodriguez R. M., Ferrero C., Martinez-Camblor P., Obaya A. J., Bernal T., Parra-Blanco A., Rodrigo L., Santacana M., Matias-Guiu X., Soldevilla B., Dominguez G., Bonilla F., Cal S., Lopez-Otin C., and Fraga M. F. (2013). Immune-Dependent and Independent Antitumor Activity of GM-CSF Aberrantly Expressed by Mouse and Human Colorectal Tumors. *Cancer Res.* **73**, 395–405. doi:10.1158/0008-5472.CAN-12-0806

Vainio H., and Boffetta P. (1994). Mechanisms of the combined effect of asbestos and smoking in the etiology of lung cancer. *Scand. J. Work. Environ. Health* **20**, 235–242. doi:10.5271/sjweh.1402

Valacca C., Bonomi S., Buratti E., Pedrotti S., Baralle F. E., Sette C., Ghigna C., and Biamonti G. (2010). Sam68 regulates EMT through alternative splicing–activated nonsense-mediated mRNA decay of the SF2/ASF proto-oncogene. *J. Cell Biol.* **191**, 87–99. doi:10.1083/jcb.201001073

Valkenburg K. C., de Groot A. E., and Pienta K. J. (2018). Targeting the tumour stroma to improve cancer therapy. *Nat. Rev. Clin. Oncol.* **15**, 366–381. doi:10.1038/s41571-018-0007-1

Vara J. Á. F., Casado E., de Castro J., Cejas P., Belda-Iniesta C., and González-Barón M. (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treat. Rev.* **30**, 193–204. doi:10.1016/j.ctrv.2003.07.007

Vega F. M., and Ridley A. J. (2008). Rho GTPases in cancer cell biology. *FEBS Lett.* **582**, 2093–2101. doi:10.1016/j.febslet.2008.04.039

Velikova T., Kyurkchiev D., Ivanova-Todorova E., Spassova Z., Stanilova S., and Altankova I. (2016). Cytokines in Inflamed Mucosa of IBD Patients. 'New Insights into Inflammatory Bowel Disease'. (Ed S Huber)(InTech) doi:10.5772/64286

Venables J. P., Klinck R., Koh C., Gervais-Bird J., Bramard A., Inkel L., Durand M., Couture S., Froehlich U., Lapointe E., Lucier J.-F., Thibault P., Rancourt C., Tremblay K., Prinos P., Chabot B., and Elela S. A. (2009). Cancer-associated regulation of alternative splicing. *Nat. Struct. Mol. Biol.* **16**, 670–676. doi:10.1038/nsmb.1608

Verhoeckx K., Cotter P., López-Expósito I., Kleiveland C., Lea T., Mackie A., Requena T., Swiatecka D., and Wichers H. (Eds) (2015). 'The Impact of Food Bioactives on Health.' (Springer International Publishing: Cham) doi:10.1007/978-3-319-16104-4

Visser K. E., Korets L. V., and Coussens L. M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* **7**, 411–423. doi:10.1016/j.ccr.2005.04.014

Vogelstein B., and Kinzler K. W. (2004). Cancer genes and the pathways they control. *Nat. Med.* **10**, 789–799. doi:10.1038/nm1087

Vogelstein B., Papadopoulos N., Velculescu V. E., Zhou S., Diaz L. A., and Kinzler K. W. (2013). Cancer Genome Landscapes. *Science* **339**, 1546–1558. doi:10.1126/science.1235122

Volodko N., Reiner A., Rudas M., and Jakesz R. (1998). Tumour-associated macrophages in breast cancer and their prognostic correlations. *The Breast* **7**, 99–105. doi:10.1016/S0960-9776(98)90065-0

Voronov E., and Apte R. N. (2015). IL-1 in Colon Inflammation, Colon Carcinogenesis and Invasiveness of Colon Cancer. *Cancer Microenviron.* **8**, 187–200. doi:10.1007/s12307-015-0177-7

Voronov E., Shouval D. S., Krelin Y., Cagnano E., Benharroch D., Iwakura Y., Dinarello C. A., and Apte R. N. (2003). IL-1 is required for tumor invasiveness and angiogenesis. *Proc. Natl. Acad. Sci.* **100**, 2645–2650. doi:10.1073/pnas.0437939100

Wahl M. C., Will C. L., and Lührmann R. (2009). The Spliceosome: Design Principles of a Dynamic RNP Machine. *Cell* **136**, 701–718. doi:10.1016/j.cell.2009.02.009

Wakabayashi O., Yamazaki K., Oizumi S., Hommura F., Kinoshita I., Ogura S., Dosaka-Akita H., and Nishimura M. (2003). CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. *Cancer Sci.* **94**, 1003–1009. doi:10.1111/j.1349-7006.2003.tb01392.x

Wan P. T. C., Garnett M. J., Roe S. M., Lee S., Niculescu-Duvaz D., Good V. M., Project C. G., Jones C. M., Marshall C. J., Springer C. J., Barford D., and Marais R. (2004). Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF. *Cell* **116**, 855–867. doi:10.1016/S0092-8674(04)00215-6

Wang H., Luo Q., Feng X., Zhang R., Li J., and Chen F. (2018). NLRP3 promotes tumor growth and metastasis in human oral squamous cell carcinoma. *BMC Cancer* **18**, 500. doi:10.1186/s12885-018-4403-9

Wang E. T., Sandberg R., Luo S., Khrebtukova I., Zhang L., Mayr C., Kingsmore S. F., Schroth G. P., and Burge C. B. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**, 470–476. doi:10.1038/nature07509

Wang S.-W., and Sun Y.-M. (2014). The IL-6/JAK/STAT3 pathway: Potential therapeutic strategies in treating colorectal cancer. *Int. J. Oncol.* **44**, 1032–1040. doi:10.3892/ijo.2014.2259

Wang Z.-Y., Zhang J.-A., Wu X.-J., Liang Y.-F., Lu Y.-B., Gao Y.-C., Dai Y.-C., Yu S.-Y., Jia Y., Fu X.-X., Rao X., Xu J.-F., and Zhong J. (2016). IL-6 Inhibition Reduces STAT3 Activation and Enhances the Antitumor Effect of Carboplatin. *Mediators Inflamm.* **2016**, 1–8. doi:10.1155/2016/8026494

Wang M., Zhao J., Zhang L., Wei F., Lian Y., Wu Y., Gong Z., Zhang S., Zhou J., Cao K., Li X., Xiong W., Li G., Zeng Z., and Guo C. (2017). Role of tumor microenvironment in tumorigenesis. *J. Cancer* **8**, 761–773. doi:10.7150/jca.17648

Waugh D. J. J., and Wilson C. (2008). The Interleukin-8 Pathway in Cancer. *Clin. Cancer Res.* **14**, 6735–6741. doi:10.1158/1078-0432.CCR-07-4843

Weg-Remers S. (2001). Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway. *EMBO J.* **20**, 4194–4203. doi:10.1093/emboj/20.15.4194

Wennerberg K. (2005). The Ras superfamily at a glance. *J. Cell Sci.* **118**, 843–846. doi:10.1242/jcs.01660

Wheeler K. C., Jena M. K., Pradhan B. S., Nayak N., Das S., Hsu C.-D., Wheeler D. S., Chen K., and Nayak N. R. (2018). VEGF may contribute to macrophage recruitment and M2 polarization in the decidua (X Ye, Ed.). *PLOS ONE* **13**, e0191040. doi:10.1371/journal.pone.0191040

Wilson T. R., Fridlyand J., Yan Y., Penuel E., Burton L., Chan E., Peng J., Lin E., Wang Y., Sosman J., Ribas A., Li J., Moffat J., Sutherlin D. P., Koeppen H., Merchant M., Neve R., and Settleman J. (2012). Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* **487**, 505–509. doi:10.1038/nature11249

Witte D., Otterbein H., Förster M., Giehl K., Zeiser R., Lehnert H., and Ungefroren H. (2017). Negative regulation of TGF-β1-induced MKK6-p38 and MEK-ERK signalling and epithelial-mesenchymal transition by Rac1b. *Sci. Rep.* **7**, 17313. doi:10.1038/s41598-017-15170-6

Wittinghofer A. (2014). 'Ras superfamily small g proteins: biology and mechanisms.' (Springer: New York) 10.1007/978-3-7091-1806-1_1

Wu H., Larribère L., Sun Q., Novak D., Sachindra S., Granados K., Umansky V., and Utikal J. (2018). Loss of neural crest-associated gene FOXD1 impairs melanoma invasion and migration via RAC1B downregulation: FOXD1 regulates melanoma invasion and migration. *Int. J. Cancer* **143**, 2962–2972. doi:10.1002/ijc.31799

Wu C., Wang S., Chao M., and Chi C. (1996). Serum interleukin-6 levels reflect disease status of gastric cancer. *Am J Gastroenterol* **91**, 1417–22.

Xue X., and Falcon D. M. (2019). The Role of Immune Cells and Cytokines in Intestinal Wound Healing. *Int. J. Mol. Sci.* **20**, 6097. doi:10.3390/ijms20236097

Yaeger R., and Saltz L. (2012). BRAF Mutations in Colorectal Cancer: Clinical Relevance and Role in Targeted Therapy. *J. Natl. Compr. Canc. Netw.* **10**, 1456–1458. doi:10.6004/jnccn.2012.0148

Yang W., and Lu Z. (2015). Pyruvate kinase M2 at a glance. *J. Cell Sci.* **128**, 1655–1660. doi:10.1242/jcs.166629

Yang J., Nie J., Ma X., Wei Y., Peng Y., and Wei X. (2019). Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol. Cancer* **18**, 26. doi:10.1186/s12943-019-0954-x

Yoshida T., Kato J., Inoue I., Yoshimura N., Deguchi H., Mukoubayashi C., Oka M., Watanabe M., Enomoto S., Niwa T., Maekita T., Iguchi M., Tamai H., Utsunomiya H., Yamamichi N., Fujishiro M., Iwane M., Takeshita T., Ushijima T., and Ichinose M. (2014). Cancer development based on chronic active gastritis and resulting gastric atrophy as assessed by serum levels of pepsinogen and *Helicobacter pylori* antibody titer: Cancer Development in *H. Pylori* -Associated Gastritis. *Int. J. Cancer* **134**, 1445–1457. doi:10.1002/ijc.28470

Yoshimura A. (2003). Negative regulation of cytokine signaling influences inflammation. *Curr. Opin. Immunol.* **15**, 704–708. doi:10.1016/j.coi.2003.09.004

Zabron A., Edwards R. J., and Khan S. A. (2013). The challenge of cholangiocarcinoma: dissecting the molecular mechanisms of an insidious cancer. *Dis. Model. Mech.* **6**, 281–292. doi:10.1242/dmm.010561

Zhang K., Hornef M. W., and Dupont A. (2015). The intestinal epithelium as guardian of gut barrier integrity: The epithelium as a barrier to infection. *Cell. Microbiol.* **17**, 1561–1569. doi:10.1111/cmi.12501

Zhao Z., and Manser E. (2012). PAK family kinases: Physiological roles and regulation. *Cell. Logist.* **2**, 59–68. doi:10.4161/cl.21912

Zhou C., Licciulli S., Avila J. L., Cho M., Troutman S., Jiang P., Kossenkov A. V., Showe L. C., Liu Q., Vachani A., Albelda S. M., and Kissil J. L. (2013). The Rac1 splice form Rac1b promotes K-ras-induced lung tumorigenesis. *Oncogene* **32**, 903–909. doi:10.1038/onc.2012.99

Zhou G., Yang J., and Song P. (2018). Correlation of ERK/MAPK signaling pathway with proliferation and apoptosis of colon cancer cells. *Oncol. Lett.* doi:10.3892/ol.2018.9857

Zimta A.-A., Cenariu D., Irimie A., Magdo L., Nabavi S. M., Atanasov A. G., and Berindan-Neagoe I. (2019). The Role of Nrf2 Activity in Cancer Development and Progression. *Cancers* **11**, 1755. doi:10.3390/cancers11111755

Zinn R., Otterbein H., Lehnert H., and Ungefroren H. (2019). RAC1B: A Guardian of the Epithelial Phenotype and Protector Against Epithelial-Mesenchymal Transition. *Cells* **8**, 1569. doi:10.3390/cells8121569