UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Exploring MEK5/ERK5 Signaling and miRNAs as Therapeutic Strategies in Colon Cancer

André Gonçalo do Espírito Santo Simões

Mestrado em Bioquímica

2011

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Exploring MEK5/ERK5 Signaling and miRNAs as Therapeutic Strategies in Colon Cancer

André Gonçalo do Espírito Santo Simões

Mestrado em Bioquímica

Dissertação de Mestrado orientada por:

Prof.ª Doutora Cecília M. P. Rodrigues

Prof.ª Doutora Luísa Cyrne

Lisboa

2011

The studies presented in this thesis were performed in the Molecular and Cell Biology of Eukaryotic Systems research group, at the Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculty of Pharmacy, University of Lisbon, under the supervision of Cecília M. P. Rodrigues, Ph.D.

This work was supported by grant PTDC/SAU-GMG/099161/2008 from FCT (to C.M.P.R.).

The studies presented in this thesis were developed under the frame of the following publications:

Paper:

Borralho PM, **Simões AE**, Gomes SE, Lima RT, Carvalho T, Ferreira DM, Vasconcelos MH, Castro RE, Rodrigues CM. MiR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation. *PLoS One*2011; 6(8): e23787.

Abstracts:

Borralho PM, **Simões AES**, Gomes SE, Lima RT, Carvalho T, Castro RE, Vasconcelos MH,Rodrigues CMP. O microRNA-143 aumenta a apoptose e reduz a proliferação de células de cancro colorectal *in vivo. Jornal Português de Gastrenterologia* 2011; 18: 4.
[Selected oral communication]

Borralho PM, **Simões AES**, Gomes SE, Lima RT, Carvalho T, Ferreira DMS,Vasconcelos MH, Castro RE,Rodrigues CMP. miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation.*19thEuroconference on Apoptosis "Metabolism, Epigenetics and Cell Death"*, Stockholm, Sweden, 2011.
[Poster presentation]

Borralho PM,**Simões AES**, Gomes SE, Lima RT, Carvalho T, Vasconcelos MH, Castro RE,Rodrigues CMP.miR-143 overexpression reduces the growth of xenograft Tumors from colon carcinoma cells, increasing tumor cell apoptosis and decreasing proliferation. *XX Porto Cancer Meeting, Drug Resistance in Cancer: from Biology to Molecular Drugs and Targets, Porto, Portugal, 2011.*
[Poster presentation]
Best Poster Award for the Basic Research session.

Agradecimentos

Em primeiro lugar, gostava de agradecer à professora Cecília Rodrigues pela oportunidade que me proporcionou, ao aceitar-me no seu grupo, e me continua a proporcionar, ao permitir-me continuar, contribuindo muito directamente para a minha realização profissional e pessoal. Não podendo deixar de referir que a sua dedicação e capacidade de trabalho espelham o percurso a percorrer para o sucesso neste mundo da investigação.

Queria também agradecer à professora Luísa Cyrne, minha orientadora interna, pela sua disponibilidade em possibilitar-me a realização da minha tese de mestrado fora da instituição em que me encontro inscrito. Contribuindo directamente na minha demanda pela minha concretização no mundo profissional.

Gostava também de expressar a minha gratidão ao professor João Gonçalves e ao seu grupo por nos terem fornecido o anticorpo α-FITC-protamine, fulcral para o desenvolvimento e progressão de um dos objectivos do presente estudo.

Ao Pedro, o "chefe", quero agradecer-lhe a dobrar e com um grande abraço forte! Em primeiro lugar, agradeço-te Pedro pela pessoa fantástica que és e que tens sido comigo, pela amizade que sinto termos desenvolvido, que transcedeu muito a relação meramente profissional. Acho que nunca te disse, mas trabalhar contigo foi uma experência da qual estou enormemente grato, não conseguindo sequer imaginar de que forma poderia ter tido mais sorte. Para além de seres um exemplo que considero a seguir a nível profissional, és também uma pessoa que sempre se monstrou disponível, que da melhor forma que sabes procuraste sempre ajudar e que espalha e espelha boa disposição e sorrisos em todos os que te rodeiam. Muito agradecido por tudo Pedro.

Desejo também fazer um agradecimento especial aos meus compinchas do lab, Duarte, Beni e Daniela, que muito perto estiveram quando muito precisei, que mostrando-me quem são, fizeram-me reconstruir novamente e mais forte. Duarte, agradeço-te pela tua incondicionalidade, paciência e amizade. Beni, agradeço-te o teu sorriso, a tua força e a tua paz. Daniela, agradeço-te o teu abraço, o teu bem-estar e alegria. Fizeram um amigo!

Ao chavalo, o Miguel, agradeço a louca boa disposição e a serenidade. A tua contagiante boa disposição e a serenidade com que sempre te mostraste disponível para ajudar fazem de ti...um chavalo especial! E eu pergunto.."Are you gonna be my girl?"!

Quero com um grande sorriso agradecer aos restantes, mas não menos importantes, membros do grupo molcellbiol, Filipa, Rita, Joana Amaral, Joana Xavier, Maria, Rui, Susana, Elsa, Maria João e Andreia, destacando também a Simone que durante uns tempos também nos pertence. Agradeço o incrível bom ambiente, existente no laboratório, que por vocês é suscitado e pela prestabilidade que todos demonstraram em ajudar-me quando necessário.

Destaco agora um agradecimento também muito especial ao gangue da cave, que mesmo na ausência de janelas, tornou este lugar mais luminoso. Não esquecendo de destacar os sempre característicos espirros e massagens da menina Cátia, que desejo que consiga alcançar os seus objectivos, e a boa disposição e carinho da Filipa Sol.

À Ana, a minha oliverinha pgeta, canto uma cantiga especial...mas não agora! Agradeço-te a tua capacidade de me fazer rir e de dançares comigo! As palavras fogem-me para descrever como a amizade que se criou é especial e não agradeço nada perder-te daqui da minha beira. Mas é com grande desejo que procurarei sempre ajudar-te a encontrares aquilo que procuras. Agradeço-te minha amiga.

Inês, a ti agradeço-te toda a partilha...todo o apoio, carinho, bem-estar, amizade e amor. Guardo tudo o que me deste, tudo o que me proporcionás-te e fizeste sentir. Ajudáste-me a voltar a acreditar...em mim e na vida e só espero ter conseguido e conseguir mostrar e fazer-te sentir tão especial, como tu me tens feito sentir. Especialmente te sinto. Também gosti.

Agradeço também, sem esquecer, a dois amigos muito especiais. À minha amiga do coração, Mariana, que já me acompanha à 11 anos e que por mais conflitos que já tenhamos vivido é uma amizade que perdura e perdurará. E ao Cadota, o alentejano, que é das pessoas que mais paz e sossego dá em cada passo, mostrando-me que na vida está sempre tudo bem.

Por último, quero agradecer aos meus pais e ao meu irmão. Agradeço todo o apoio incondicional que me têm dado e todo o amor com que o têm feito. Mãe agradeço-te o amor que transmites em cada toque, em cada palavra e em cada preocupação tua. Pai agradeço-te a tua presença constante, a tua paz transcendente e as nossas longas conversas. E por fim, agradeço-te a ti mano, por irremediavelmente me conseguires fazer abstrair de tudo e todos, assim como me tirar do sério! És o meu puto, o ser que mais valor dou na minha vida, por isso porta-te bem! Amo-vos!

Table of Contents

Resumo

O cancro é, actualmente, a segunda causa de morte no mundo ocidental, sendo o cancro colorectal, a terceira forma de cancro mais commumente diagnosticada, tanto no homem, como na mulher. Mais ainda, o cancro colorectal é a terceiraprincipal causa de morte relacionada com o cancro.A transformação do tecido colónico normal e o desenvolvimento do cancro colorectal é considerado um processo faseado, com acumulação de diferentes alterações genéticas e epigenéticas, que levam à transformação do epitélio normal em adenoma e, mais tarde, em carcinoma. Adenomas ou pólipos são neoplasmas que, embora apresentem vantagem proliferativa inerente e redução de diferenciação, progridem para formas malignas em apenas 10% dos casos. Um dos principais eventos moleculares que pode conduzir ao desenvolvimento e progressão do cancro é a perda de estabilidade genómica que, no caso do cancro colorectal, está associada à instabilidade de microssatélites e à instabilidade cromossómica. A instabilidade de microssatélites é caracterizada pela presença de mutações em genes do sistema de reparação de *mismatches*no DNA, as quais podem resultar na inserção ou delecção de repetições em microssatélites. Os microssatélites, por sua vez, são pequenas repetições de sequências de nucleótidos, contendo cerca de uma dúzia de repetições de 4 a 6 sequências nucleótidicas. As mutações geradas em cancros com instabilidade de microssatélites são, inicialmente, aleatórias, afectando qualquer repetição de microssatélite. Alguns clones podem, no entanto, adquirir mutações em genes chave, conferindo à célulavantagem proliferativa. A instabilidade cromossómica é o tipo de instabilidade genómica mais comum, ocorrendo em cerca de 80 a 85% dos tumores colorectais esporádicos, que não apresentam deficiências no sistema de reparação de *mismatches* noDNA. Tumores com instabilidade cromossómica estão associados a uma elevada frequência de aneuploidias e desequilíbrios alélicos, apresentando em geral, um prognóstico pior do que tumores com deficiências no sistema de reparação *demismatches*noDNA. Nos neoplasmas colorectais é, ainda, comum a instabilidade epigenética, a qual resulta da metilação aberrante de genes supressores de tumores. Dentro dos tumores esporáricos com instabilidade de microssatélites, a instabilidade epigenética manifesta-se através da metilação do promotor de genes envolvidos na reparação do DNA, nomeadamente do gene *MLH1*.

Várias vias de sinalização que regulam o ciclo celular e a apoptose encontram-se desreguladas na transformação, desenvolvimento e progressão tumoral, levando a um aumento descontrolado da proliferação celular e à quase completa insensibilidade a estímulos pro-apoptóticos. Destacam-se a sobreactivação das vias de sinalização das MAPKs, do NF-κB e do PI3/AKT, que promovem a proliferação celular, bem como a mutação do p53, que inibe a apoptose numa elevada percentagem de carcinomas.

A desregulação da expressão de microRNAs (miRNAs) tem sido associada ao desenvolvimento de vários tipos de cancro, incluindo o cancro colorectal, através da alteração dos níveis de produção dos vários mediadores envolvidos nestas vias de sinalização celular. Os miRNAs são pequenas moléculas de RNA não codificante que regulam negativamente a expressão génica, a nível pós-transcricional, através da repressão da tradução do mRNA alvo, ou da clivagem e degradação do mesmo, estando este processo dependente do grau de complementaridade entre o miRNA maduro e o mRNA alvo. A acção dos miRNAs está

envolvida na regulação de inúmeros processos biológicos, nomeadamente na proliferação celular e na apoptose. Curiosamente, observou-se que o miRNA-143 (miR-143) apresenta expressão reduzida no cancro colorectal, assim como em outros tipos de cancro, sendo que um dos seus principais alvos biológicos é a ERK5. A acção do miR-143 na inibição do crescimento tumoral, com modulação da expressão de ERK5, foi já demonstrada pelo nosso grupo, tanto *in vitro,* como *in vivo*.

Não existe, actualmente, qualquer informação disponível na literatura relativa à produção da proteína ERK5, ou da sua proteína activadora directa, a MEK5, no cancro colorectal. No entanto, o aumentonos níveis destas duas cinases foi já observado em outros tipos de cancro, estando associado ao aumento de proliferação, progressão tumoral e resistência à terapêutica,para além de se correlacionar com um pior prognóstico da doença. Um dos objectivos do presente trabalho foi analisar a produçãodas proteína ERK5 e MEK5 durante o desenvolvimento e progressão do cancro colorectal, por forma a determinar se esta via de sinalização pode constituir um novo alvo terapêutico no cancro colorectal. Foi estudado um conjunto bem definido de 197 amostras humanas, incluindo tecido normal, adenomas e carcinomas, com e sem instabilidade de microsatélites. Os resultados demonstram, inequivocamente, que as cinases ERK5 e MEK5 se encontram aumentadas no cancro colorectal e que, possivelmente, a sinalização via ERK5 é um evento importante na iniciação da transformação tumoral. De facto, verificou-se um aumento significativo de ERK5 e MEK5 em adenomas, bem como em carcinomas, de forma independente da integridade do sistema de reparação de *mismatches*no DNA, relativamente ao tecido colónico normal. Sugere-se, assim, que a ERK5 poderá representar um alvo terapêutico novo e relevante no cancro colorectal, possivelmente através da redução da sua produção induzida pelo miR-143. O estudo de mediadores de outras vias de sinalização, importantes no desenvolvimento tumoral, como o NF-κB e o AKT, revelou que estão alteradosdurante o desenvolvimento do cancro colorectal, tanto nos pólipos, como nos carcinomas com e sem instabilidade de microsatélites. Confirmou-se, ainda, que a perda de função da proteína p53 está envolvida na transição de adenoma para carcinoma e que a frequência de ocorrência de mutações no p53 está relacionada com a funcionalidade do sistema de reparação de *mismatches* no DNA.

Tendo em conta o conhecimento actual do potencial anti-proliferativo, pro-apoptótico e quimiosensibilizador do miR-143, possivelmente através da repressão da via de sinalização da ERK5, outro objectivo do presente trabalho consistiu no desenvolvimento de uma estratégia eficiente de entrega do miR-143, tendo em vista o seu uso como ferramenta terapêutica no tratamento do cancro colorectal. O maior obstáculo na aplicação terapêutica dos miRNAs está relacionado com o seu direcionamento para as células alvo*in vivo*, bem como com a sua estabilidade, uma vez que os miRNAs, não modificados e desprotegidos, são instáveis em circulação. O sistema de entrega em desenvolvimento neste estudo baseia-se na conjugação do miR-143 com o cetuximab, já utilizado no cancro colorectal como terapia dirigida. O cetuximab é um anticorpo monoclonal que se liga ao receptor EGFR, frequentemente aumentado no cancro colorectal, promovendo a sua internalização e degradação. O tratamento com este anticorpo melhora significativamente a sobrevivência dos doentes e a progressão livre de doença, sendo, no entanto, a sua eficácia dependenteda ausência de mutações no KRAS.

Sabendo que o miR-143 apresenta efectores específicos a jusante na via do EGFR, KRAS e ERK5, como principais alvos, o potencial terapêutico do miR-143 é elevado e poderá aumentar a eficácia do cetuximab. O objectivo último é tirar partido da especificidade do cetuximab para direccionar o miR-143 para as células com produção aumentada de EGFR. A estratégia de entrega desenvolvida baseou-se na conjugação do cetuximab com fluoresceína 5(6)-isotiocianato (FITC). De seguida, o anticorpo conjugado foi complexado com um anticorpo anti-FITC, conjugado com protamina (anti-FITC-protamina), numa proporção superior ao número de moléculas de FITC conjugadas com o cetuximab, para garantir que todos os resíduos de FITC no cetuximab estejam ocupados e, desta forma, maximizar a carga de miRNA em cada molécula de cetuximab. Por fim, o miR-143 será marcado com o fluoróforo Cy3 e carregado nas protaminas. A protamina é uma proteína carregada positivamente, com afinidade para ácidos nucleícos, que apresenta um grande potencial para a entrega de pequenos RNAs em células de cancro, já demonstrado *in vitro* e *in vivo*. O progresso conseguido no desenvolvimento desta ferramenta permitiu demonstrar que o cetuximab conjugado com FITC manteve a capacidade de ligação ao EGFR, para além de que o complexo terapêutico constituído por cetuximab-FITC e anti-FITC-protamina foi entregue em células de cancro colorectal com níveis elevados de EGFR. Uma vez que a eficiência desta ferramenta depende, em grande medida, da ligação e internalização do complexo nas células alvo, os resultados obtidos até agora reforçam o potencial desta estratégia de entrega, para além de estimularem estudos futuros com vista à sua utilização terapêutica .

Palavras-chave: Cancro colorectal; Cetuximab; ERK5; Ferramenta de entrega*;* miR-143

Abstract

Colorectal cancer (CRC) is a major health problem worldwide, with high incidence, representing a major cause of cancer related deaths. The development of CRC is considered a stepwise process, with the accumulation of genetic and epigenetic alterations, which contribute to transformation of normal colonic epithelium into adenoma, and eventually to carcinoma. The overexpression of ERK5 and MEK5 have been reported in many types of human cancer and correlated with increased cell survival, proliferation, chemoresistance and poor disease prognosis. Curiously, no information is available on ERK5 or MEK5 expression profiles in CRC. However, ERK5 expression is directly regulated by microRNA-143 (miR-143), typically downregulated in CRC. The aim of the present work was to evaluate the expression levels of ERK5 and MEK5 in normal colon, colon adenomas, and colon carcinomas. In addition, we aimed to develop an efficient delivery strategy for future use of miR-143 in CRC therapy, using cetuximab as a delivery vehicle. Our results provide the first human data demonstrating the aberrant overexpression of ERK5 and MEK5 in adenomas and, to a lesser extent, carcinomas. This suggests ERK5 as a potential target for the development of novel cancer therapies. Further, we confirmed a significant deregulation of NF-κB and AKT signaling pathways, thought to be involved in cancer development and progression. p53, a key player in adenoma-carcinoma transition was also deregulated. Finally, we built an effective delivery complex, delivered to target cells with high affinity, highlighting the potential of this strategy for cancer cell delivery of miRNA therapeutics.

Keywords: Cetuximab; Colorectal cancer; Delivery tool;ERK5; miR-143

Abbreviations

5-FU	5-fluorouracil
AGO	argonaute
APC	adenomatous polyposis coli
BSA	bovine serum albumin
CRC	colorectal cancer
DMEM	Dulbecco's modified Eagle's medium
dMMR	defective DNA mismatch repair
DPBS	Dulbecco's phosphate-buffered saline
EGFR	epidermal growth factor receptor
ERK	extracellular-regulated protein kinase
FACS	fluorescence activated cell sorting
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FITC	fluorescein 5(6)-isothiocyanate
IgG	immunoglobulin G
IκB	nuclear factor κB inhibitor
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen-activated protein kinase
Mdm-2	mouse double minute 2
MEK	MAPK/ERK kinase
MEKK	MEK kinase
miR-143	microRNA-143
miR/miRNA	microRNA
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli)*
MMR	DNA mismatch repair
MSH2	mutS homolog 2
MSI	microsatellite instability
MSS	microsatellite stability
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NF-κB	nuclear factor κB
p-bodies	processing bodies
PBS	phosphate buffered saline
PI3K	phosphatidylinositide 3'-OH kinase
pMMR	proficient DNA mismatch repair
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homologue
Ras	rat sarcoma
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
scFv	single chain variable fragment
SDS	sodium dodecyl sulfate
TNM	tumor-node-metastasis
Wnt	wingless

I. INTRODUCTION

1. Colorectal Cancer

1.1. Colon Structure

The large intestine is the last section of the gastrointestinaltract,and is composed by four sequential sections: cecum, colon, rectum and anus. The colon is the longest portion of the large intestine, a muscular tube that extends for about 1.5 m, followed bythe rectum, approximately0.15 m long.The main biological function of the colon is toabsorb water and minerals, and to conduct the waste products (feces) into the rectum, where they will be expelled from the anus. The colon is further divided in four sections, although histologically indistinct; the first section is the ascending colon, which connects tothe cecum and extends upward in the right abdomen;the second section, the transverse colon, crosses the abdomen from the right to the left side; the third section, the descending colon, continues downward in the left side of the abdomen; and the final section, the sigmoid colon, connects to the rectum, which in turn connects to the anus(American Cancer Society, 2011).

The human colon is composed by four functionally distinct layers: mucosa, submucosa, muscularispropria and serosa (Fig. 1). The mucosa is composed by the colonic epithelium at the luminal surface, the supporting lamina propria, and the smooth muscle layer called muscularis mucosa. The submucosais a thin layer of loose collagenous tissue, supporting the mucosa, comprised of blood vessels, lymphatics and nerves. The muscularispropria is a layer of smooth muscle arranged as an inner circular layer and an outer longitudinal layer. Finally, the serosa is an outer layer of connective tissue, containing the major nerves and vessels.The colonic epithelium at the luminal surface is composed by a single sheet of cells, which form finger-like invaginations into the underlying tissue, the lamina propria. These colonic invaginations are the basic structural units of the colon and are called crypts of Lieberkühn(Young and Heath, 2000).

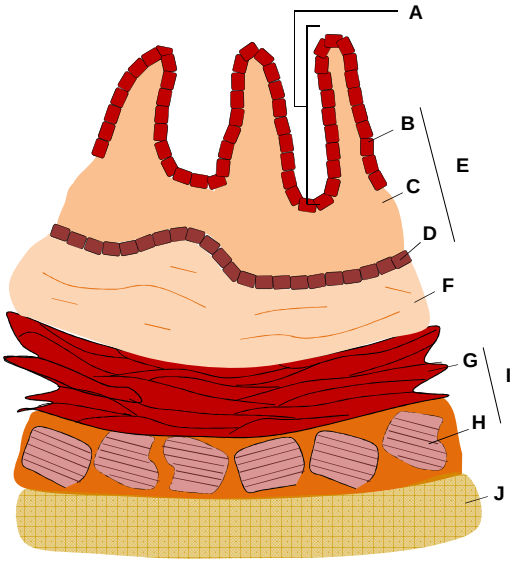

Fig.1. Schematic representation of colon structure.(A) Lieberkühn crypt; (B) colonic epithelium; (C) lamina propria; (D) muscularis mucosa; (E) mucosa; (F) submucosa; (G) circular muscle; (H) longitudinal muscle; (I) musculares propria; and (J) serosa.Adapted from (www.cancerquest.org/colon-rectal-cancer-anatomy).

Colonic epithelium lining and maintenance is assured by stem cells located near or at the bottom of each crypt, within the stem cell niche. It is characterized by a high rate of cellular turnover, with complete cellular replacement every week, except for stem cells that are long-lived (Potten et al., 1992).The structure of the crypt and the dynamics of cell replication ensure that, under normal conditions, crypt stem cells and immediate daughter cells replicate in the lowest third of the crypt. Subsequently, they terminally differentiate into colonocytes or absorptive cells, the mucus-secreting goblet cells, and peptide hormone-secreting endocrine cells, as they migrate to the top of the crypt(Wright, 2000). Once cells reach the top of the crypt,they have stopped dividing and differentiating, and may begin to undergo apoptosis. This highly ordered series of events contributes to the notion that any mutagenic alterations acquired in non-stem cells would have no major impact on the integrity of the crypt cell population. However,alterations acquired by crypt stem cells or very early daughter cells will contribute to the onset of cancer(Potter, 1996).

1.2. Cancer Development

Cancer is the second most common cause of death in the United States and Europe.Colorectal cancer (CRC)is the third most commonly diagnosednon-cutaneous cancer in both sexes, after lung and prostate cancer in men, and lung and breast cancer in women.Importantly, CRCis also the third leading cause of cancer-related death(American Cancer Society, 2011).

CRC usually develops slowly over the course of many years. Thisis considered a stepwise process, with the accumulation of different genetic and epigenetic alterations, leading to the transformation from normal colonic epithelium to adenoma, and later to carcinoma. Polyps (adenomas) are neoplasms, whichresult from increasedproliferationand reduced differentiation of the epithelial cells lining the colon or rectum, as they migrate to the top of the crypts. However, despite this gain of proliferative advantage and loss of differentiation capacity, only fewer than 10% of adenomas actually become cancerous. Curiously, more than half of all individuals will eventually develop one or more adenomasthroughout life (American Cancer Society, 2011).Polyp type, size, and degree of dysplasia are correlated with the malignancy potential of an adenoma; higher grades of dysplasia or size greater than 1 cm in diameter are associated with increased risk of developing into carcinoma(Winawer et al., 2006).

The sequential events that lead to CRC development are associated with aprogressive accumulation of genetic and epigenetic changes, which affect signaling pathways that regulate key hallmarks of cancer, thus creating a clonal growth advantage that leads to the outgrowth of malignant cells.These genetic changesare associated with the inactivation of tumor suppressor genes, activation of oncogenes andacquisition of mismatch repair defects, viagene mutations or gene amplification, and epigenetic silencing, viaaberrant DNA methylation or chromatin modifications(Gayet et al., 2001; Grady and Carethers, 2008). However, to date, the molecular events leading to CRC initiation arestill not fully elucidated, particularly those that underlie adenoma progression to carcinoma.Nevertheless, these events have been the target of intense research in the last decades.

1.2.1. Genomic instability

Important contributions for the current knowledge of the pathogenesis of CRC derived from studies on hereditary colorectal cancer syndromes, such as familial adenomatous polyposis (FAP) and Lynch syndrome (also called HNPCC or hereditary non-polyposis colon cancer).These autosomal dominant diseases represent a small proportion of total CRC cases, (1 and 2-5% for FAP and Lynch syndrome, respectively), but are not considered rare events, due to the high incidence of CRC (Lynch et al., 2006).

FAP is an autosomal dominant disease, associated with agermline mutation in the adenomatous polyposis coli (APC) gene.FAP patients usually develop over 100 colorectal polyps, typically during puberty, which carries a risk of developing colorectal cancer of 100% by the second or third decade of life, if prophylactic colectomy is not performed (Al-Sukhni et al., 2008). TheAPC gene is a tumor suppressor involved in the control of many biological processes related with cell division and cell motility. Furthermore, there isstrong evidence that the increased potential for cells presenting APC mutation to progress to CRC may not only result from the overactivation of the Wingless(Wnt) signaling pathway, but also from the induction of chromosome instabilitydue to the function of APC protein in spindle formation(Al-Sukhni et al., 2008; Grady and Carethers, 2008; Nathke, 2004).The elucidation of the role of APC mutations in the development of FAP, led to the discovery that somatic APC gene mutations are also an important early event in 60 to 80% of sporadic CRC (Al-Sukhni et al., 2008).The loss of functional APC can lead to increased proliferation and lack of differentiation, mainly due to decreasedβ-catenin degradation, one of the major functions of the APC protein. The resulting accumulation and increased nuclear translocation of β-catenin leads to the transcriptional activation of many target genes involved in the regulation of cellular proliferation and differentiation, including c-myc andcyclin-D1(Nathke, 2004).

The Lynch syndrome is an autosomal dominant condition with incomplete penetrance,characterized by mutations in mismatch repair (MMR) genes, primarily in MLH1, MSH2, MSH6 and PMS2.Mutations in MLH1 and MSH2 genes have a higher incidence inCRC and are associated with a more severe phenotype, when compared to MSH6 and PMS2, presenting an incidence between 80 to 90% in Lynch syndrome patients (Al-Sukhni et al., 2008; Lynch et al., 2006). Importantly, MMR genes have a major role in correcting and recognizing errors during DNA replication.Mutations in these genes lead to deletion or insertion of repeats in microsatellites, polymorphic stretches of DNA consisting of up to a dozen repeats of 4 to 6 nucleotide sequences(Al-Sukhni et al., 2008).This form of genomic instability, termed microsatellite instability (MSI), occurs either through the aberrant methylation of CpG islands of MLH1 gene or by point mutations in MLH1, MSH2 and other members of the MMR family.TheseDNA mutations are initially random in MSI cancers, affecting any susceptible microsatellite repeat. However, some clones ultimately acquire mutations in key regulatory genes that induce growth imbalances,leading to growth advantage(Grady and Carethers, 2008).MSI status of a particular tumor may be evaluated through the analysis of the expansion or contraction of microsatellites,in comparison to non-tumor DNA from the same individual.It may also be ascertained through the evaluation of expression of protein products of MMR genes. From this analysis, a tumor may then be categorized as MSI-high (MSI-H), if over 30 to 40% of the markers are unstable;MSI-low (MSI-L), if under 30 to 40% of the markers are unstable; or microsatellite stable (MSS), if none of the markers display instability(Boland et al.,

1998). Importantly, colon tumors presenting defective DNA mismatch repair (dMMR) represent 15 to 20% of sporadic human colon tumors, and more than 95% of colon cancer arising in patients with Lynch syndrome(Grady and Carethers, 2008).

Therefore, it becomes clear that one of the key molecular steps that may lead to cancer formation is the loss of genomic stability. In this case, gain of mutations is increasingly facilitated, and the acquisition of a sufficient number of gene alterationsthat may transform cells and promote tumor progression is facilitated.So far, two predominant forms of genomic instability have been identified in colon cancer: MSIand chromosome instability (CIN).CIN is the most common type of genomic instability and occurs in 80 to 85% of sporadic colorectal tumors, which displayproficient DNA mismatch repair (pMMR) system. In addition, sporadic pMMRtumors are associated with high frequency of aneuploidy and allelic imbalance, and present an overall poorer prognosis compared to dMMRtumors (Grady and Carethers, 2008).In turn, sporadic dMMR colon tumors display distinct clinicopathologic features compared to sporadic pMMRcolon tumors, including proximal colon predominance, lymphocytic infiltration, mucinous histology, diploid DNA content, and have a better overall survival (Baudhuin et al., 2005).

1.2.2. Epigenetic instability

In addition to genomic instability, another common feature observed in colorectal neoplasms is epigenetic instability. This phenomenon results, among others, from the aberrant methylation of tumor suppressor genes. Within MSI sporadic tumors, the most common mechanism of MLH1 gene inactivation is promoter hypermethylation, which occurs in more than 80% of cases. The recovery of MLH1 expression and function can be achieved by demethylation of the MLH1 promoter (Grady and Carethers, 2008).

1.3. Cancer Progression

Colon tumors represent a heterogeneous group of neoplasms, since not all tumors present the same genetic defects/alterations (Gayet et al., 2001). Importantly, in FAP, in contrast with Lynch syndrome, tumor initiation is significantly accelerated due to the inheritance of a germline APC mutation. In turn, in Lynch syndrome, tumor initiation can be normal or slightly accelerated,with tumor progression greatly accelerated due to the hypermutable phenotype that results from the loss of function of the DNA MMR system.In marked contrast, sporadic colon cancers develop and progress at a slower pace when compared with hereditary CRC syndromes, FAP and Lynch (Fig. 2) (Grady and Carethers, 2008).

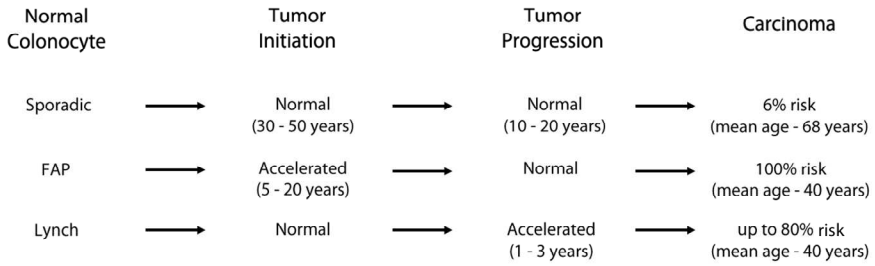

Fig.2. Colorectal cancer progression. Tumor initiation and progression is depicted in sporadic and high-risk genetic syndromes.Adapted from (Grady and Carethers2008).

Considering the current knowledge, the consensusto represent the adenoma to carcinoma progression is an improved version of the Vogelstein model (Fig.3). In this model, the genetic alterations that lead to tumor initiation are mutations in the components of the Wnt signaling pathway, such as APC and/or β-catenin, and loss of heterozigozity of APC. The consequentderegulation of c-myc and cyclin-D1 expression leads to imbalance between cellular proliferation and apoptosis.Therefore, APC mutations initiate the neoplastic process and lead to the formation of dysplastic aberrant crypt foci.In turn, tumor progression results from the accumulation of other genetic alterations, such as activation of proto-oncogene KRAS (k-Ras), loss of heterozigozity at chromosome 18, more precisely of the tumor suppressor Smad-4, which leads to the disruption of thetransforming growth factor beta(TGF-β) signaling pathway. This disruption enhances cell cycle division and promotes growth advantage, due to the previously deranged Wnt signaling. Importantly, mutations in p53 with the subsequent loss of heterozigozity are also common. Cells may become almost completely insensitive to apoptosis and allowed to accumulate many additional genetic alterations(Arends, 2000; Kinzler and Vogelstein, 1996).

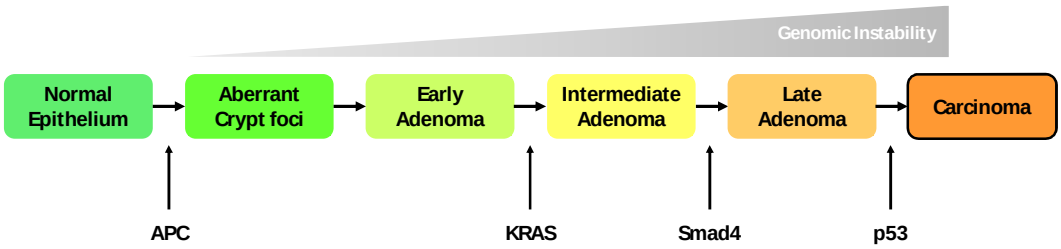

Fig.3.Schematic representation of the Vogelstein modelfor the adenoma to carcinoma progression in human colorectal carcinogenesis. Mutations in components of the Wnt signaling pathway, such as APC or β-catenin initiate the neoplastic process.Tumor progression results from mutations in other genes, such as mutational activation of KRAS, loss of heterozigosity of Smad4 (at chromosome 18) and mutation of p53 with subsequent loss of heterozigosity.Adapted from(Ricci-Vitiani et al., 2008).

Without treatment, CRC can become more aggressive, eventually growing beyond the mucosa,invading the underlying tissues of the colonic wall, and ultimately,metastasizing to distant sites.For the development of proper treatment strategies, determination of the precise pathologic tumor staging is essential, currently representingthe most important prognosis

factor in CRC. In addition,MSI and loss of heterozygosity at chromosome 18q are relevantprognostic markers, and the two best characterizedthus far (Wolpin and Mayer, 2008). Tumors with increased MSI have a more favorable prognosis than MSS tumors. Further, tumors with loss of heterozygosity at chromosome 18q present a poorer prognosis(Walther et al., 2009).

Currently, the tumor-node-metastasis (TNM) system is the most commonly used for colon cancer staging. This system grades tumors based on the extent of invasion of the bowel wall (T), extent of the involvement of regional lymph nodes (N), and presence of metastatic cancerous cells in distant sites (M)(Wolpin and Mayer, 2008).Tumors are usually further categorized by stage grouping 0-IV. Stage 0 is the earliest cancer stage, also defined as carcinoma i*n situ* (T_{is}), with tumor growth confined to the mucosa. Stage I is associated with tumor growth through the mucosa into the submucosa (T_1), or into the muscularispropria (T_2). At this stage, there is no spread into nearby lymph nodes (N_0) or distant sites (M_0). In stage II, the tumor has grown into the outermost layers of the colorectal wall (T_3), or through it intoneighboring tissues (T_4), without spreading to nearby lymph nodes(N_0),nor distant sites (M_0).At stage III, the tumor has grown into all the layers of the colorectal wall or even into nearby tissues (T_{1-4}). In addition, the tumor has also spread to 1-3, or 4 or more,nearby lymph nodes (N_{1-2}), but not to distant sites (M_0). The last stage, stage IV, is the most advanced stage of CRC and besides the invasion of all colorectal wall layers and nearby tissues (T_{1-4}), and the spread to nearby lymph nodes (N_{1-2}), metastasis at distant sites are also detectable (M_1)(Wolpin and Mayer, 2008) (Fig.4).

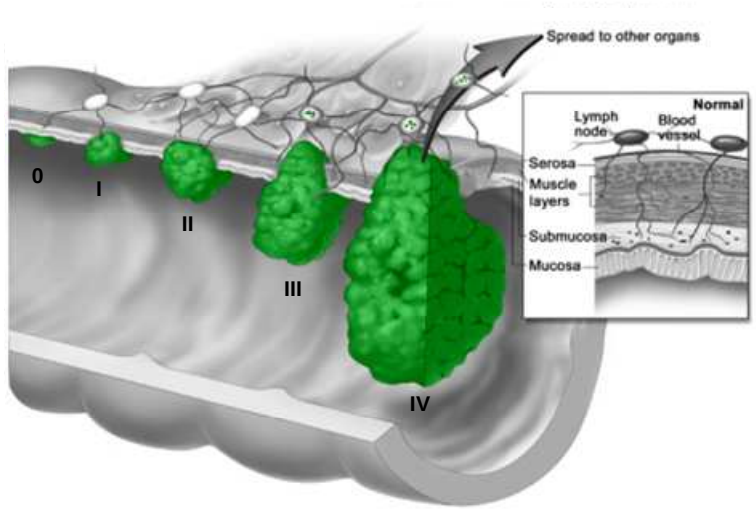

Fig.4.Representation of colorectal cancer growth. Tumor progression from stage 0 to IV, with sequential invasion of nearby tissues, spread to lymph nodes and ultimately to other organs.Adapted from (American Cancer Society, 2011)**.**

As CRCprogresses from stage I to IV, the overall survival rates decrease abruptly due to increased aggressiveness of the disease (Fig. 5).

Primary tumor (T)

T_{is}	Carcinoma *in situ*
T_1	Tumor invades submucosa
T_2	Tumor invades muscularis propria
T_3	Tumor invades through the muscularis propria into the subserosa
T_4	Tumor directly invades other organs or structures, or perforates visceral peritoneum

Regional lymph nodes (N)

N_0	No regional lymph node metastases
N_1	Metastases in 1-3 regional lymph nodes
N_2	Metastases in ≥ 4 regional lymph nodes

Regional lymph nodes (N)

M_0	No distant metastases detected
M_1	Distant metastases detected

Stage grouping and 5-year survival

Stage	TNM classification	5-year survival
I	T_{1-2}, N_0, M_0	≥ 90%
IIA	T_3, N_0, M_0	80 - 85%
IIB	T_4, N_0, M_0	70 - 80%
IIIA	T_{1-2}, N_1, M_0	65 - 70%
IIIB	T_{3-4}, N_1, M_0	50 - 65%
IIIC	T_{1-4}, N_2, M_0	25 - 50%
IV	T_{1-4}, N_{0-2}, M_1	5 - 8%

Fig. 5.TNM staging system for CRC and overall 5-year survival. Adapted from (Wolpin and Mayer, 2008).

1.4. Signaling Pathways in Cancer

Apoptosis is a cellular event that plays a major role in maintenance of the epithelium homeostasis, by ensuring the elimination of epithelial cells at the top of the crypt (Potter, 1999). The expression of anti-apoptotic effectors, such as Bcl-2, is maximal at the bottom of the crypt, and minimal at the top (Huerta et al., 2006). Imbalance in cell renewal and death is considered important for the development of CRC. In addition, loss of sensitivity to undergo apoptosis, even after an insult, increases the malignancy of the tumor, contributing to tumor progression and chemotherapy resistance.

Therefore, normal apoptosis is a major intended cellular process to prevent cancer onset, and also to treat cancer. This cellular programmed event may proceed through several pathways that cross-talk and influence each other. The best characterized pathways are the intrinsic pathway or mitochondrial pathway, and the extrinsic pathway or death receptor pathway. The intrinsic pathway is triggered by a cascade of intracellular events in which mitochondrial permeabilization plays a crucial role. In turn, the extrinsic pathway is triggered by activation of ligand-induced death receptors at the cell surface.

Loss of function of p53 makes cells almost insensitive to apoptosis, allowing the accumulation of additional genetic alterations. p53 is transcriptionally active as a homotetramer, functioning as a transcription factor in mediating DNA damage responses to a variety of cellular stresses. In addition, it induces cell-cycle arrest, senescence and apoptosis to maintain genomic stability (Amaral et al., 2010). Since p53 is a powerful inhibitor of cell proliferation, the tight control of its activity is essential during normal growth and development. In this sense, p53 activity is tightly regulated by the Mdm-2 protein and is continuously degraded in healthy cells (Amaral et al., 2010; Balint and Vousden, 2001).

TheMdm-2 protein binds to p53 transactivation domain and restrains p53 activity, thus targeting p53 for ubiquitination, by inhibiting its acetylation and by shuttling p53 to cytoplasm. Importantly, Mdm-2 inhibition, mainly during stress conditions, allows p53 activation and stabilization in the active conformation (Balint and Vousden, 2001). The engagement of p53 signaling pathway occurs in response to cellular stress, which stabilizes and alters p53 by several post-translational modifications. Phosphorylation is a classical and crucial first step in p53 activation, whereas acetylation has recently been shown to prevent interaction with Mdm-2, leading to increased p53 stability. Finally methylation, sumoylation and neddylation have also been associated with regulation of p53 stability and transcriptional activation (Amaral et al., 2010).

Induction of apoptosis driven by p53 is an important event in the maintenance of homeostasis in response to multiple toxic stimuli, and occurs mainly through the mitochondrial pathway. Nevertheless, p53 can also modulate this process via the death receptor pathway (Fig.6). The permeability of the mitochondrial outer membrane is a tightly regulated process effected by proteins of the Bcl-2 family, with some of its members being regulated by p53, via transcription-dependent and independent events (Elmore, 2007).

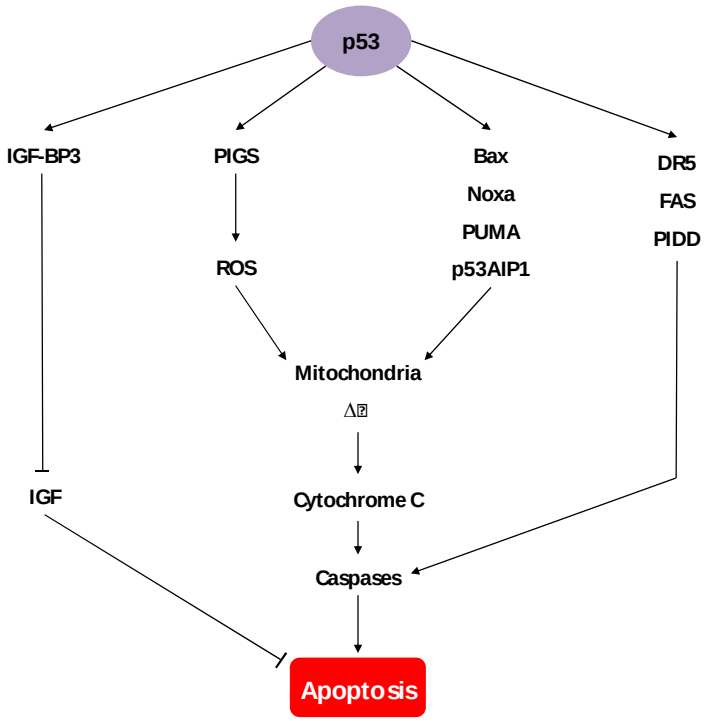

Fig. 6.Main routes of p53-induced apoptosis.p53 induces apoptosis by activating target genes such as p53-induced genes (PIGs) that modulate reactive oxygen species (ROS) production, Bax, Noxa, p53 upregulated modulator of apoptosis (PUMA), p53-regulated apoptosis-inducing protein 1 (p53AIP1), death receptor 5 (DR5), FAS, p53-induced protein with death domain (PIDD) and insulin-like growth factor-binding protein 3 (IGF-BP3). p53-induced apoptosis involves changes in mitochondrial membrane potential (ΔΨ), cytochrome C release, caspase activation and prevention of apoptosis inhibition.Adapted from (Balint and Vousden 2001).

p53 has the ability to activate the transcription of several pro-apoptotic genes, such as those coding for theBcl-2 homology domain 3 (BH-3) only proteins Bax, Noxa and PUMA. Additionally, p53 can promote apoptosis by repression of anti-apoptotic genes, thus promoting caspase activation. p53 is also able to increase cell surface levels of FAS, further promoting caspase activation and apoptosis (Amaral et al., 2010). The TP53 gene is frequently mutated in sporadic cancers. Germlinemutations in TP53 are associated with the Li-Fraumeni syndrome, an hereditary cancer susceptibility syndrome predisposing individuals to sarcomas, lymphomas, breast, brain and other tumors. Curiously, malignancies that retain wild-type p53 gene have often acquired other mechanisms to compromise p53 function. Importantly, inactivation of p53 function seems to be a general mechanism in tumor development and might be a common feature of all cancers, including colon cancer (Balint and Vousden, 2001).

In addition to apoptosis inhibition, aberrant activation of survival pathways, by overexpression, overactivation or mutation, is also a common feature observed in cancer. Importantly, the abnormal activation of epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), NF-κB and AKT signaling pathways, can promoteuncontrolled cell growth, survival and chemotherapy resistance(Altomare and Testa, 2005; Grandis and Sok, 2004; Yu et al., 2005).

1.4.1. EGFR pathway

The EGFR/ErbB1 is one of four ErbB family members. It is a transmembrane protein activated by EGF and transforming growth factor-α. Importantly, EGFR is overexpressed or constitutively activated in many cancer types, including in up to 80% of colorectal tumors,and is associated with a poorer prognosis (Grandis and Sok, 2004; Wolpin and Mayer, 2008).EGFR activation triggers several downstream signaling pathways, including MAPK, phosphoinositide phospholipase C γ(PLCγ), phosphatidylinositol 3-kinase/AKT (PI3K/AKT),and signal transducers and activators of transcription(STATs) (Fig.7).

In cancer cells, overactivation of EGFR signaling pathways leads to increased proliferation, survival, invasion and angiogenesis (Grandis and Sok, 2004). Importantly, one of the major EGFR signaling pathways proceeds through Ras-Raf-MEK-ERK-MAPK (ERK MAPK pathway). Adding to the deregulation of EGFR observed in many types of cancer, several members of this pathway are also commonly deregulated in cancer, leading to a greater potentiation of EGFR aberrant signaling. Further, one of the members most frequently activated by mutation is Ras, occurring in 30% of all cancers and, specifically, in 50% of colon tumors (Roberts and Der, 2007). Ras is a small GTPase protein, whose function is regulated by a GDP/GTP switch. Typically, mutated and activated Ras is unresponsive to this regulation, becoming constitutively GTP-bound, and thereby, constitutively activated. Another downstream effector also mutated in cancer is Raf, including B-Raf, which occurs in ~10% of colorectal tumors (Roberts and Der, 2007).

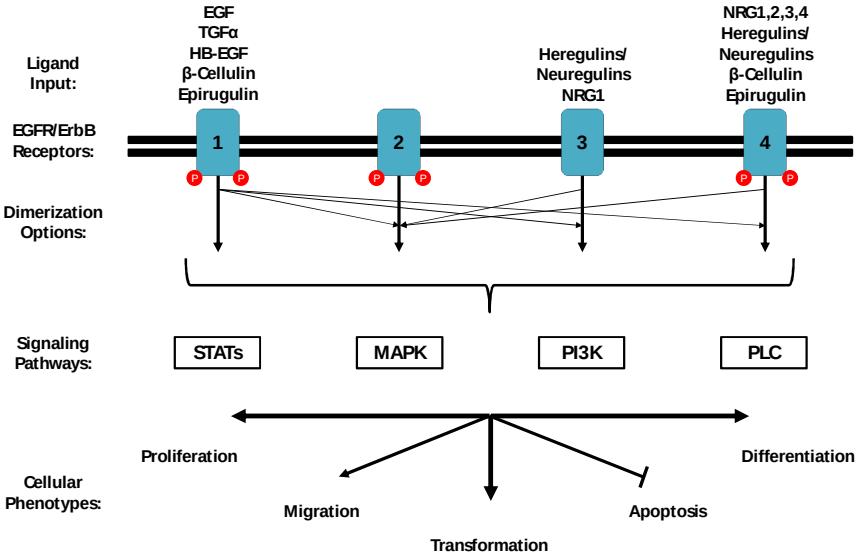

Fig.7.ErbB family members and major signaling routes. The EGFR transduction cascade is a highly complex network. Ligand input and receptor engagement occur in the extracellular domain. No direct ligand has been isolated for ErbB2 to date. Receptor engagement leads to tyrosine phosphorylation and several dimerization options.ErbB3 lacks intrinsic tyrosine kinase activity. Finally, the output signal is involved in a variety of cell responses.Adapted from (GrandisandSok 2004).

1.4.2. PI3K/AKT pathway

The PI3K/AKT pathway stimulates cell proliferation and promotes cell survival(Altomare and Testa, 2005).Ras is an upstream regulator of thissignaling pathway(Tokunaga et al., 2008). Abnormal activation of AKT and further nuclear translocationis an important step toward cell transformation, contributing to both metabolic changes in cell energetics and metabolic requirements for human tumor maintenance(Kim et al., 2005; Mosca et al., 2011;Tokunaga et al., 2008). In addition, the PI3K/AKT pathway regulates multiple cellular functions, including proliferation, growth, survival and mobility.AKTis a serine-threonine kinase, ubiquitously expressed and located downstream of the phosphatase and tensin homologue (PTEN)/PI3K. There are three family members (AKT1, AKT2 and AKT3) encoded by three different genes.Activation of AKTresults in the suppression of apoptosis induced by stress conditions, such as growth factor withdrawal, detachment of extracellular matrix, UV irradiation and FASsignaling(Carnero et al., 2008).In addition, PTEN is one of the major regulators of AKT, discovered as a tumor suppressor mutated in several tumors. Further, the loss of PTEN activity was shown to induceAKT activation (Tokunaga et al., 2008).

AKTleads to apoptosis inhibition and cell cycle progression by direct phosphorylation of transcription factors that regulate the expression of pro- and anti-apoptotic genes, or by direct phosphorylation of pro-apoptotic proteins.Furthermore, AKTnegatively regulates the expression of cell death genes, such as Forkhead transcription factors, and activates transcription factors that upregulate anti-apoptotic genes such IκB kinase that phosphorylates IκB and leads to the activation of NF-κB signaling pathway, and cyclic-AMP response element protein.Also, AKT promotes cell survival due to the direct phosphorylation and inactivation of

Bad and procaspase-9, and p53 degradation by phosphorylation and activationof Mdm-2(Tokunaga et al., 2008) (Fig.8).

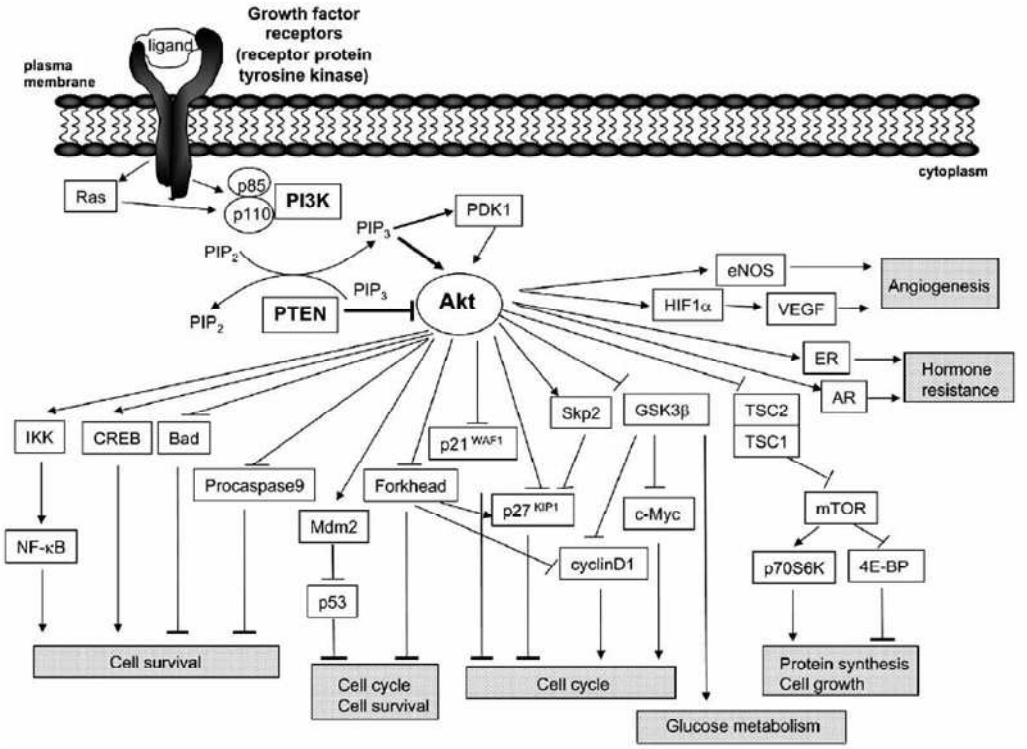

Fig.8.PI3K/AKT signaling pathways. In human cancer, activation of the AKTsignaling pathway results from various mechanisms, including PI3K activation and loss of PTEN function. Once activated, AKT regulates a wide range of target proteins and has multiple cellular functions including: cell survival, cell cycle progression and cell growth.Adapted from (Tokunaga *et al*2008).

The PI3K/AKT signaling pathway has been frequently reported overactivated in cancer resulting in uncontrolled cell growth, survival, proliferation, angiogenesis and migration (Holand et al., 2011; Tokunaga et al., 2008), including in CRC (Johnson et al., 2010). Importantly, overactivation usually results from overexpression or activating mutations of receptor tyrosine kinases, mutations or deletions of PTEN tumor suppressor gene, and/or due to alterations in Ras signaling(Holand et al., 2011).The induction of tumor metastasis by the PI3K/AKT signaling pathway was already reported in several cancers, and mainly results from the loss and downregulation of E-cadherin and β-catenin, which leads to the disassembly of the cell adhesion complex(Sheng et al., 2009).In turn, the loss of the cell adhesion complex and the upregulation of angiogenesis, mainly throughvascular endothelial growth factor(VEGF), critically facilitate the migration oftumor cells(Kim et al., 2005).Importantly, AKT overexpression and activation was implicated as an early event during sporadic colon carcinogenesis, but it seems to beless common in colon cancers with MSI(Roy et al., 2002; Wang and Basson, 2011).Finally,AKT was also demonstrated as a good target to prevent cancer cell adhesion and metastasis in colon cancer(Wang and Basson, 2011).

1.4.3. NF-κB pathway

The NF-κB signaling pathway is involved in the regulation of immune response and inflammation.Further, there is growing evidence supporting a major role for this pathway in carcinogenesis.

NF-κB comprises a family of transcription factors involved in several biological responses. NF-κB dimers are predominantly present in the cytoplasm due to their interaction with IκB.The phosphorylation of IκB by IκB kinases (IKKs) targetsIκB for degradation by the proteasome, leading tothe release and nuclear translocation of NF-κB (Fig.9).NF-κB activation is a very tightly regulated event, inducible and transient(Dolcet et al., 2005). Further NF-κB promotes its own inactivation by promoting IκB expression(Quivy and Van Lint, 2004).

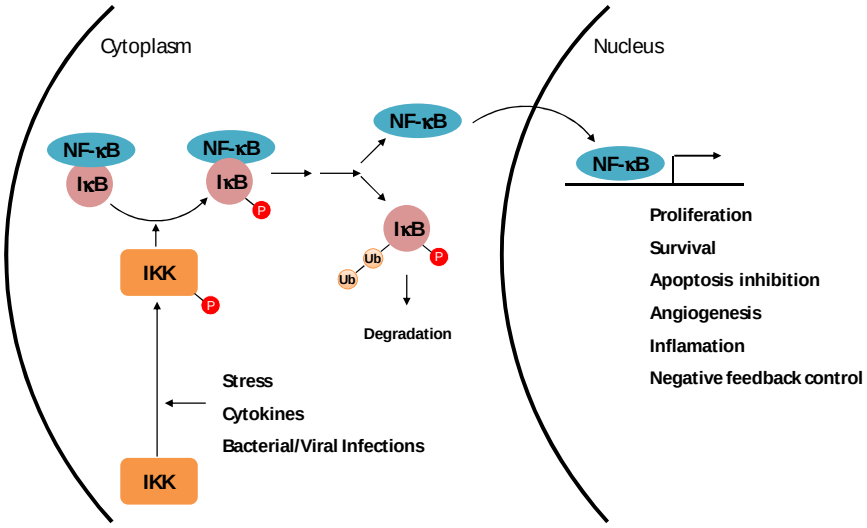

Fig. 9.Canonical pathway of NF-κB activation through IκB inactivation. Phosphorylation of IκB by IKK leads to IκB degradation and consequent NF-κB release and nuclear translocation. Once in the nucleus, NF-κB promotes expression of target genes involved in proliferation, survival, apoptosis inhibition, angiogenesis, and inflammation, as well as negative feedback control by increasing IκB expression. Adapted from (Marcu et al., 2010).

Under certain pathological conditions, such as cancer, NF-κB is often constitutively activated, leading to deregulated expression of genes involved in apoptosis and cell cycle control, adhesion and migration (Aranha et al., 2007; Karin and Lin, 2002). It is generally accepted that NF-κB activation isresponsible for apoptosis resistance and cell survival; however, the clear correlation between NF-κB and apoptosis is yet to be firmly established (Aranha et al., 2007; Dolcet et al., 2005). Nevertheless, the involvement of NF-κB in tumor initiation and progression is well established.

NF-κB is constitutively active in several malignant cells, including colon cancer,lymphoma, leukemia, breast cancer,pancreatic adenocarcinoma and gastric carcinoma (Sakamoto et al., 2009).In addition, tumors with increased NF-κB activity usually present increased resistance to chemotherapy.Importantly, it has already been reported that inhibition of NF-κB activity sensitizes colon cancer cells to daunomycin(Yu et al., 2005) and to 5-

fluorouracil (5-FU)(Voboril et al., 2004). Importantly, the overexpression and overactivation of NF-κB have been reported in colon cancer, and have been associated with colon tumor progression. The regulation of this transcription factor is also considered to be useful for therapeutic managementof colon cancer (Kojima et al., 2004; Sakamoto et al., 2009).

1.4.4. MAPK pathways

MAPK signaling pathways are alsocommonly deregulated in cancer.In fact, MAPK/ERK pathwaysare keysignaling routes in cell proliferation,with several key growth factors and proto-oncogenes transducing signalsthatact through these cascades. Therefore, all kinases involved are potential targets in the treatment of CRC.So far, four major MAPK subfamilies have been identified; the extracellular-regulatedprotein kinases 1/2 (ERK1/2), the c-Jun N-terminal kinase (JNK), p38 MAPKs, and more recently, ERK5(Fang and Richardson, 2005; Nishimoto and Nishida, 2006).ERK1/2 and ERK5 pathways are activated by growth factors and mitogens, including EGF, and the activation of their downstream effectors leads to activation of several transcription factors that regulate cell survival, differentiation and proliferation.The ERK1/2 pathway is stimulated by sequential activation of Ras, Raf-1 and MEK1/2, finally leading to ERK1/2 activation. In turn, activated ERK1/2 translocates to the nucleus activating several transcription factors, such as NF-κB and Myc.Activation of ERK5 proceeds through sequential activation of upstream kinases MEKK2/3 and MEK5. Importantly, ERK5 transcriptionally activates cyclin-D1(Nishimoto and Nishida, 2006), and regulates G2-M progression and timely mitotic entry by activating NF-κB(Cude et al., 2007).In addition to this pro-survival and pro-proliferative role, ERK5 prevents apoptosis by phosphorylating pro-apoptotic proteinsBim and Bad(Girio et al., 2007), and downregulatingFAS ligand (Fig. 10)(Wang et al., 2006). ERK5 also plays an important role in angiogenesis (Wang and Tournier, 2006).

ERK1/2 overactivation has already been reported in several different primary tumors and cell lines, including in CRC (Hoshino et al., 1999). In addition, ERK5 aberrant expression has been reported in many cancer types, including breast (Montero et al., 2009), prostate (McCracken et al., 2008), oral squamous cell carcinoma (Sticht et al., 2008), and hepatocellular carcinoma (Zen et al., 2009). Nevertheless, the precise molecular mechanisms regulated by ERK5 in cancer are not entirely clear. Importantly, ERK5 cascade has been implicated in proliferation and chemoresistance of breast cancer cells (Montero et al., 2009), and in cell survival of human lung cancer cells (Linnerth et al., 2005) and leukemic T cells (Garaude et al., 2006). ERK5 overexpression is also associated with bone metastasis, and poor prognosis in human prostate cancer (McCracken et al., 2008).

The reported overexpression and/or overactivation in many types of human cancer, the involvement in cell survival and the association to a poorer disease survival, makes ERK5 a desirable target for the development of additional novel cancer therapies. To date, there is no available information on the expression of ERK5, or its direct activator MEK5, in CRC. However, ERK5 expression is directly regulated by microRNA-143 (miR-143), and forced expression of miR-143 is associated with decreased ERK5 expression (Akao et al., 2010; Borralho et al., 2009). Further, the expression of miR-143 has been reported downregulated in CRC patients (Akao et al., 2006), and persistent downregulation of miR-143 may directly

accelerate tumorigenesis(Akao et al., 2009; Akao et al., 2007; Akao et al., 2006). Finally, miR-143 overexpression decreases colon cancer xenograft growth (Borralho et al., 2011).

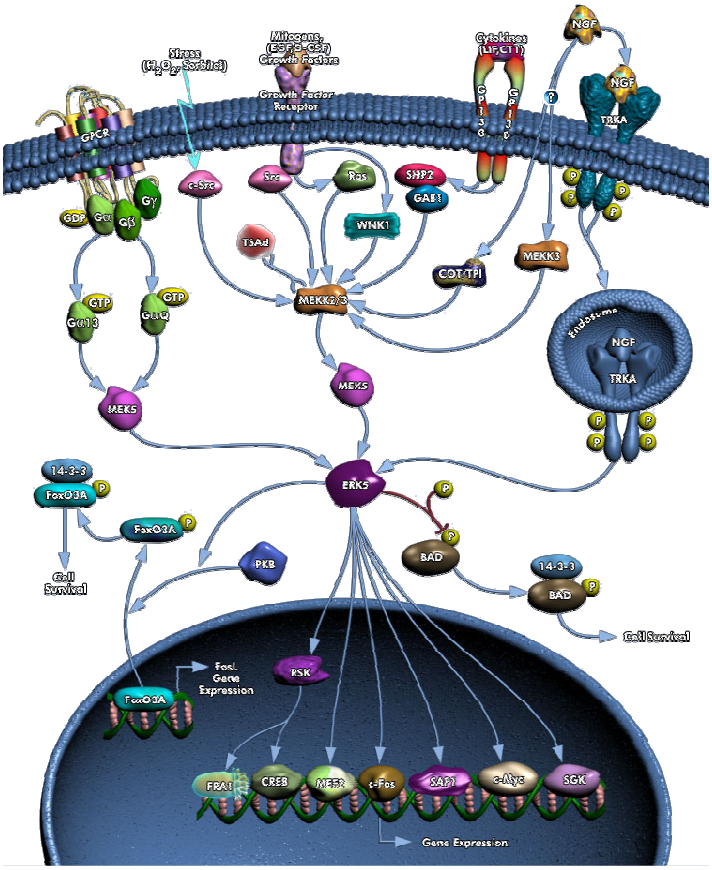

Fig. 10.ERK5 signaling pathway. Activation of ERK5 through sequential activation of MEKK2/3 and MEK5, leads to the activation of several transcription factors and other protein effectors that promote cell survival and proliferation.Adapted from (GeneGlobe Pathways, QIAGEN).

The elucidation of ERK5 expression in CRC may be useful as an additional prognostic marker, similarly to ERK5 expression in prostate and breast cancers. It may also prove to be an interesting target for therapeutic approachesto treat CRC, namely by miR-143 direct targeting of ERK5 mRNA *in vivo*, In fact, we have already demonstrated that miR-143 overexpression increases CRC cell line sensitivity to 5-FU, through modulation of ERK5 and NF-κB expression (Borralho et al., 2009).

1.5. Colorectal Cancer Treatment

Despite all the improvements already accomplished in CRC treatment, the metastatic form of the disease remains incurable. Treatments currently applied depend on tumor stage, which supports the importance of determining the correct tumor stage. Surgical intervention is considered sufficient to treat stage I and II colorectal tumors, despite the controversial use of adjuvant therapy for the treatment of stage II tumors. The use of adjuvant therapy might

help stage II CRC patients at high risk of recurrence, but the real benefit has not been prospectively examined. In contrast, for the treatment of stage III tumors, the use of adjuvant therapies is well defined, with clear benefits in improved overall survival of patients (Wilkes and Hartshorn, 2009; Wolpin and Mayer, 2008). Finally, for the treatment of stage IV patients, systemic chemotherapy is used, in hopes of prolonging life and palliating symptoms (Wolpin and Mayer, 2008).

The improvements achieved in the treatment of colon cancer are associated with the development and introduction of new therapeutic agents for systemic treatment. These agents include cytotoxic agents, like capecitabine, irinotecan and oxaliplatin, and targeted agents, like cetuximab, bevacizumab and panitumumab. Cytotoxic agents mainly act through the induction of DNA damage and apoptosis, while targeted agents are usually monoclonal antibodies targeting proteins aberrantly expressed in colon cancer. The actions developed by these agents contribute to the inhibition of tumor growth and to tumor cell apoptosis.

Despite the progress already achieved with new therapeutic agents, 5-FU has been the drug of choice for the treatment of CRC for several decades. Due to its rapid and efficient absorption into the cells, and due the action of its active metabolites, disrupting RNA synthesis, leading to DNA damage and interfering in the assembly and function of small nuclear RNAs, 5-FU ultimately leads to apoptosis (Borralho et al., 2007; Longley et al., 2003). Despite the large use of 5-FU, its efficacy is limited and has benefitted from the combination with newer chemotherapeutic agents (Longley et al., 2003). Thus, there is still a high demand for novel, efficacious therapies for the management of this disease.

Cetuximab is a targeted agent used in the adjuvant therapy for patients with stage III colon cancer (Wilkes and Hartshorn, 2009).It is a recombinant murine immunoglobulin G1 antibody, which binds to the extra-cellular domain of EGFR that is overexpressed in several tumor types. Importantly, cetuximab treatment has been associated with decreased cell proliferation and angiogenesis, and increased apoptosis (Bouali et al., 2009).Cetuximab binding to EGFR prevents receptor activation, and leads to its internalization and degradation, thus abrogating several EGFR-regulated pathways leading toapoptosis inhibition, cell cycle progression, angiogenesis, cell motility and metastasis (Vincenzi et al., 2008). However, the sensitivity of colon cancer cells to cetuximab differs between tumors, according to the presence of mutations in KRAS gene, and the expression of PTEN (Bouali et al., 2009). Activating KRAS mutations and decreased expression of PTEN are associated with resistance to cetuximab. Since KRAS is a downstream effector of one of the EGFR signaling pathways, the Ras-Raf-MEK-ERK pathway, which is one of the most important signaling pathways for proliferation and survival, activating KRAS mutations prevents the upstream repression effect of cetuximab, and are associated with a worse prognosis (Lievre et al., 2006). Additionally, PTEN is a tumor suppressor protein that regulates de PI3/AKT signaling pathway, another downstream signaling pathway activated by EGFR. The loss or reduction of expression of PTEN leads to the overactivation of the AKT pathway, and ultimately to resistance to the upstream effect of cetuximab. Thus, the KRAS mutation status and the PTEN expression evaluation might be valuable predictive markers for cetuximab sensitivity (Frattini et al., 2007; Karapetis et al., 2008).

2. MicroRNAs

2.1. MicroRNAs in Cancer

MicroRNAs (miRNAs) comprise an abundant class of endogenous, small (19-25 nucleotides), single-stranded, non-coding RNAs that repress protein translation through direct binding to target mRNAs. Further, miRNAs are highly conserved, suggesting their involvement in many essential biological processes, including development, differentiation, proliferation and apoptosis (Kim et al., 2009; Slaby et al., 2009).Nevertheless, the biological function of the majority of miRNAs remains unknown.

Due to the multiple regulatory functions of miRNAs, their contribution for cancer onset/progression is well established and thus far,every tumoranalyzed for miRNA profiles is significantly different when compared to normal cells from the same tissue. In addition, analysis ofmiRNA expression profiles in human tumorshas identified several signatures associated with diagnosis, staging, prognosis and response to treatment (Calin and Croce, 2006).Downregulated miRNAs are believed to function as tumor suppressors and prevent tumor development, by negatively regulating tumor oncogenes and/or genes that control cell differentiation or apoptosis.In contrast, miRNAs that are usually overexpressed in tumor samples are considered to be oncogenes, by promoting tumor growth via negative regulation of tumor suppressor genes and/or genes that regulate differentiation or apoptosis (Manikandan et al., 2008).Curiously, one particular miRNA may act on different mRNA targets, while one particular mRNA may be targeted by several different miRNAs, in different cellular contexts, which increases the complexity of miRNA-gene regulatory networks (Calin and Croce, 2006).

2.1.1. miRNA biogenesis

miRNAs undergo several processing steps until the mature and functional form of the miRNA is produced. Biogenesis of miRNAs starts with the synthesis of a long precursor transcript known as primary miRNA (pri-miRNA).Generally, the pri-miRNA transcriptsare generated by RNA polymerase II and retain mRNA features, such as 5'-cap structure and 3'-poly(A) tail. Nevertheless, some miRNAs found interspersed among repetitive elements are transcribed by RNA polymerase III(Borchert et al., 2006; Lee and Dutta, 2009).The primary transcripts generated areprocessed in the nucleus by the RNase III enzyme Drosha and its cofactor, the double-stranded-RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8).Drosha performs the enzymatic cleavage reaction of pri-miRNAs by recognition and cut of the stem hairpin structure, thus releasing a small RNA hairpin of 60-70 bp, called precursor miRNA (pre-miRNA). After this first cleavage, the pre-miRNA is exported to the cytoplasm by exportin-5, where it will be ultimately converted to a mature duplex miRNA by RNase III enzyme Dicer.Once in the cytoplasm, Dicer cleaves near the terminal loop, at the opposing end from Drosha, releasing approximately 22-nucleotide mature miRNA duplexes.After Dicer cleavage, the miRNA duplexes are transferred to Argonaute (AGO) proteins, which in turn interact with other proteins, to form the effector complex, the RNA-induced silencing complex (RISC). Importantly, only one of the strands of the RNA duplex (guide strand) remains in the complex, while the other (passenger strand) is released and usually rapidly degraded.After RISC complex assembly, the miRNA purpose is to direct the RISC

complex to target mRNAs that are subsequently cleaved or translationally repressed (Fig. 11)(Bartel, 2004; Kim et al., 2009; Lee and Dutta, 2009).

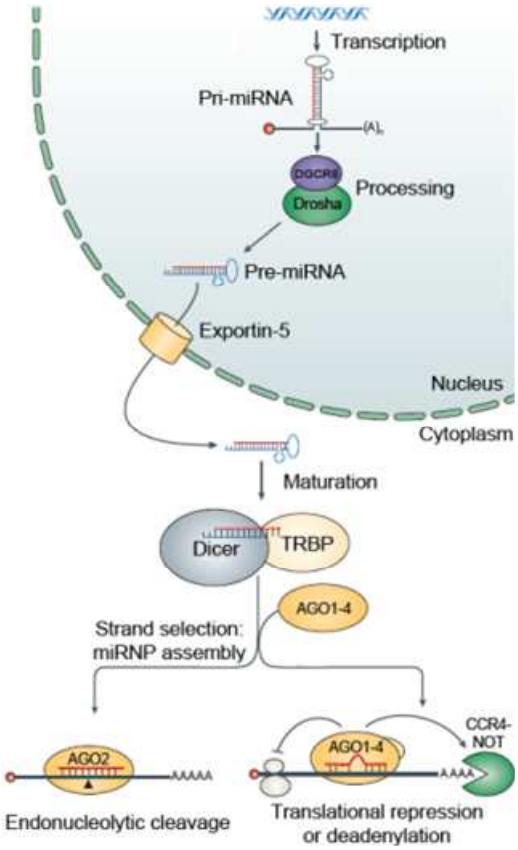

Fig. 11.Schematic overview of miRNA biogenesis and general mechanism of action.miRNAs are transcribed into long precursor pri-miRNAs molecules. Pri-miRNAs are cleaved by the RNAse III endonuclease Drosha and its cofactor DGCR8, originating pre-miRNAs. Pre-miRNAs are transported to the cytoplasm by binding to Exportin-5. In the cytoplasm, pre-miRNAs are processed by the RNAse III endonuclease Dicer. miRNA duplexes are then transferred to AGO, and one strand is then selected as the mature miRNA, while the other is degraded. miRNAs assemble in effector ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complex (miRISC). miRNA RISC loading is a dynamic process, usually coupled with pre-miRNA processing by Dicer, but its details are unknown. Target mRNAs may also be deadenylated, decapped and exonucleolyticalydegradaded. CCR4-NOT, deadenylation complex.Adapted from(Filipowicz et al., 2008).

2.1.2. miRNA function

The fate of the target mRNA, whether it is cleaved and degraded, or translationaly repressed, is determined by the degree of base pair complementaritybetween the mature miRNA and the target mRNA.Full complementarity leads to the cleavage of the target mRNA by RISC, while on the other hand, an incomplete complementarity leads to translational repression.However,miRNAs may also interfere with mRNA stability, without cleavage by AGO proteins, by leading to deadenylation, decapping and exonucleolytic degradation of the mRNA.This process involves the recruitment of cellular decapping and deadenylation machinery, the CCR4:NOTdeadenylase and the DCP1:DCP2 decapping complexes, and occurs

on processing bodies (P-bodies). P-bodies are intracellular sites, where translationally inactive mRNAs are often stored and may undergo decay. Further, the presence of miRNAs, targeted mRNAs and AGO proteins in P-bodies has already been demonstrated (Carthew and Sontheimer, 2009; Liu et al., 2008).

2.2. Role of miRNA-143

It has been shown that many miRNAs are located at genomic sites, which in cancer are frequently deleted, amplified or rearranged, suggesting the critical role of miRNA expression forcancer onset (Calin et al., 2004). Interestingly,miR-143 is increasingly viewed as a tumor suppressor, since it isdownregulated in several types of cancer, including colon cancer (Michael et al., 2003), prostate cancer (Clape et al., 2009), gastric cancers (Takagi et al., 2009), B-cell malignancies (Akao et al., 2007) and esophageal squamous cell carcinoma (Wu et al., 2011). In addition, downregulation of miR-143 was reported in an early phase of adenoma development, and therefore, decreased levels of miR-143 appear to be closely related with the initiation of tumorigenesis. This led to the suggestionof miR-143 as an overall marker for colon tumors(Akao et al., 2010).

The transfection of mature miR-143 in human colon cancer DLD-1 and SW480 cells induced a significant growth inhibition (Akao et al., 2006).Importantly, our group has already shown the anti-proliferative, pro-apoptotic and chemosensitizer effect of miR-143 *in vitro* (Borralho et al., 2009), as well as a marked decrease in tumor growth *in vivo* (Borralho et al., 2011).It has also been demonstrated that miR-143 directly targets the mRNA of ERK5 (Akao et al., 2009), KRAS (Chen et al., 2009), DNMT3A (Ng et al., 2009) and Bcl-2 (Zhang et al., 2010) in CRC. The knowledge that ERK5 plays an important influence in FAS-dependent signaling, reducing FASL expression, suggests that miR-143 promotes FAS-mediated apoptosis, by enabling FASL expression(Akao et al., 2009). Additionally, miR-143 was also shown to interact with KRAS, with their levels showing an inverse correlation in CRC *in vivo*.Thus, miR-143 downregulation might contribute to tumorogenesis also by leading to upregulation ofKRAS expression (Chen et al., 2009).Activating KRAS mutations are a common feature in CRC and they lead to the aberrant activation of the Ras-Raf-MEK/ERKsignaling pathway, involved in enhanced proliferation. KRAS inhibition by miR-143 directly results in decreased phosphorylation of ERK1/2, thus showing the promising therapeutic role for miR-143 in CRC (Chen et al., 2009). Recently, the anti-apoptotic protein Bcl-2 was identified as a new direct target of miR-143 (Zhang et al., 2010), suggesting that the pro-apoptotic effect of miR-143 may also derive from direct targeting Bcl-2 expression. In fact, our laboratoryhas reported that Bcl-2 isdownregulated upon miR-143 overexpression i*n vitro* and*in vivo*(Borralho et al., 2009; Borralho et al., 2011). Therefore, there is growing evidence onthe potential protective role of miR-143 against colorectal carcinogenesis.

3. Aims

In the present study, our aim was to evaluate the expression of ERK5, MEK5, NF-κB, IκB, AKT, p-AKT and p53, in human colon polyps and cancers, compared to normal colon tissue. We established the aberrant expression of ERK5 pathway members in colon cancer, thus highlighting the relevance of this particular pathway for putative therapeutic targeting. ERK5 and MEK5 overexpression has been shown in other cancer types. However, to our knowledge, we are first to evaluate the expression of these proteins in colon cancer, and using well-defined sample sets of colon polyps, carcinomas, and normal colon. Further, our evaluation of NF-κB, IκB, AKT, p-AKT and p53 expression provides solid additional information on the deregulation of these pathways in human colon polyps and cancer, when compared to normal colon tissue.

An increasing body of evidence arising from studies in prostate and colon cancer *in vitro*, and *in vivo* models, indicates that ERK5 knock-down via miR-143 overexpression may play a role in the control of tumor growth, and also in increased tumor cell chemosensitization. In addition, due to the current knowledge on the potential deleterious role of miR-143 downregulation in carcinogenesis and/or progression of CRC, and due to the growing evidence of its anti-proliferative, pro-apoptotic and chemosensitizer role, possibly through ERK5 signaling, our second aim was to develop miR-143 as a putative therapeutic tool. Therapeutic applications of miRNAs will rely on the development of efficient delivery strategies, since unmodified naked miRNAs are often unstable in circulation. The system under development is based on the conjugation of miR-143 to cetuximab, a targeted agent in CRC therapy, and offers the basis for the development of an efficient modular alternative to deliver miRNAs to colon cancer cells.

II. MATERIALS AND METHODS

1. Colon cancer samples

CRC, polyps and normal colonbiospecimens were obtained from Mayo Clinic (Rochester, MN, USA) patients, and all samples were anonymized. Pathologic tumor staging was carried out using the tumor-node-metastasis (TNM) system (Kehoe and Khatri, 2006). Overall, the complete set of samples used in this study was composed by 197 samples, which included 28 normal colonic mucosa, 40 adenomas, 94 adenocarcinomas with pMMR selected for stage I-IV, and 35 sporadic adenocarcinomas with dMMR.

2. DNA mismatch repair status

The mismatch repair status of the samples was evaluated by the presence or absence of microsatellite instability (MSI-H) and/or the presence or absence of *hMLH1* expression. dMMR samples were defined by the presence of microsatellite instability and/or the absence of MLH1 expression. pMMR samples were defined by the absence of microsatellite instability (MSS/MSI-L) and the presence of normal MLH1 protein expression. MSI in CRC cases was compared with paired normal and tumor DNA isolated from formalin fixed, paraffin-embedded (FFPE) material, using the Qiagen DNA extraction kit (Chatsworth, CA). Tumors were classified as MSI-H if > 30% of markers demonstrated instability and as MSS/MSI-L if < 30% demonstrated MSI (Boland et al., 1998; Thibodeau et al., 1998). Immunohistochemical analysis of hMLH1 expression was performed on FFPE samples, as previously described (Lindor et al., 2002).

3. Total protein isolation

Following harvest, tissue samples were immediately snap frozen and stored at -80^{o}C. For each case, frozen tumor tissue was cut on a cryostat to generate hematoxylin and eosin (H&E) stained slides. Tumors were then evaluated for content, and areas containing at least 70% tumor were macro-dissected. Tissue equivalent to 7 mm^2 and 10-µm thick was then sectioned and placed in a vial containing 400 µL of RTL buffer (Qiagen) including 4 µL β-mercaptoethanol. The vial was then stored at -80ºC until utilized for RNA extraction using TRIzol® LSTrizol© (Invitrogen, Corp., Carlsbad, CA), according to the manufacturer's instructions. RNA extractions took place in September and October 2008. At this time, following removal of the aqueous phase for RNA extraction, the trizol-chloroform phases were snap frozen and stored at -80^{o}C for subsequent protein extraction. Total protein isolation from these samples was performed 2 years later, starting in September 2010, following TRIzol® LSTrizol© manufacturer's instructions. Briefly, the tube was centrifuged at 12,000 x g for 15 min at 4^{o}C; then, the remaining aqueous phase supernatant was removed and discarded. Subsequently, 100% ethanol was added to precipitate DNA. Tubes were vigorously shaken and centrifuged at 2,000 x g for 5min, at 4^{o}C. The phenol-ethanol supernatant was removed to 2 mL tubes, for protein extraction. The DNA pellet obtained was washed twice with 0.1 M sodium citrate in 10% ethanol, for 30 min at room temperature, followed by centrifugation of 2,000 x g for 5 min at 4^{o}C. After the second wash, 75% ethanol was added to the pellets, and samples were stored at -20^{o}C for later DNA extraction. Subsequently, the 2 mL tubes

containing the phenol-ethanol supernatant were processed for protein extraction. Protein was precipitated with isopropanol, followed by mixing, and 10 min incubation at room temperature. Subsequently, samples were centrifuged at 12,000 x g for 10 min and the supernatant discarded. Protein pellets were next washed three times with 0.3 M guanidine-HCl in 95 % ethanol. In each wash, tubes were vigorously shaken, incubated at room temperature for 20 min followed by centrifugation of 7,500 x g for 5 min at 4°C. After the final wash and spin, 100% ethanol was added, and samples incubated at room temperature for 20 min, followed by a final centrifugation at 7,500 x g for 5 min at 4°C. The supernatant was removed and the protein pellet obtained was ressuspended in an adequate volume of 8 M Ureia (in Tris-HCl 1 M, pH 8.0) and 1% SDS solution (1:1), a modification from the 1% SDS ressuspension solution recommendedin the manufacturer's protocol.

4. Western blot

The steady-state levels of all proteins were determined by immunoblot analysis. Briefly, 40 µg of total protein extracts were separated on 8% SDS-polyacrylamide electrophoresis gels. After electrophoretic transfer onto nitrocellulose membranes, immunoblots were blocked with 5% milk solution for 30 min. Subsequently, blots were incubated overnight at 4°C with primary rabbit anti ERK5 (#3372; Cell Signaling, Beverly, MA), p-AKT, AKT, NF-κB (p65) and IκB-α (#sc-7985-R, #sc-8312, #sc-372 and #sc-371, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and with primary mouse antiMEK5 and -p53 (#sc-135986 and #sc-126, respectively; Santa Cruz Biotechnology). Next, immunoblots were incubated with anti-rabbit or -mouse secondary antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories) for 3 h at room temperature. Finally, blots were processed for protein detection using Super Signal™ substrate (Pierce, Rockford, IL, USA). Ponceau S dye staining of immunoblots was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit, according to the manufacturer's instructions. Bovine albumin was used as standard (New England Biolabs Inc., New England). Absorbance was measured in a UNICAM UV/Vis 2 spectrophotometer (Speck & Burke Analytical, Clackmannanshire, Scotland) at 595 nm. The protein concentrations were determined after a linear regression curve determination and absorbance value interpolation.

5. Cell culture

HCT116 cells were cultured in DMEM supplemented with 10 % fetal bovine serum (Invitrogen, Grand Island, NY) and 1% antibiotic/antimycotic solution (Sigma Chemical Co., St Louis, MO), and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were seeded at 5000 cells/well, in 96-well plates for cell viability assays and at 75000 cells/well in 12-well plates containing sterile glass coverslips, for immunocytochemistry, at 150000 cells/well in 12-well plates for fluorescence activated cell sorting (FACS) assays and at 400000 cells/well in 6-well plates for α-FITC-protamine binding evaluation assays.

6. Evaluation of cell viability

Cetuximab was serially diluted in sterile tissue culture media at 0.5-10 μM, final concentration. Twenty-four hours after plating, media was removed from cells and replaced with fresh media containing either cetuximab, at the above mentioned concentrations, or vehicle PBS (control), for 48 and 72 h.

After 48 and 72 h of cell incubation in the presence of cetuximab or vehicle, cell viability was evaluated using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacturer's instructions. Briefly, the MTS assay is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The CellTiter 96® AQ_{ueous} Assay is composed of solutions of MTS and an electron coupling reagent phenazinemethosulfate (PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product at 490 nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product was measured in a Bio-Rad microplate reader Model 680 (Bio-Rad, Hercules, CA) as the amount of 490 nm absorbance, which is directly proportional to the number of living cells in culture.

7. Cetuximab-FITC labeling

Before labeling, the commercial cetuximab solution (5 mg / ml) was submitted to buffer exchange to PBS 1X, using PD-10 Desalting Columns (#17-0851-01, GE Healthcare) and following the gravity protocol, according to the manufacturer's instructions. PD-10 desalting columns are prepacked for rapid clean-up of proteins and other large biomolecules (MW > 5000 Da). Briefly, the column was opened and the storage solution was allowed to flow by gravity and discarded. Subsequently, the column was filled with PBS 1X pH 7.4 (equilibration buffer) and allowed to enter the packed bed completely. This step was repeated four times, always discarding the flow-through. After equilibration, 2.5 mL of commercial cetuximab was added to the column, and allowed to enter the packed bed completely, discarding the flow-through. Subsequently, cetuximab was eluted by gravity flow-through from the columns by adding 3.5 mL of PBS 1X pH 7.4, and collected for further use.Subsequently, cetuximab labeling with fluorescein 5(6)-isothiocyanate (FITC) was performed with the FluoReporter® FITC protein labeling kit (# F6434; Invitrogen, Inc.), following the manufacturer's instructions. Briefly, two parallel labeling reactions were performed by adding 20 μL of 1 M sodium bicarbonate solution to 200 μL of cetuximab in PBS 1X pH 7.4, per reaction. Subsequently, FITC was dissolved in dimethyl sufoxide (DMSO), immediately before starting the reaction, and added to cetuximab solution, at a molecular ratio of FITC:cetuximab of 100:1. The mixture was incubated at room temperature for approximately 2h, protected from light and with stirring, for covalent conjugation. Unreacted FITC was removed using a spin column. Briefly, the spin columns were prepared by adding purification resin, placing the column in a collection tube, and centrifuging at 1,100 x g for 3min, discarding the flow-through. The labeling reaction was then placed on

the center of the purification resin within the spin column. Finally, the spin column was placed in an empty collection tube and centrifuged at 1,100 x g for 5 min. After this final centrifugation, the flow-through in the collection tube contains the FITC labeled cetuximab (Cetuximab-FITC). Next, the two parallel labeling reactions were pooled and further purified using a new spin column with fresh purification resin, as described above. The flow through in the final collection tube contains the FITC labeled cetuximab (cetuximab-FITC). Cetuximab concentration and cetuximab-FITC labeling ratio in this solution were determined by measuring the absorbance at 280 and 494 nm using a Nano drop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and by using the following equations: Cetuximab concentration (M) = $[A_{280} - (A_{494} \times 0.3)] \times$ dilution factor/ε (where ε is the molar extinction coefficient of the protein at 280 nm, for most IgGs, ε = 203,000 $cm^{-1}M^{-1}$); and FITC:cetuximab labeling ratio (number of dye molecules per antibody molecule) = dilution factorx A_{494} / [68,000 ($cm^{-1}M^{-1}$) xcetuximabconcentration (M)] (where 68,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the dye at pH 8.0 at 494 nm. The FITC:cetuximab ratio obtained in the cetuximab-FITC labeling solution was approximately11(10.74).

8. Immunocytochemistry and fluorescence microscopy

For the evaluation of unlabeled cetuximab binding to EGFR expressed by HCT116 cells, cells were grown on glass coverslips and incubated with 50 nMcetuximab in complete culture media in a humidified incubator at 37°C, for 1 h. Cells were then fixed with paraformaldehyde (4% w/v) in 1X PBS and permeabilized for 20 min at room temperature in PBS containing 0.1 % Triton-X-100. Subsequently, cells were incubated for 30 min at room temperature in blocking solution containing 0.05 % Tween-20 and 5 % normal goat serum in 1X PBS. Cells were next incubated with secondary anti-human FITC-conjugated antibody (#109-095-003, FITC-conjugated affinipure goat anti-human IgG; Jackson Immuno Research Laboratories, Inc.) diluted 1:200 in blocking solution, for 1 h at room temperature, followed by counterstaining with Hoechst at 5 µg/mL, for 10 min, also at room temperature. Finally, glass coverslips were mounted on glass slides using Fluoromount-G™ (Beckman Coulter Inc., Fullerton, CA, USA) for fluorescence microscopy evaluation of cetuximab binding to EGFR in HCT116 cells. For the evaluation of cetuximab-FITC binding to EGFR on HCT116 cells, cells grown on glass coverslips were incubated with 50 nM FITC-cetuximab in complete culture media, and incubated in a humidified incubator at 37°C, for 1 h.Cells were then fixed, permeabilized, counterstained, and mounted as described above for fluorescence microscopy evaluation of cetuximab binding to EGFR in HCT116 cells. Fluorescence microscopy observation of samples was performed by using a Leica DM5000B microscope (Leica Microsystems CMS Gmb H, Germany). Images were acquired, under 400x and 630x magnification, using a Leica DC 350F camera (Leica Microsystems CMS Gmb H, Germany) with the IM50 software for image acquisition (Leica Microsystems, version 1.20, Release 19).

9. Fluorescence activated cell sorting (FACS) analysis

Cetuximab-FITC binding to EGFR on HCT116 cells and dose-response curves were performed by FACS analysis, to determine the percentage of FITC positive cells resulting from exposure to increasing concentrations of cetuximab-FITC. For this purpose, cells were seeded in 12-well plates at 150000 cells/well. Twenty-four hours after plating, media was removed from cells and replaced with fresh media containing cetuximab-FITC, at concentrations ranging 0.01–100 nM, cetuximab unlabeled, cetuximab unlabeled with secondary anti-human FITC-conjugated antibody, secondary anti-human FITC-conjugated antibody, or with vehicle PBS (control), and incubated at 37^{o}C in a humidified incubator, for 1h. Cells were then washed twice with DPBS and detached with StemPro®Accutase® (A11105-01, Invitrogen, Inc.). Subsequently, harvested cells were collected in FACS tubes and centrifuged at 700 x g for 5 min. All following steps were performed on ice. The supernatant was discarded and the cells washed and ressuspended twice in DPBS containing 2% FBS, followed by centrifugation at 700 x g for 5 min. Subsequently, cells were ressuspended and fixed with paraformaldehyde (4% w/v in 1X PBS), for 20 min. After fixation, cells were centrifuged at 700 x g for 5 min and washed again with DPBS containing 2% FBS. For FACS data acquisition, cells were ressuspended in DPBS 2% FBS and data acquired using a BD LSRFortessa Cell Analyser flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software (Tree Star Inc, version 6.4.7).

10. Cloning, expression and purification of α-FITC-protamine scFv

The anti-FITC scFv was obtained from Dr. Carlos Barbas III (Scripps Research Institute). The protamine sequence was derived from (Peer et al., 2007)and cloned by overlay PCR in pComb 3x,provided by Dr. Carlos Barbas III, with anti-FITC scFv. The anti-FITC-protamine single chain variable fragment (scFv) antibody in the original vector was subjected to PCR using the primers: FITC pET21-Nhe-F andProt-Xho-His-R (FITC-pET21-Nhe-F: 5'- CTA GCT AGC TAC CCC TAC GAC GTG CCT GAT TAC GCC GGA GGA TCC GGA GGA-3'; Prot-Xho-His-R: 5'-CCG CTC GAG TCA TTA ATG GTG ATG GTG ATG GTG ATG GTG GCT ACC TCC ACC TCC AGA ACG ACG GCG ACG GCG-3').The resulting DNA fragment was cloned into the expression vector pET21a (Novagen) using the restriction enzymes NheI/XhoI (Nzytech) and then expressed in *E. coli* Tuner (Tuner™(DE3)pLacI Competent Cells, Novagen). Bacterial cells were grown at 37ºC in LB medium until mid-exponential phase (Abs_{600nm}=0.4). Cells were then induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), and further incubated at 16ºC for 16h. Subsequently, cells were collected by centrifugation (2636 x g for 15 min) and ressuspended in 10 mL of 50 mM HEPES pH 7.5, 1 M NaCl, 10 mM imidazole, 5 mM $CaCl_2$ and protease inhibitior cocktail (Roche). Ressuspended cells were next disrupted by ultra-sonication during 20 min. The soluble fraction was collected by centrifugation at 10000 x g for 30 min, and purified by nickel affinity chromatography using a His GraviTrap column (#11-0033-99, GE Healthcare). Following purification, the purified scFv α-FITC-protamine was submitted to buffer exchange to 20 mM of HEPES pH 7.4, 100 mM of NaCl, 0.1 mM EDTA and 5% (v/v) glycerol, using disposable P-10 Desalting Columns (#17-0851-01, GE Healthcare). scFv α-FITC-

protamine was next quantitated with NanoDropND-1000 spectrophotometer (Thermo Scientific) and stored at 4ºC.

11. ELISA assay for the evaluation of α-FITC-protamine scFv binding to cetuximab-FITC

Antigens (cetuximab-FITC, anti-human FITC-conjugated antibody, and unlabeled cetuximab as a negative control) were diluted in PBS 1X and incubated in 96-well plates for 1h at 37^{o}C, followed by a blocking step with bovine serum albumin(BSA) 3% for 1h at 37^{o}C. After incubation and blocking, the wells are washed once with ddH_2O, and incubated with α-FITC-protamine in BSA 1% for 1 h at 37^{o}C. Next, each well was washed 5 times with PBS 1X containing 0.05% Tween-20, followed by incubation with α-HA-HRP (1:1000) in BSA 1%, for 1 h at 37^{o}C. Finally, each well was washed 5 times with ddH_20 ant incubated with 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (#194430, Calbiochem) containing 0.2% H_2O_2, for 1h, at 37^{o}C. The relative intensities at 405 with reference at 492 nm were then measured in a Tecan Infinite® M200 multimode microplate reader (Tecan, Grodig, Austria).

12. Delivery of α-FITC-protamine scFv conjugated with cetuximab-FITC to HCT116 cells

The levels of α-FITC-protamine scFv in HCT116 cells were determined by Western blot using total protein extracts from cells exposed to the delivery complexes, and controls. Firstly, the delivery complex (cetuximab-FITC plus α-FITC-protamine scFv) and the controls (no addition, cetuximab, cetuximab-FITC, α-FITC-protamine scFv and cetuximab plus α-FITC-protaminescFv) were incubated *in vitro*, in DMEM, for 2h at room temperature, in a rocking platform. The ratio ofα-FITC-protamine scFv to cetuximab-FITC used was 15:1. Subsequently, the delivery complexes were concentrated using Vivaspin 500 centrifugal concentrators (Vivascience AG, Hannover, Germany), with a cut-off of 100 KDa, to remove free uncomplexedα-FITC-protamine scFv. Final retentate volume containing the delivery complexes was adjusted with DMEM to 1 ml, and added to cells for 4h. Cells were then collected and processed for total proteins extracts, and subsequently used for Western blot analysis. Detection of α-FITC-protamine scFv in immunoblots was performed with anti-HA-Peroxidase (3F10) (#12013819001,Roche Applied Science) and anti-His-Peroxidase (#11965085001, Roche Applied Science) antibodies, to detect α-FITC-protamine presents an N-terminal HA tag and C-terminal His tag, respectively.

13. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the densitometric analysis program Quantity One Version 4.6 (Bio-Rad Laboratories). All data are expressed as the mean ± SEM for all CRC samples within the sample sets defined, or from at least three independent experiments. Protein band intensities for each sample, in all blots, were normalized to the mean protein band intensities from duplicate HCT116 total protein sample

present in the same blot, and similarly included in all blots. HCT116 protein extracts used as internal normalization control in each blot were all from the same batch. Statistical significance was evaluated using Student's t-test. Values of $p < 0.05$ were considered statistically significant.

III. RESULTS AND DISCUSSION

1. Selected Protein Expression Profiles in Human Colon Cancer

1.1. ERK5 and MEK5

ERK5 is one of the four major MAPK subfamilies, and is involved in the regulation of several key cellular processes, including cell survival, proliferation, differentiation and motility, by relaying extracellular signals to intracellular responses (Cargnello and Roux, 2011). The crucial involvement of ERK5 in early embryonic development and in normal development of the vascular system have already been reported (Roberts et al., 2009). Further, ERK5 activity is increased in response to multiple stimuli, such as growth factors or oxidative stress, via dual phosphorylation by its direct activator MEK5. MEK5 physiological significance has been demonstrated by gene ablation studies in mice, and the resulting phenotype was similar to that of ERK5 silencing (Turjanski et al., 2007; Wang and Tournier, 2006).

ERK5 and MEK5 overexpression has been reported in several cancer types and associated with enhanced proliferation, chemoresistance, metastasis and angiogenesis, correlating with poorer disease prognosis. In CRC, no information is available on ERK5 and MEK5 expression profiles. To clarify the significance of these two kinases in CRC development, we examined steady-state levels of ERK5 and MEK5 by Western blot. For this purpose, total protein extracts were collected from a large set of 197 well-defined human colon samples, including normal colonic mucosa, adenomas (polyps), stage-divided pMMR adenocarcinomas, and sporadic dMMR adenocarcinomas.

The results clearly show for the first time that ERK5 is overexpressed in colon polyps, and in both dMMR and pMMR colon carcinomas. Further, steady-state levels of ERK5 were significantly increased in adenomas ($p < 0.01$), stage 1-4 pMMR carcinomas ($p < 0.05$), and also in dMMR carcinomas ($p< 0.01$), compared to normal colonic mucosa (Fig. 1A). This is in accordance with the current knowledge on ERK5 overexpression in multiple human cancers, including breast (Montero et al., 2009), prostate (McCracken et al., 2008), oral squamous cell carcinoma (Sticht et al., 2008) and hepatocellular carcinoma (Zen et al., 2009). Our results add colon cancer to the growing list of cancer types with aberrant ERK5 expression, thus increasing the relevance of this kinase as a putative novel therapeutic target in cancer.

Survival and proliferation routes usually acquire enhanced signaling capabilities, as the expression and/or activation of relevant effectors in these pathways become deregulated. Our data provides clear evidence that ERK5 is overexpressed early in the transition from normal colon to adenoma. In addition, at the adenoma stage,ERK5 displayed significantly increased steady-state expression levels, compared to all pMMR tumor stages ($p < 0.05$ for stage 1 and 2, and $p < 0.01$ for stage 3 and 4) (Fig. 1A). In addition, steady-state levels of ERK5 are not significantly altered between dMMR tumors and each individual pMMR tumor stages (1-4). Taken together, our results clearly show aberrant expression of ERK5 in polyps and all pMMR tumor stages, suggesting the involvement of ERK5 in tumor initiation and progression. Importantly, these results also suggest that ERK5 overexpression is independent of MMR system status, therefore representing a common event in colorectal tumorigenesis.

Next, we evaluated MEK5 expression in colon cancer, which is also unreported in the literature. The results show that MEK5 presents an expression profile similar to that observed for ERK5. Steady-state levels of MEK5 are clearly increased in polyps ($p < 0.01$), stage 1-4

pMMR carcinomas ($p < 0.05$), and also in dMMR carcinomas ($p< 0.05$), compared to normal colon tissue (Fig. 1B). In addition, MEK5 steady-state levels are higher in polyps than in pMMR and dMMR carcinomas ($p < 0.05$), and unchanged between pMMR and dMMR carcinomas.

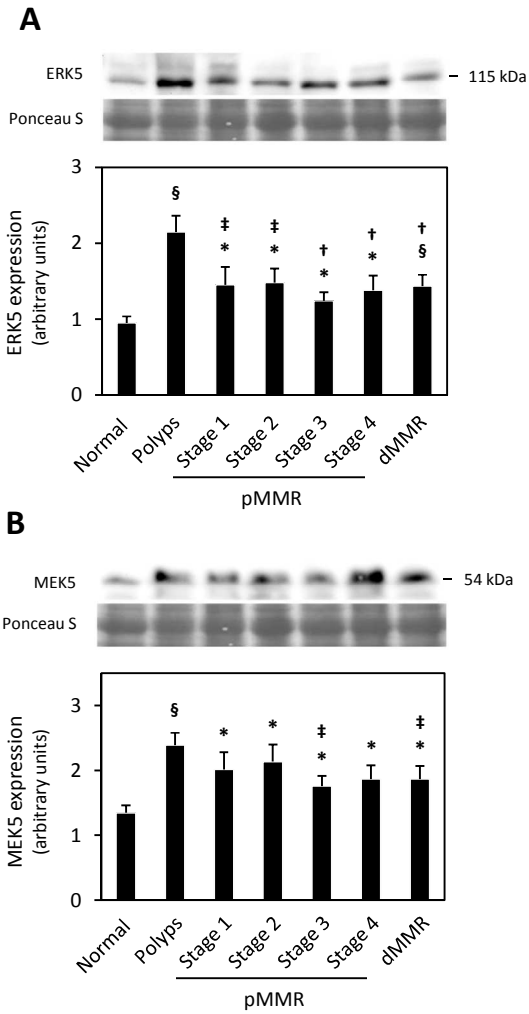

Fig.3. ERK5 and MEK5 protein levels in human colon cancer.(A) ERK5 and (B) MEK5 steady-state levels in normal colon samples, polyps, stages 1-4 pMMR, and dMMR tumors. Total proteins were extracted and used forWestern blot analysis. Results are expressed as mean ± SEM. §$p< 0.01$ and *$p < 0.05$ from normal colon; †$p< 0.01$ and ‡$p < 0.05$ from colon polyps.

MEK5 and ERK5 are overexpressed in colon adenomas and carcinomas, suggesting that overexpression and possibly overactivation of ERK5 signaling occurs in CRC, and may be relevant in disease onset and progression. In other cancer types, ERK5 overexpression was associated with enhanced proliferation, migration, tumor growth and chemoresistance(Linnerth et al., 2005; McCracken et al., 2008; Montero et al., 2009; Sticht et al., 2008; Zen et al., 2009). MEK5 overexpression has also been associated with enhanced proliferation and invasive potential (Mehta et al., 2003; Ramsay et al., 2011). Interestingly, ERK5 is one of the major biological targets of miR-143, whose expression is downregulated in colon adenomas and carcinomas (Akao et al., 2010; Michael et al., 2003). miR-143 expression

was suggested to be lost in the normal to adenoma transition, and is therefore considered closely associated with tumor initiation (Akao et al., 2010). Since the expression of miR-143 is inversely correlated with ERK5, downregulation of miR-143 is probably responsible for the relief of ERK5 suppression, which in turn may directly accelerate CRCtumorigenesis(Akao et al., 2009; Akao et al., 2007; Akao et al., 2006; Borralho et al., 2009; Borralho et al., 2011). Considering the reduced expression reported for miR-143 in colon adenomas and carcinomas, it would be expected that ERK5 steady-state levels would be increase in adenomas and carcinomas, as evidenced by our results. To complement this data, it would be important to correlate miR-143 expression levels with those of ERK5 in this group of samples.

Nevertheless, our data provides evidence that ERK5 may be a novel and relevant therapeutic target in CRC, which can be modulated via the use of miR-143 to potentially inhibit tumor growth and progression.

1.2. NF-κB and IκB

The NF-κB signaling pathway is frequently deregulated in several types of cancer, including CRC. NF-κB is important for cellular transformation, and NF-κB overexpression and activation may lead to uncontrolled cell growth and survival, apoptosis suppression, angiogenesis and metastasis (Chaturvedi et al., 2011). Importantly, NF-κB activation may occur in response to activating stimuli propagated via several signaling pathways, including AKT (Kane et al., 1999), ERK5 (Garaude et al., 2006), and ERK1/2 (Wu et al., 2011).

For assessment of the NF-κB signaling pathway in human colon cancer, we evaluated the expression of NF-κB and its inhibitor IκB in colon polyps, carcinomas and normal colonic mucosa. The results clearly show that NF-κB is overexpressed in colon polyps ($p < 0.01$), in stage 1-4 pMMR carcinomas ($p < 0.01$ for stage 1, and $p < 0.05$ for stage 2-4), and also in dMMR carcinomas ($p < 0.01$), compared to normal colon tissue (Fig. 2A). Interestingly, NF-κB displayed increased steady-state levels in colon polyps, compared to stage 1-4 pMMR carcinomas ($p < 0.05$ for stage 1, and $p < 0.01$ for stage 2-4) and also to dMMR carcinomas ($p< 0.01$) (Fig. 2A). IκB steady-state levels were increased in polyps compared to normal colon tissue and pMMRand dMMR tumors ($p< 0.01$), with no significant expression changes in pMMR and dMMRfrom normal colon (Fig. 2B).Further, to ascertain NF-κB activation, we determined the ratio of NF-κB over IκB for each sample. NF-κB was aberrantly overactivated in polyps and stage 1-4 pMMR tumors ($p < 0.05$), and also in dMMR tumors ($p < 0.01$), compared to normal tissue. Curiously, there were no significant differences in NF-κB over IκB ratio from polyps to any stage pMMR tumors, nor to dMMR tumors (Fig. 2C). Our results confirm aberrant NF-κB signaling in colon polyps and carcinomas, and suggest that the NF-κB pathway is not influenced by MMR system status, although NF-κB over IκB ratio is slightly higher in dMMR versus pMMR tumors and also versus polyps.

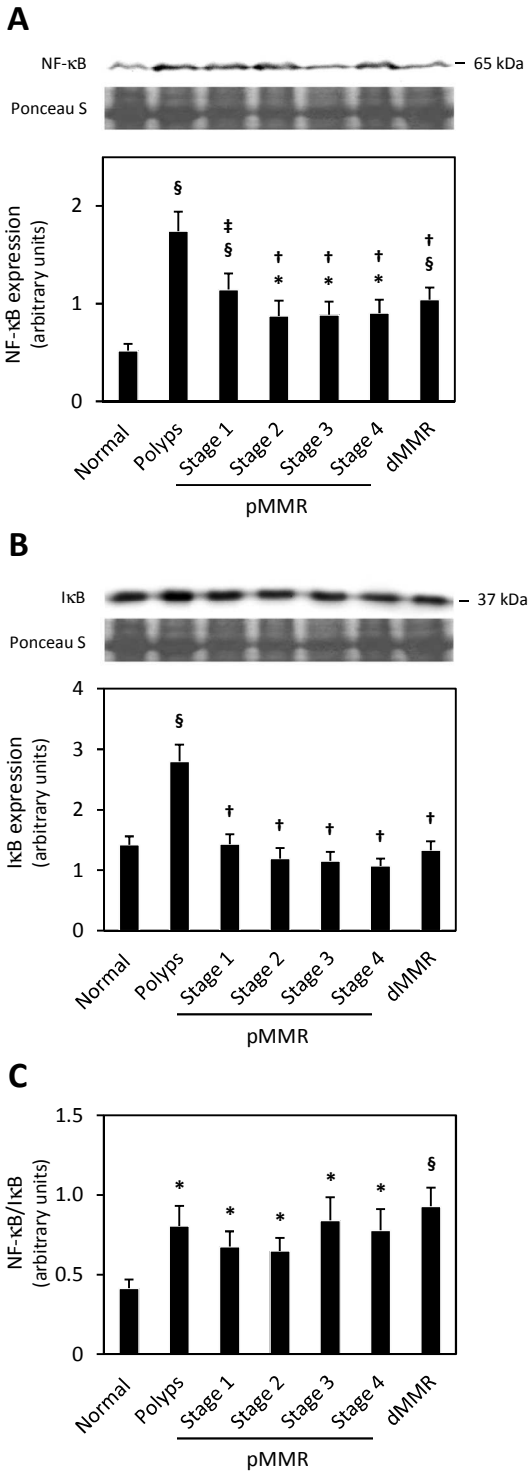

Fig. 2.NF-κB and IκB protein levels and NF-κB/IκB ratio in human colon cancer.(A) NF-κB and (B) IκB steady-state levels, and (C) NF-κB/IκB ratiosin normal colon samples, polyps, stages 1-4 pMMR, and dMMR tumors. Total proteins were extracted and used for Western blot analysis. Results are expressed as mean ± SEM. §p< 0.01 and *p < 0.05 from normal colon; †p< 0.01 and ‡p < 0.05 from colon polyps.

1.3. p-AKT and AKT

The PI3K/AKT signaling pathway is deregulated in several types of cancer, including CRC. Abnormal AKT activation has been demonstrated as an important step for cell transformation and tumor maintenance, due to its role in the promotion of cell proliferation, growth, survival and mobility (Mosca et al., 2011; Tokunaga et al., 2008). Interestingly, Rasacts as an upstream regulator of AKT(Tokunaga et al., 2008). AKT overexpression is also observed in various human cancers. However, in CRC, the overactivation of this signaling pathway is a much more common event then its overexpression, mainly due to the typical overexpression of EGFR in this type of cancer (Tokunaga et al., 2008).

For the evaluation of the AKT signaling pathway, we analyzed p-AKT and AKT steady-state levels, and assessed AKT activation by calculating the ratio of p-AKT over AKT in colon polyps, carcinomas and nomal colonic mucosa. Our results showed that p-AKT was significantly higher in polyps compared to normal colon tissue ($p< 0.01$). There was also a trend for increase in pMMR and dMMR tumors relatively to normal colon tissue, although without statistical significance (Fig. 3A). In addition, AKT steady-state levels were increased in polyps, without reaching statistical significance, and in pMMR and dMMR tumors versus normal colon tissue ($p< 0.05$) (Fig. 3B). AKT activation was markedly increased in polyps compared to normal tissue ($p< 0.01$), and higher than that of carcinomas ($p < 0.01$ for pMMR; and $p < 0.05$ for dMMR tumors). However, the increase in AKT activation found in pMMR and dMMR carcinomas compared to normal tissue did not reach statistical significance (Fig. 3C). Our data suggests that overexpression and overactivation of AKT signaling occurs in CRC, represents an early event, and proceeds independently of tumor MMR system status. The involvement of AKT in early events of sporadic colon carcinogenesis has already been reported (Roy et al., 2002).

1.4. p53

Loss of p53 function by mutation is a common event in cancer, at rates varying between 10% and close to 100%. These mutational events have been reported to occur at different phases of the multistep process of malignant transformation. The question whether p53 mutations are involved in the initiation of malignant transformation, or perhaps only in more advanced stages of cancer is still an open question (Rivlin et al., 2011). However, the loss of p53 function in CRC is usually associated with adenoma to carcinoma transition (Arends, 2000; Baker et al., 1990). In most cases, the p53 gene mutation gives rise to a stable mutant protein, which tends to accumulate in the cell (Rivlin et al., 2011), and may lead to loss of p53 tumor suppressor activity, increased cellular proliferation and inhibition of apoptosis (Tan et al., 2005). Loss of heterozygosity is believed to be the main inactivation event on one of the alleles of tumor suppressor genes in cancer, where approximately 70% of CRC display 17p LOH. In most cases of CRC, the main target of 17p LOH is the p53 gene. Approximately 85% of the somatic mutations carried in the remaining p53 allele are missense mutations (Fearon, 2011). Further, only a small minority of CRC cases lack 17p LOH and have somatic mutations in both p53 alleles, whereas most adenomas usually lack 17p LOH and p53 mutations. Thus, adenomas usually present wild-type functional p53, in contrast to carcinomas, where p53 is frequently mutated (Baker et al., 1990).

Our results showed p53 overexpression in polyps as well as in pMMR and dMMR tumors (p< 0.01). Importantly, p53 levels in polyps and dMMR carcinomas are much lower than those found in pMMR carcinomas (Fig. 4).

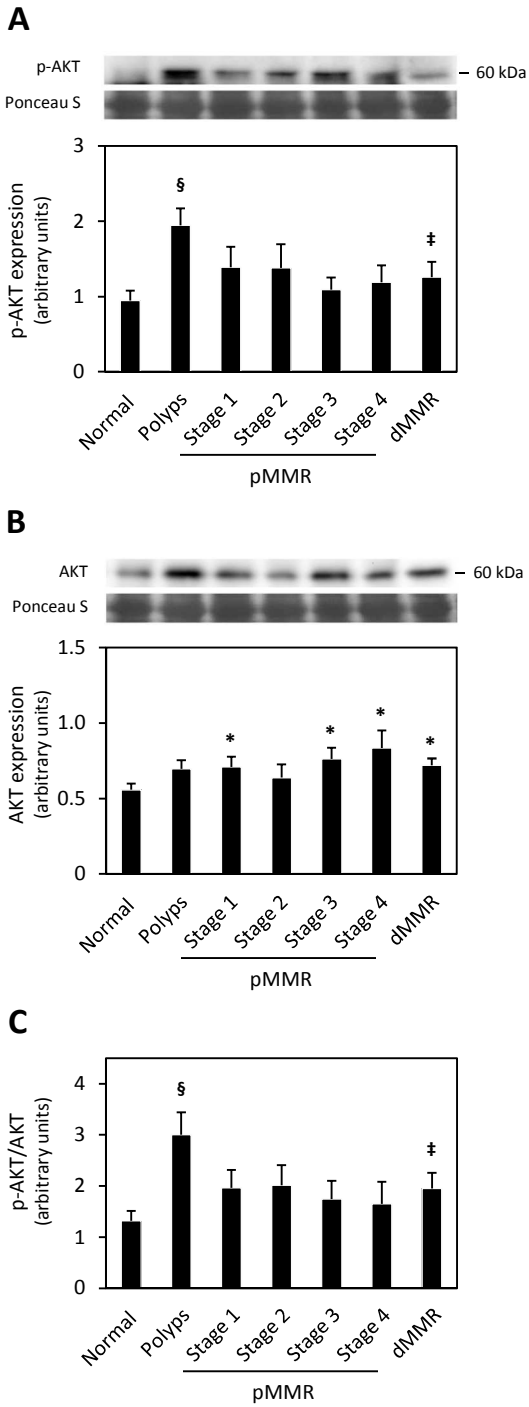

Fig. 3.p-AKT and AKT protein levels, and p-AKT/AKT ratio in human colon cancer.(A) p-AKT and (B) AKT steady-state levels, and (C) p-AKT/AKT ratioin normal colon samples, polyps, stages 1-4 pMMR, and dMMR tumors.Total proteins were extracted and used forWestern blot analysis. Results are expressed as mean ± SEM. §p< 0.01 and *p < 0.05 from normal colon; ‡p < 0.05 from colon polyps.

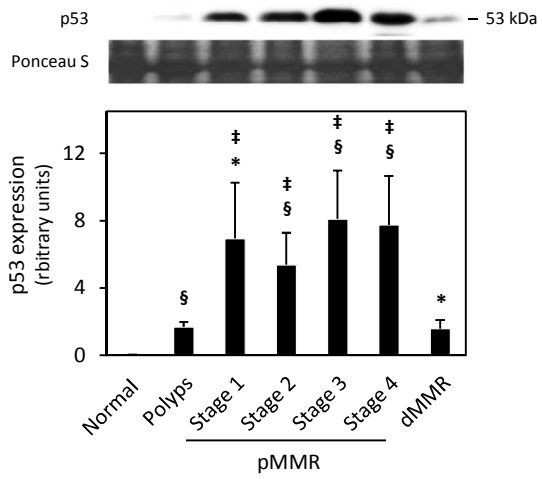

Fig. 4.p53protein levels in human colon cancer.p53 steady-state levels in normal, polyps, stages 1-4 pMMR, and dMMR tumors. Total proteins were extracted and used for Western blot analysis. Results are expressed as mean ± SEM. §p< 0.01 and *p < 0.05 from normal colon; ‡p < 0.05 from colon polyps.

The apparent p53 overexpression may result from stable mutant proteins that accumulate in cells, which is a common event in several types of cancer, including CRC (Iacopetta, 2003). In addition, the loss of functional p53 in adenomas represents a minority of cases (Baker et al., 1990). Therefore, the increased p53 steady-state levels found in adenomas might be associated with an attempt to control cell growth, rather than derive from p53 mutations. Nevertheless, this requires further experiments to characterize p53 status. In dMMR carcinomas, it has been reported that mutations in p53 are infrequent (Zaanan et al., 2010), and our data apparently illustrates this aspect. The lower rate of p53 mutation might also be related with the better prognosis associated with CRC presenting MSI in contrast to CRC presenting intact mismatch repair system (Popat et al., 2005).

The present results are in accordance with the literature and suggest that the loss of functional p53 is indeed involved in the adenoma to carcinoma transition, and that the status of the mismatch repair system is strongly correlated with the frequency of p53 mutations (Grady and Carethers, 2008).

2. Development of a Small Non-coding RNA Delivery Tool

Despite the use of 5-FU as the drug of choice for the treatment of CRC (Borralho et al., 2007; Longley et al., 2003), major improvements in the treatment of colon cancer have been associated with the development and introduction of new systemic therapeutic agents. These might be either cytotoxic or targeted agents. Cytotoxic agents such as irinotecan, oxaliplatin and fluoropyrimidines, and targeted agents including bevacizumab, the antibody against vascular endothelial growth factor A, have increased the survival of patients with advanced CRC. Nevertheless, in most patients, the disease remains incurable (Jonker et al., 2007), and more specific agents with reduced side-effects are in high demand.

The main goal in cancer therapy is to develop a systemic therapeutic agent that could improve overall and progression-free survival. This led to the development of agents that specifically inhibit tumor growth, such as cetuximab. Cetuximab is a monoclonal antibody targeted agent that binds to the extracellular domain of EGFR, frequently upregulated in CRC, leading to its internalization and degradation. Cetuximab treatment improves overall and progression-free survival in patients with CRC, in whom other treatments have failed (Jonker et al., 2007). Cetuximab prevents the activation of EGFR signaling that is responsible for tumor enhancement by promoting apoptosis inhibition, proliferation, angiogenesis and metastasis (Vincenzi et al., 2008). Importantly, CRC cell sensitivity to cetuximab is affected according to the presence of mutations in downstream effectors, such as KRAS and PTEN. Mutations in KRAS and loss of PTEN expression are negative predictive markers for cetuximab response (Frattini et al., 2007; Karapetis et al., 2008).

The discovery that miR-143 is commonly downregulated in CRC(Akao et al., 2010; Akao et al., 2006; Michael et al., 2003), in association with the demonstration that miR-143 forced expression significantly reduces tumor growth and proliferation, while increasing tumor cell apoptosis (Borralho et al., 2011), and chemosensitizing to 5-FU(Borralho et al., 2009), have revealed the promising therapeutic usefulness of miR-143 mimetics in CRC. Importantly, there is also better knowledge on its biological targets involved in the regulation of these cellular events, which include ERK5, KRAS and Bcl-2, among other relevant targets in CRC. Curiously, recent data from our laboratory indicates that miR-143 overexpression leads to NF-κB downregulation(Borralho et al., 2009) and reduced activation (Borralho et al., 2011). Interestingly, Ras is an upstream regulator of AKT (Tokunaga et al., 2008), which is also directly targeted by miR-143 inCRC(Chen et al., 2009). Importantly, in prostate cancer cell lines, mutant KRAS reduced miR-143 expression, whereas miR-143 overexpression reduced ERK1/2 and AKT activation. Therefore, the activity of the AKT signaling pathway might also be regulated by miR-143 in colon cancer (Kent et al., 2010). In accordance with this notion, AKT downregulation by miR-143 tumor suppression function was very recently reported in human bladder cancer cell lines (Noguchi et al., 2011). In addition, maturation of several miRNAs, including miR-143, is enhanced by p53, while mutant forms of p53 hamper miRNA maturation, leading to reduced mature miRNA production (Suzuki et al., 2009). Therefore, loss of functional p53 in CRC might also contribute to the maintenance of low miR-143 levels. Collectively, these findings offer the basis, and strengthen the rationale behind the potential use of miR-143 as a therapeutic tool.

The knowledge of the potential for miR-143 to target downstream effectors of the EGFR signaling pathway may prove to be effective in overcoming apoptosis inhibition, and reducing tumor growth and progression. However, the therapeutic application of miRNAs will rely on the development of efficient delivery strategies, since naked unmodified small RNAs are often unstable in circulation. Therefore, we are attempting to develop a delivery strategy based in the conjugation of miR-143 to cetuximab. With this approach, we aim to take advantage of the benefits offered by both cetuximaband miR-143.

The strategy under development consists in the conjugation of cetuximab with FITC. This conjugation will be accomplished by the FITC reaction with primary amines on cetuximab, resulting in covalently bound protein-dye conjugates. Subsequently, an anti-FITC-protamine single chain Fv (scFv) antibody will be bound to FITC-labeled cetuximab (cetuximab-FITC), and

Cy3-labeled miR-143 will then be loaded onto protamine moyeties. Protamine presents high affinity for nucleic acids and has already been used for *in vitro* and *in vivo* delivery of small non-coding RNAs to cancer cells (Song et al., 2005). If successful, this strategy will allow an efficient delivery of miR-143 (and other small RNAs) to cancer cells overexpressing EGFR, without requiring covalent coupling of miRNAs. The efficacy of this delivery strategy will rely on the efficiency of targeting, and cellular internalization, provided by cetuximab and EGFR overexpression. This tool is still under development; however, relevant progress has been made, which will be discussed in the following sub-sections.

2.1. Cell Sensitivity to Cetuximab

To evaluate the effect of miR-143 without masking from the therapeutic effect of cetuximab, it is necessary to evaluate cellular sensitivity to cetuximab. HCT116 cells overexpress EGFR and display mutant KRAS, whose activating mutations are associated with lack of response to cetuximab(Lievre et al., 2006). HCT116 cells were exposed to increasing concentrations of cetuximab from 0.5 to 10 μM, and cell viability was evaluated by the MTS metabolism assay, at 48 and 72 h of exposure. The results showed that cetuximab has no effect on cell viability at concentrations bellow 2 μM. Even at concentrations up to 4 μM, cell viability was reduced only by < 15% (Fig. 5).

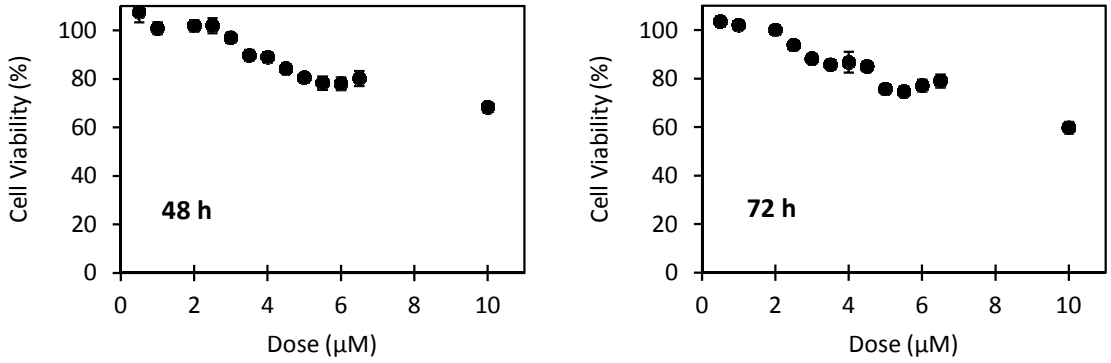

Fig. 5.Effect of cetuximab on HCT116 cell viability. Cells were exposed to increasing concentrations of cetuximabfor 48 (left) and 72 h (right). Cell viability was evaluated by the MTS metabolism assay. The results are expressed as mean ± SEM of at least three independent experiments.

2.2. Cetuximab-FITC Labeling and Function

Cetuximab-FITC conjugation was possible thanks to the reactivity of FITC to primary amine groups present at the surface of cetuximab. The number of FITC molecules per cetuximab molecule was found to be of approximately 10.74. With the knowledge that the molecular weight of FITC is approximately 390 Da, cetuximab-FITC was expected to be approximately 4 kDa heavier than unlabelledcetuximab, and to migrate slower in Western blot. Figure 6 clearly shows this effect.

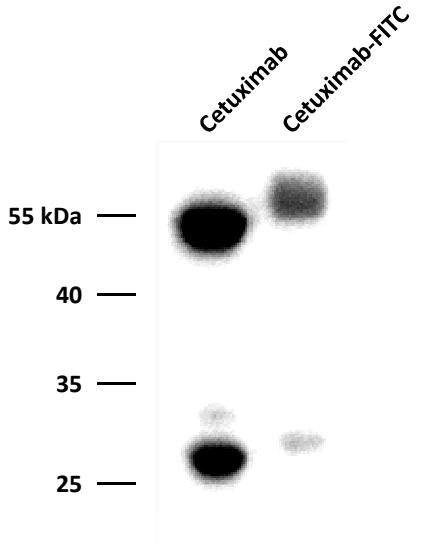

Fig. 6.Labeling of cetuximab with FITC. Labeling was performed with the FluoReporter® FITC protein labeling kit, Invitrogen, Inc.), following the manufacturer's instructions. The labeling ratio FITC/cetuximab was 10.74. Cetuximab and cetuximab-FITC were used to evaluate FITC labeling by Western blot, with anti-Human-IgG-HRP.

One of the critical steps in the construction of this delivery tool is the functionality of labeled cetuximab. Therefore, the maintenance of the binding capacity of cetuximab to EGFR after FITC labeling is of great importance. The binding capacity of cetuximab-FITC to EGFR was evaluated through immunocytochemistry (Fig. 7). As expected, no significant fluorescence was detected in negative controls, including untreated cells, cells treated with cetuximab, and cells processed for immunohistochemistry with secondary α-Human-FITC alone. In addition, significant fluorescence, mostly located at the cell membrane, was detected in the positive control cells treated with cetuximab and processed for immunohistochemistry detection of cetuximab with α-Human-FITC, and in cells treated with cetuximab-FITC. This demonstrated that FITC did not significantly alter the binding capacity of cetuximab to EGFR. Importantly, HCT116 cells exposed to cetuximab-FITC clearly showed higher signal intensity, suggesting that there is indeed a high number of FITC molecules delivered at EGFR in the cell membrane. This will be crucial for the efficacy of the delivery tool, since loading capacity is directly related to the ratio of FITC moieties per cetuximab antibody.

We next titrated FITC-positive cells after cetuximab-FITC treatment by FACS analysis to choose the cetuximab dose to use in the delivery-tool. We found that from concentrations greater than 0.5 nM, all cells in the population are FITC-positive (Fig. 8A and B).

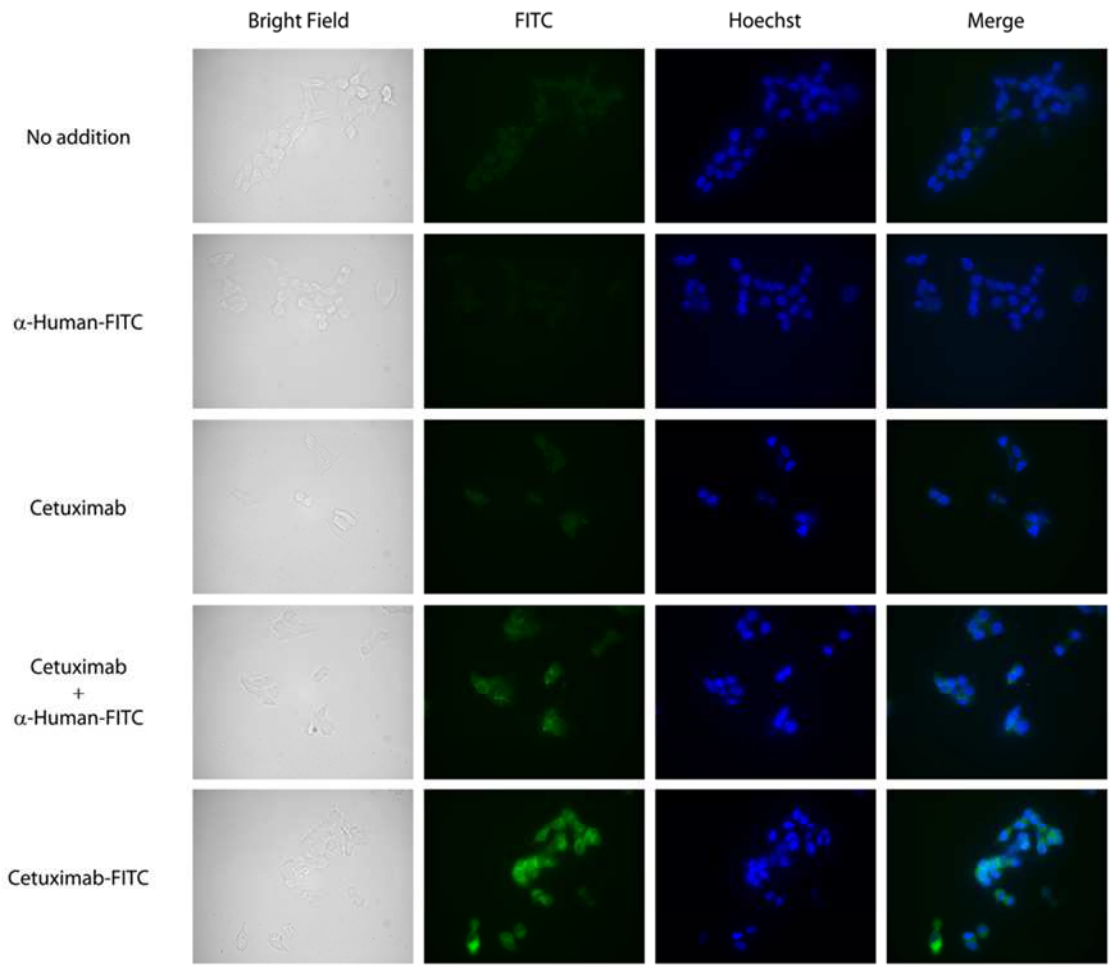

Fig. 7.Evaluation of cetuximab-FITC delivery to HCT116 cells. Cetuximab-FITC binding to EGFR receptors in HTC116 was evaluated by immunocytochemistry and fluorescence microscopy. Cells were incubated with 50 nM of cetuximab, 50 nMcetuximab-FITC and/or α-Human-FITC-conjugated secondary antibody (1:200). Representative images of imunocytochemistry at 400x magnification.

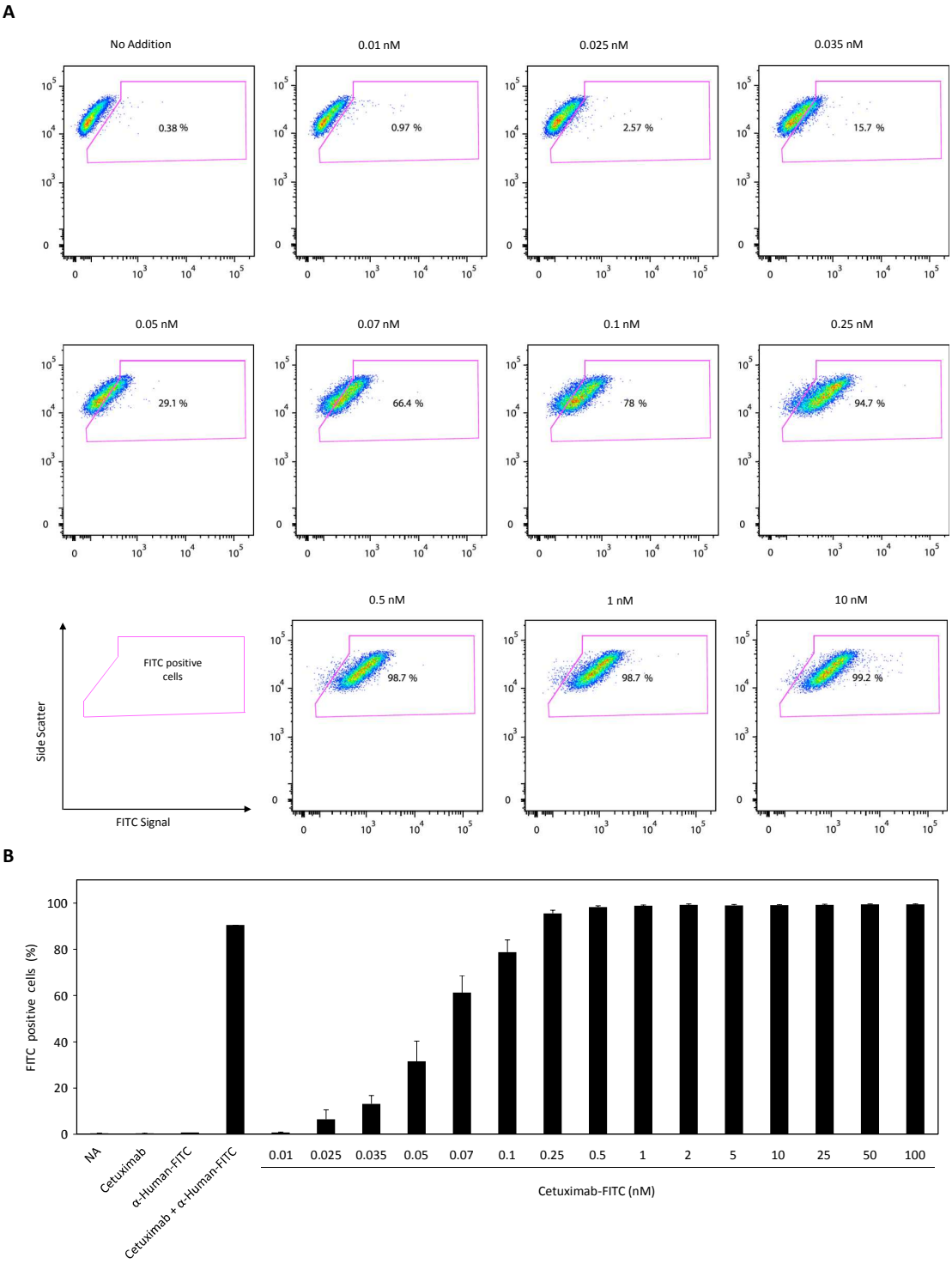

Fig. 8.Titration of cetuximab-FITC.(A) Evaluation of FITC-positive cells after cetuximab-FITC treatment by FACS analysis. (B) Percentage of FITC-positive cells. Cells were incubated with media containing cetuximab-FITC, at concentrations ranging from 0.01 to 100 nM. Unlabeledcetuximab (100 nM), unlabeledcetuximab (100 nM) with secondary α-Human-FITC conjugated antibody, α-Human-FITC conjugated antibody or vehicle PBS (control), were used as controls. The results are expressed at mean ± SEM from at least three independent experiments.

2.3. α-FITC-protamine scFVBinding to Cetuximab-FITC

The next step in the construction of the delivery complex was to evaluate binding of the α-FITC-protamine fusion protein to cetuximab-FITC. This was performed by ELISA, using an anti-HA-HRP antibody for the detection of HA tagged protein. The results showed enrichment in α-FITC-protamine scFv binding to cetuximab-FITC, compared to unlabeled cetuximab (Fig. 9, column 4versus column 3)($p < 0.05$). However, the overall signal intensities measured in these experiments were quite low, suggestive of possible problems in α-FITC-protamine scFv. We later confirmed that the α-FITC-protamine in this batch was highly degraded, which explains the low signal (data not shown).

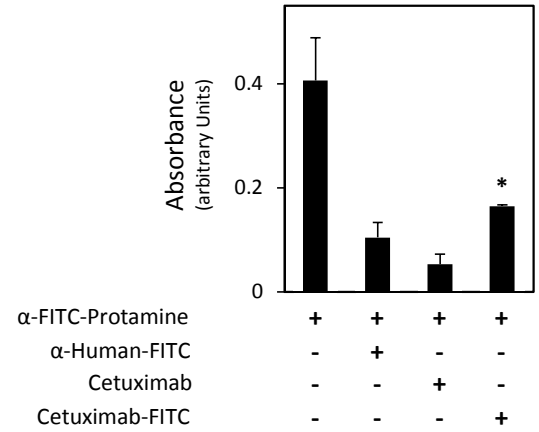

Fig. 9.Evaluation of α-FITC-protamine scFV binding to cetuximab-FITC.α-FITC-protamine scFV binding to cetuximab-FITC was evaluated by ELISA for HA, using α-HA-HRP and ABTS with 0.2% H_2O_2 as substrate. Cetuximab and α-Human-FITC conjugated antibody were used as negative and positive controls, respectively. Results are displayed as absorbance at 405 nm with reference at 492nm. Results are expressed at mean ± SEM from at least three independent experiments.*$p < 0.05$ from cetuximab.

2.4. Delivery of α-FITC-protamine scFv Conjugated with Cetuximab-FITC Complex

The delivery of α-FITC-protamine scFv to cells was performed after *in vitro* complex formation with cetuximab-FITC. For these experiments, we used 5 nMcetuximab-FITC and α-FITC-protamine scFv at a molecular ratio to cetuximab-FITC of 15:1, to allow all FITC residues on cetuximab to be bound by α-FITC-protamine scFv. Subsequently, complexes were delivered to HCT116 cells and α-FITC-protamine scFv cellular delivery was evaluated by Western blot using total protein extracts. Since α-FITC-protamine has an N-terminal HA tag and a C-terminal His tag, we detected both tags by immunobloting. The results clearly show the presence of a duplet band displaying α-HA reactivity in the positive control for α-FITC-protamine scFv (Fig. 10, upper panel, lane 7). This indicates that α-FITC-protamine scFv is to some extent degraded. In addition, a relevant fraction corresponding to the lower band in the doublet may be devoided of protamine. Further dissecting these results, the N-terminal region appears to remain intact since both bands in the doublet display α-HA reactivity. In contrast, anti-His immunoblotting showed that the C-terminal region of α-FITC-protamine scFv was degraded, since the lowest band in the doublet did not display α-His reactivity (Fig. 10, middle panel, lane

7). A possible explanation is the fact that the linker attaching protamine to the scFV is located at the C-terminal of the construction, and is probably being targeted by proteases. Since protamine has approximately 3 kDa, and the lowest band with α-HA reactivity has approximately 3 kDa less, these results suggest that the degradation of the C-terminal region is leading to the loss of α-FITC-protamine. This will compromise downstream applications, since miRNAs cannot be loaded onto the complex.

Neverthless, α-HA immunoblotting clearly showed that our delivery complexes are reaching HCT116 cells with high specificity, since α-FITC-protamine scFV was significantly enriched in cells treated with the delivery complex cetuximab-FITC:α-FITC-protamine, compared to those treated with the delivery complex controls (Fig. 10, upper panel, lane 6 versus lanes 5 and 4). However, in the HA tag immunoblot, it is clearly visible that the lowest band of the α-FITC-protamine scFv is highly enriched, in comparison to the upper band. This suggests that α-FITC-protamine scFvdevoided of protamine is being enriched and more efficiently binding to cetuximab-FITC. In turn, this may reflect a possible interference of protamine in the scFv antigen recognition. To overcome this technical issue, changes in the structure of α-FITC-protamine scFv construction are currently being made.

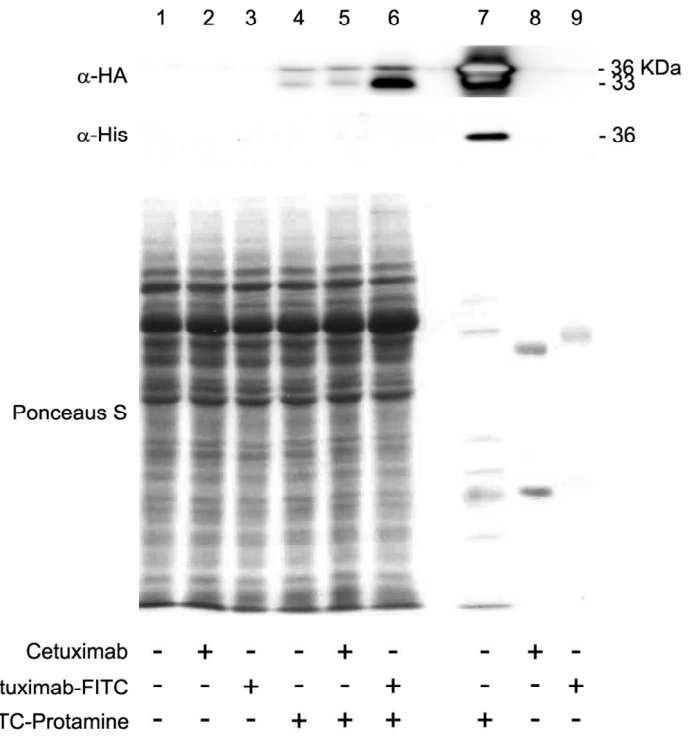

Fig. 10. Evaluation of α-FITC-protamine scFvconjugated with cetuximab-FITCdelivery to HCT116 cells. The delivery complex and respective controls were incubated *in vitro* for 2 h. HCT116 cells were then exposed to the delivery complexes and controls for 4 h. Total proteins were extracted and scFv steady-state levels were evaluated by Western blot. Representative immunoblots for HA and His tags present in scFv are displayed.

3. Conclusions and Future Work

Our results are the first to demonstrate overexpression of two main kinase effectors of the ERK5 signaling pathwayin CRC, ERK5 and its direct activator MEK5. Our data strongly suggest that ERK5 overexpression may be a relevant event in the transition of normal colon to adenoma, and also for the progression of adenoma to carcinoma. This, in turn, may reflect loss of miR-143 expression, which is known to occur early in the transition of normal colon to adenoma and to persist in CRC tumors. The evaluation of miR-143 expression and the correlation with the present ERK5 expression data would more properly allow the testing ofthis suggested correlation. In addition, our results indicate that accompanying aberrant ERK5 and MEK5 expression, NF-κB and PI3K/AKT signaling pathways are also aberrantly activated in CRC, independently of MMR system status. The activation of these particular signaling routes might be stimulated by ERK5, or by other upstream pathway commonly deregulated in cancer. The activation of AKT seems to be particularly important as an early event in CRC development, whereas NF-κB activation seems to be important in tumor development and maintenance. Further, the loss of functional p53 may be involved in the adenoma to carcinoma transition, and contribute to uncontrolled cell growth. However, the loss of functional p53 is not a common event in dMMR carcinomas and that might be related with the better prognosis of this type of tumors.

The known capacity of miR-143 to target downstream effectors of the EGFR signaling pathway, particularly ERK5, might constitute a relevant novel therapeutic tool for CRC management. The present study suggests that ERK5 targeting, possibly via miR-143 overexpression, might be promising and beneficial in the treatment of CRC, thus deserving further investigation. Here, we propose to prepare a delivery tool that combines cetuximab and miR-143. The great enthusiastic advantage of the development of this particular delivery tool arises from the combination of a therapeutic agent already in clinical use for CRC treatment, featuring very good targeting specificity and increased overall and progression-free survival, together with the high potential provided by RNAinterference. Particularly, miRNAs may target multiple deleterious factors in cancer cells. Therefore, the complementary actions of cetuximab and miR-143 in CRC therapy is highly promising in the management of CRC, and expected to significantly improve the efficacy of current therapeutic agents.

IV. REFERENCES

Akao, Y., Y. Nakagawa, I. Hirata, A. Iio, T. Itoh, K. Kojima, R. Nakashima, Y. Kitade, and T. Naoe. 2010. Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. *Cancer Gene Ther*. 17:398-408.

Akao, Y., Y. Nakagawa, A. Iio, and T. Naoe. 2009. Role of microRNA-143 in Fas-mediated apoptosis in human T-cell leukemia Jurkat cells. *Leuk Res*. 33:1530-1538.

Akao, Y., Y. Nakagawa, Y. Kitade, T. Kinoshita, and T. Naoe. 2007. Downregulation of microRNAs-143 and -145 in B-cell malignancies. *Cancer Sci*. 98:1914-1920.

Akao, Y., Y. Nakagawa, and T. Naoe. 2006. MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol Rep*. 16:845-850.

Al-Sukhni, W., M. Aronson, and S. Gallinger. 2008. Hereditary colorectal cancer syndromes: familial adenomatous polyposis and lynch syndrome. *Surg Clin North Am*. 88:819-844, vii.

Altomare, D.A., and J.R. Testa. 2005. Perturbations of the AKT signaling pathway in human cancer. *Oncogene*. 24:7455-7464.

Amaral, J.D., J.M. Xavier, C.J. Steer, and C.M. Rodrigues. 2010. Targeting the p53 pathway of apoptosis. *Curr Pharm Des*. 16:2493-2503.

American Cancer Society. 2011. Colorectal cancer facts &figures 2011-2013. *Atlanta: American Cancer Society*.

Aranha, M.M., P.M. Borralho, P. Ravasco, I.B. Moreira da Silva, L. Correia, A. Fernandes, M.E. Camilo, and C.M. Rodrigues. 2007. NF-kappaB and apoptosis in colorectal tumourigenesis. *Eur J Clin Invest*. 37:416-424.

Arends, J.W. 2000. Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol*. 190:412-416.

Baker, S.J., A.C. Preisinger, J.M. Jessup, C. Paraskeva, S. Markowitz, J.K. Willson, S. Hamilton, and B. Vogelstein. 1990. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res*. 50:7717-7722.

Balint, E.E., and K.H. Vousden. 2001. Activation and activities of the p53 tumour suppressor protein. *Br J Cancer*. 85:1813-1823.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 116:281-297.

Baudhuin, L.M., L.J. Burgart, O. Leontovich, and S.N. Thibodeau. 2005. Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome. *Fam Cancer*. 4:255-265.

Boland, C.R., S.N. Thibodeau, S.R. Hamilton, D. Sidransky, J.R. Eshleman, R.W. Burt, S.J. Meltzer, M.A. Rodriguez-Bigas, R. Fodde, G.N. Ranzani, and S. Srivastava. 1998. A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*. 58:5248-5257.

Borchert, G.M., W. Lanier, and B.L. Davidson. 2006. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol*. 13:1097-1101.

Borralho, P.M., B.T. Kren, R.E. Castro, I.B. da Silva, C.J. Steer, and C.M. Rodrigues. 2009. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. *FEBS J*. 276:6689-6700.

Borralho, P.M., I.B. Moreira da Silva, M.M. Aranha, C. Albuquerque, C. Nobre Leitao, C.J. Steer, and C.M. Rodrigues. 2007. Inhibition of Fas expression by RNAi modulates 5-fluorouracil-induced apoptosis in HCT116 cells expressing wild-type p53. *Biochim Biophys Acta*. 1772:40-47.

Borralho, P.M., A.E.S. Simões, S.E. Gomes, R.T. Lima, T. Carvalho, D.M.S. Ferreira, M.H. Vasconcelos, R.E. Castro, and C.M.P. Rodrigues. 2011. miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation. *PLoS One*. 6:1-11.

Bouali, S., A.S. Chretien, C. Ramacci, M. Rouyer, S. Marchal, T. Galenne, P. Juin, P. Becuwe, and J.L. Merlin. 2009. P53 and PTEN expression contribute to the inhibition of EGFR downstream signaling pathway by cetuximab. *Cancer Gene Ther*. 16:498-507.

Calin, G.A., and C.M. Croce. 2006. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 6:857-866.

Calin, G.A., C. Sevignani, C.D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini, and C.M. Croce. 2004. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A*. 101:2999-3004.

Cargnello, M., and P.P. Roux. 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev*. 75:50-83.

Carnero, A., C. Blanco-Aparicio, O. Renner, W. Link, and J.F. Leal. 2008. The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. *Curr Cancer Drug Targets*. 8:187-198.

Carthew, R.W., and E.J. Sontheimer. 2009. Origins and mechanisms of miRNAs and siRNAs. *Cell*. 136:642-655.

Chaturvedi, M.M., B. Sung, V.R. Yadav, R. Kannappan, and B.B. Aggarwal. 2011. NF-kappaB addiction and its role in cancer: 'one size does not fit all'. *Oncogene*. 30:1615-1630.

Chen, X., X. Guo, H. Zhang, Y. Xiang, J. Chen, Y. Yin, X. Cai, K. Wang, G. Wang, Y. Ba, L. Zhu, J. Wang, R. Yang, Y. Zhang, Z. Ren, K. Zen, J. Zhang, and C.Y. Zhang. 2009. Role of miR-143 targeting KRAS in colorectal tumorigenesis. *Oncogene*. 28:1385-1392.

Clape, C., V. Fritz, C. Henriquet, F. Apparailly, P.L. Fernandez, F. Iborra, C. Avances, M. Villalba, S. Culine, and L. Fajas. 2009. miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice. *PLoS One*. 4:e7542.

Cude, K., Y. Wang, H.J. Choi, S.L. Hsuan, H. Zhang, C.Y. Wang, and Z. Xia. 2007. Regulation of the G2-M cell cycle progression by the ERK5-NFkappaB signaling pathway. *J Cell Biol*. 177:253-264.

Dolcet, X., D. Llobet, J. Pallares, and X. Matias-Guiu. 2005. NF-kB in development and progression of human cancer. *Virchows Arch*. 446:475-482.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 35:495-516.

Fang, J.Y., and B.C. Richardson. 2005. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol*. 6:322-327.

Fearon, E.R. 2011. Molecular genetics of colorectal cancer. *Annu Rev Pathol*. 6:479-507.

Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 9:102-114.

Frattini, M., P. Saletti, E. Romagnani, V. Martin, F. Molinari, M. Ghisletta, A. Camponovo, L.L. Etienne, F. Cavalli, and L. Mazzucchelli. 2007. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer*. 97:1139-1145.

Garaude, J., S. Cherni, S. Kaminski, E. Delepine, C. Chable-Bessia, M. Benkirane, J. Borges, A. Pandiella, M.A. Iniguez, M. Fresno, R.A. Hipskind, and M. Villalba. 2006. ERK5 activates NF-kappaB in leukemic T cells and is essential for their growth in vivo. *J Immunol*. 177:7607-7617.

Gayet, J., X.P. Zhou, A. Duval, S. Rolland, J.M. Hoang, P. Cottu, and R. Hamelin. 2001. Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. *Oncogene*. 20:5025-5032.

Girio, A., J.C. Montero, A. Pandiella, and S. Chatterjee. 2007. Erk5 is activated and acts as a survival factor in mitosis. *Cell Signal*. 19:1964-1972.

Grady, W.M., and J.M. Carethers. 2008. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology*. 135:1079-1099.

Grandis, J.R., and J.C. Sok. 2004. Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol Ther*. 102:37-46.

Holand, K., F. Salm, and A. Arcaro. 2011. The phosphoinositide 3-kinase signaling pathway as a therapeutic target in grade IV brain tumors. *Curr Cancer Drug Targets*.[Epub ahead of print]

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

Huerta, S., E.J. Goulet, and E.H. Livingston. 2006. Colon cancer and apoptosis. *Am J Surg*. 191:517-526.

Iacopetta, B. 2003. TP53 mutation in colorectal cancer. *Hum Mutat*. 21:271-276.

Johnson, S.M., P. Gulhati, B.A. Rampy, Y. Han, P.G. Rychahou, H.Q. Doan, H.L. Weiss, and B.M. Evers. 2010. Novel expression patterns of PI3K/Akt/mTOR signaling pathway components in colorectal cancer. *J Am Coll Surg*. 210:767-776, 776-768.

Jonker, D.J., C.J. O'Callaghan, C.S. Karapetis, J.R. Zalcberg, D. Tu, H.J. Au, S.R. Berry, M. Krahn, T. Price, R.J. Simes, N.C. Tebbutt, G. van Hazel, R. Wierzbicki, C. Langer, and M.J. Moore. 2007. Cetuximab for the treatment of colorectal cancer. *N Engl J Med*. 357:2040-2048.

Kane, L.P., V.S. Shapiro, D. Stokoe, and A. Weiss. 1999. Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol*. 9:601-604.

Karapetis, C.S., S. Khambata-Ford, D.J. Jonker, C.J. O'Callaghan, D. Tu, N.C. Tebbutt, R.J. Simes, H. Chalchal, J.D. Shapiro, S. Robitaille, T.J. Price, L. Shepherd, H.J. Au, C. Langer, M.J. Moore, and J.R. Zalcberg. 2008. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med*. 359:1757-1765.

Karin, M., and A. Lin. 2002. NF-kappaB at the crossroads of life and death. *Nat Immunol*. 3:221-227.

Kehoe, J., and V.P. Khatri. 2006. Staging and prognosis of colon cancer. *Surg Oncol Clin N Am*. 15:129-146.

Kent, O.A., R.R. Chivukula, M. Mullendore, E.A. Wentzel, G. Feldmann, K.H. Lee, S. Liu, S.D. Leach, A. Maitra, and J.T. Mendell. 2010. Repression of the miR-143/145 cluster by

oncogenic Ras initiates a tumor-promoting feed-forward pathway. *Genes Dev*. 24:2754-2759.

Kim, C.S., V.V. Vasko, Y. Kato, M. Kruhlak, M. Saji, S.Y. Cheng, and M.D. Ringel. 2005. AKT activation promotes metastasis in a mouse model of follicular thyroid carcinoma. *Endocrinology*. 146:4456-4463.

Kim, V.N., J. Han, and M.C. Siomi. 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 10:126-139.

Kinzler, K.W., and B. Vogelstein. 1996. Lessons from hereditary colorectal cancer. *Cell*. 87:159-170.

Kojima, M., T. Morisaki, N. Sasaki, K. Nakano, R. Mibu, M. Tanaka, and M. Katano. 2004. Increased nuclear factor-kB activation in human colorectal carcinoma and its correlation with tumor progression. *Anticancer Res*. 24:675-681.

Lee, Y.S., and A. Dutta. 2009. MicroRNAs in cancer. *Annu Rev Pathol*. 4:199-227.

Lievre, A., J.B. Bachet, D. Le Corre, V. Boige, B. Landi, J.F. Emile, J.F. Cote, G. Tomasic, C. Penna, M. Ducreux, P. Rougier, F. Penault-Llorca, and P. Laurent-Puig. 2006. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res*. 66:3992-3995.

Lindor, N.M., L.J. Burgart, O. Leontovich, R.M. Goldberg, J.M. Cunningham, D.J. Sargent, C. Walsh-Vockley, G.M. Petersen, M.D. Walsh, B.A. Leggett, J.P. Young, M.A. Barker, J.R. Jass, J. Hopper, S. Gallinger, B. Bapat, M. Redston, and S.N. Thibodeau. 2002. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. *J Clin Oncol*. 20:1043-1048.

Linnerth, N.M., M. Baldwin, C. Campbell, M. Brown, H. McGowan, and R.A. Moorehead. 2005. IGF-II induces CREB phosphorylation and cell survival in human lung cancer cells. *Oncogene*. 24:7310-7319.

Liu, X., K. Fortin, and Z. Mourelatos. 2008. MicroRNAs: biogenesis and molecular functions. *Brain Pathol*. 18:113-121.

Longley, D.B., D.P. Harkin, and P.G. Johnston. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer*. 3:330-338.

Lynch, H.T., C.R. Boland, G. Gong, T.G. Shaw, P.M. Lynch, R. Fodde, J.F. Lynch, and A. de la Chapelle. 2006. Phenotypic and genotypic heterogeneity in the Lynch syndrome: diagnostic, surveillance and management implications. *Eur J Hum Genet*. 14:390-402.

Manikandan, J., J.J. Aarthi, S.D. Kumar, and P.N. Pushparaj. 2008. Oncomirs: the potential role of non-coding microRNAs in understanding cancer. *Bioinformation*. 2:330-334.

Marcu, K.B., M. Otero, E. Olivotto, R.M. Borzi, and M.B. Goldring. 2010. NF-kappaB signaling: multiple angles to target OA. *Curr Drug Targets*. 11:599-613.

McCracken, S.R., A. Ramsay, R. Heer, M.E. Mathers, B.L. Jenkins, J. Edwards, C.N. Robson, R. Marquez, P. Cohen, and H.Y. Leung. 2008. Aberrant expression of extracellular signal-regulated kinase 5 in human prostate cancer. *Oncogene*. 27:2978-2988.

Mehta, P.B., B.L. Jenkins, L. McCarthy, L. Thilak, C.N. Robson, D.E. Neal, and H.Y. Leung. 2003. MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. *Oncogene*. 22:1381-1389.

Michael, M.Z., O.C. SM, N.G. van Holst Pellekaan, G.P. Young, and R.J. James. 2003. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res*. 1:882-891.

Montero, J.C., A. Ocana, M. Abad, M.J. Ortiz-Ruiz, A. Pandiella, and A. Esparis-Ogando. 2009. Expression of Erk5 in early stage breast cancer and association with disease free survival identifies this kinase as a potential therapeutic target. *PLoS One*. 4:e5565.

Mosca, E., M. Barcella, R. Alfieri, A. Bevilacqua, G. Canti, and L. Milanesi. 2011. Systems biology of the metabolic network regulated by the Akt pathway. *Biotechnol Adv*. [Epub ahead of print]

Nathke, I.S. 2004. The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. *Annu Rev Cell Dev Biol*. 20:337-366.

Ng, E.K., W.P. Tsang, S.S. Ng, H.C. Jin, J. Yu, J.J. Li, C. Rocken, M.P. Ebert, T.T. Kwok, and J.J. Sung. 2009. MicroRNA-143 targets DNA methyltransferases 3A in colorectal cancer. *Br J Cancer*. 101:699-706.

Nishimoto, S., and E. Nishida. 2006. MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep*. 7:782-786.

Noguchi, S., T. Mori, Y. Hoshino, K. Maruo, N. Yamada, Y. Kitade, T. Naoe, and Y. Akao. 2011. MicroRNA-143 functions as a tumor suppressor in human bladder cancer T24 cells. *Cancer Lett*. 307:211-220.

Peer, D., P. Zhu, C.V. Carman, J. Lieberman, and M. Shimaoka. 2007. Selective gene silencing in activated leukocytes by targeting siRNAs to the integrin lymphocyte function-associated antigen-1. *Proc Natl Acad Sci U S A*. 104:4095-4100.

Popat, S., R. Hubner, and R.S. Houlston. 2005. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol*. 23:609-618.

Potten, C.S., M. Kellett, S.A. Roberts, D.A. Rew, and G.D. Wilson. 1992. Measurement of in vivo proliferation in human colorectal mucosa using bromodeoxyuridine. *Gut*. 33:71-78.

Potter, J.D. 1996. Nutrition and colorectal cancer. *Cancer Causes Control*. 7:127-146.

Potter, J.D. 1999. Colorectal cancer: molecules and populations. *J Natl Cancer Inst*. 91:916-932.

Quivy, V., and C. Van Lint. 2004. Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation. *Biochem Pharmacol*. 68:1221-1229.

Ramsay, A.K., S.R. McCracken, M. Soofi, J. Fleming, A.X. Yu, I. Ahmad, R. Morland, L. Machesky, C. Nixon, D.R. Edwards, R.K. Nuttall, M. Seywright, R. Marquez, E. Keller, and H.Y. Leung. 2011. ERK5 signalling in prostate cancer promotes an invasive phenotype. *Br J Cancer*. 104:664-672.

Ricci-Vitiani, L., A. Pagliuca, E. Palio, A. Zeuner, and R. De Maria. 2008. Colon cancer stem cells. *Gut*. 57:538-548.

Rivlin, N., R. Brosh, M. Oren, and V. Rotter. 2011. Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis. *Genes Cancer*. 2:466-474.

Roberts, O.L., K. Holmes, J. Muller, D.A. Cross, and M.J. Cross. 2009. ERK5 and the regulation of endothelial cell function. *Biochem Soc Trans*. 37:1254-1259.

Roberts, P.J., and C.J. Der. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene*. 26:3291-3310.

Roy, H.K., B.F. Olusola, D.L. Clemens, W.J. Karolski, A. Ratashak, H.T. Lynch, and T.C. Smyrk. 2002. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis*. 23:201-205.

Sakamoto, K., S. Maeda, Y. Hikiba, H. Nakagawa, Y. Hayakawa, W. Shibata, A. Yanai, K. Ogura, and M. Omata. 2009. Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin Cancer Res*. 15:2248-2258.

Sheng, S., M. Qiao, and A.B. Pardee. 2009. Metastasis and AKT activation. *J Cell Physiol*. 218:451-454.

Slaby, O., M. Svoboda, J. Michalek, and R. Vyzula. 2009. MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. *Mol Cancer*. 8:102.

Song, E., P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, and J. Lieberman. 2005. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol*. 23:709-717.

Sticht, C., K. Freier, K. Knopfle, C. Flechtenmacher, S. Pungs, C. Hofele, M. Hahn, S. Joos, and P. Lichter. 2008. Activation of MAP kinase signaling through ERK5 but not ERK1 expression is associated with lymph node metastases in oral squamous cell carcinoma (OSCC). *Neoplasia*. 10:462-470.

Suzuki, H.I., K. Yamagata, K. Sugimoto, T. Iwamoto, S. Kato, and K. Miyazono. 2009. Modulation of microRNA processing by p53. *Nature*. 460:529-533.

Takagi, T., A. Iio, Y. Nakagawa, T. Naoe, N. Tanigawa, and Y. Akao. 2009. Decreased expression of microRNA-143 and -145 in human gastric cancers. *Oncology*. 77:12-21.

Tan, J., L. Zhuang, H.S. Leong, N.G. Iyer, E.T. Liu, and Q. Yu. 2005. Pharmacologic modulation of glycogen synthase kinase-3beta promotes p53-dependent apoptosis through a direct Bax-mediated mitochondrial pathway in colorectal cancer cells. *Cancer Res*. 65:9012-9020.

Thibodeau, S.N., A.J. French, J.M. Cunningham, D. Tester, L.J. Burgart, P.C. Roche, S.K. McDonnell, D.J. Schaid, C.W. Vockley, V.V. Michels, G.H. Farr, Jr., and M.J. O'Connell. 1998. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res*. 58:1713-1718.

Tokunaga, E., E. Oki, A. Egashira, N. Sadanaga, M. Morita, Y. Kakeji, and Y. Maehara. 2008. Deregulation of the Akt pathway in human cancer. *Curr Cancer Drug Targets*. 8:27-36.

Turjanski, A.G., J.P. Vaque, and J.S. Gutkind. 2007. MAP kinases and the control of nuclear events. *Oncogene*. 26:3240-3253.

Vincenzi, B., G. Schiavon, M. Silletta, D. Santini, and G. Tonini. 2008. The biological properties of cetuximab. *Crit Rev Oncol Hematol*. 68:93-106.

Voboril, R., S.N. Hochwald, J. Li, A. Brank, J. Weberova, F. Wessels, L.L. Moldawer, E.R. Camp, and S.L. MacKay. 2004. Inhibition of NF-kappa B augments sensitivity to 5-fluorouracil/folinic acid in colon cancer. *J Surg Res*. 120:178-188.

Walther, A., E. Johnstone, C. Swanton, R. Midgley, I. Tomlinson, and D. Kerr. 2009. Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer*. 9:489-499.

Wang, S., and M.D. Basson. 2011. Akt directly regulates focal adhesion kinase through association and serine phosphorylation: implication for pressure-induced colon cancer metastasis. *Am J Physiol Cell Physiol*. 300:C657-670.

Wang, X., K.G. Finegan, A.C. Robinson, L. Knowles, R. Khosravi-Far, K.A. Hinchliffe, R.P. Boot-Handford, and C. Tournier. 2006. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. *Cell Death Differ*. 13:2099-2108.

Wang, X., and C. Tournier. 2006. Regulation of cellular functions by the ERK5 signalling pathway. *Cell Signal*. 18:753-760.

Wilkes, G., and K. Hartshorn. 2009. Colon, rectal, and anal cancers. *Semin Oncol Nurs*. 25:32-47.

Winawer, S.J., A.G. Zauber, R.H. Fletcher, J.S. Stillman, M.J. O'Brien, B. Levin, R.A. Smith, D.A. Lieberman, R.W. Burt, T.R. Levin, J.H. Bond, D. Brooks, T. Byers, N. Hyman, L. Kirk, A. Thorson, C. Simmang, D. Johnson, and D.K. Rex. 2006. Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US multi-society task force on colorectal cancer and the American cancer society. *Gastroenterology*. 130:1872-1885.

Wolpin, B.M., and R.J. Mayer. 2008. Systemic treatment of colorectal cancer. *Gastroenterology*. 134:1296-1310.

Wright, N.A. 2000. Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. *Int J Exp Pathol*. 81:117-143.

Wu, B.L., L.Y. Xu, Z.P. Du, L.D. Liao, H.F. Zhang, Q. Huang, G.Q. Fang, and E.M. Li. 2011. MiRNA profile in esophageal squamous cell carcinoma: downregulation of miR-143 and miR-145. *World J Gastroenterol*. 17:79-88.

Young, B., and J.W. Heath. 2000. Gastrointestinal tract, *in Wheather's Functional Histology, a text and colour atlas*. 4 ed. Edinburgh: Churchill Livingstone, 249-273.

Yu, Y.Y., Q. Li, and Z.G. Zhu. 2005. NF-kappaB as a molecular target in adjuvant therapy of gastrointestinal carcinomas. *Eur J Surg Oncol*. 31:386-392.

Zaanan, A., P. Cuilliere-Dartigues, A. Guilloux, Y. Parc, C. Louvet, A. de Gramont, E. Tiret, S. Dumont, B. Gayet, P. Validire, J.F. Flejou, A. Duval, and F. Praz. 2010. Impact of p53 expression and microsatellite instability on stage III colon cancer disease-free survival in patients treated by 5-fluorouracil and leucovorin with or without oxaliplatin. *Ann Oncol*. 21:772-780.

Zen, K., K. Yasui, T. Nakajima, Y. Zen, Y. Gen, H. Mitsuyoshi, M. Minami, S. Mitsufuji, S. Tanaka, Y. Itoh, Y. Nakanuma, M. Taniwaki, S. Arii, T. Okanoue, and T. Yoshikawa. 2009. ERK5 is a target for gene amplification at 17p11 and promotes cell growth in hepatocellular carcinoma by regulating mitotic entry. *Genes Chromosomes Cancer*. 48:109-120.

Zhang, H., X. Cai, Y. Wang, H. Tang, D. Tong, and F. Ji. 2010. microRNA-143, down-regulated in osteosarcoma, promotes apoptosis and suppresses tumorigenicity by targeting Bcl-2. *Oncol Rep*. 24:1363-1369.