# Molecular mechanisms involved in the initiation and progression of colorectal cancer

Colorectal cancer (CRC) is the third most common type of cancer and the second cause of death by cancer in the Western world. It causes around 650,000 deaths worldwide per year. Most sporadic colorectal cancers arise from adenomas that initially are benign and occur frequently: approximately 50% of the Western population develops an adenoma by the age of 70. However, the development of a full-blown malignant colorectal tumour is a progressive process that often takes several years. During this period, the progression of the disease appears to follow a precise series of molecular events, requiring the accumulation of mutations in proto-oncogenes and tumour suppressor genes in these initially benign lesions. Access to specimens of CRC at different stages of the malignancy has allowed the analysis of the molecular alterations most frequently associated with each step of the disease (reviewed in Sancho *et al*, 2004). The aim of the research in our laboratory is to decipher the molecular instructions that underlie the signalling pathways that are altered in CRC and that are responsible for the initiation and progression of the disease.
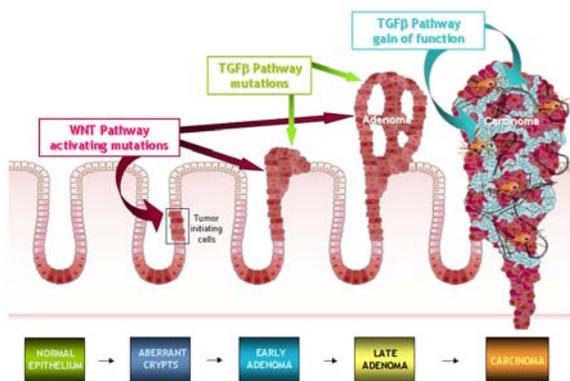

Figure 1. Genetic alterations frequently associated with CRC progression and main signalling pathways. The accessibility to specimens of CRC at different stages of the malignancy has allowed the analysis of the molecular alterations most frequently associated with each step of the disease. Our research is focused mainly on elucidating the mechanisms of CRC initiation by activating Wnt signalling mutations, and on studying the dual role played by TGF-beta signalling at later stages of the disease. TGF-beta signalling is lost in CRC epithelial cells through the acquisition of mutations as the disease progresses, but there also appears to be a gain of function in the stromal component of the tumour, which we are currently investigating.

Wnt signalling and the initiation of CRC

Around 70% of sporadic colorectal tumours show bi-allelic inactivation of the APC gene (Adenomatous polyposis Coli). A high percentage of remaining tumours show activating mutations in beta-catenin or axin. These molecules are components of the Wnt signalling pathway. Activating mutations of this signalling pathway are the only known genetic alterations present in early premalignant lesions in the intestine, such as aberrant crypt foci and small adenomas. In various animal models, activating mutations in this pathway effectively initiate tumorigenesis in the intestine in a process characterised by the formation of displastic cripts and adenomas similar to those found in humans. Therefore, it is widely accepted that constitutive activation of Wnt signalling caused by mutations in components of the pathway are responsible for the initiation of CRC (reviewed in Sancho *et al*, 2004; see Figure 1).

Mutations in Wnt signalling components that lead to CRC result in the stabilisation and accumulation of beta-catenin in the nucleus, and as a result in increased transcriptional activation mediated by the beta-catenin/TCF complex. Therefore, the trans-activation of beta-catenin/TCF target genes is a primary transforming event in CRC. A few years ago we identified the genetic programme driven by beta-catenin and TCF in CRC cells. Our studies indicated that beta-catenin/TCF target genes are expressed not only in tumours but also in healthy non-transformed intestinal progenitor cells at the bottom of the crypts (van de Wetering *et al*, 2002; see Figure 2).

Principal Investigator Elena Sancho **Postdoctoral Fellows** Alexandre Calon, Annie Rodolosse
PhD Student Elisa Espinet **Research Assistant** Sergio Palomo
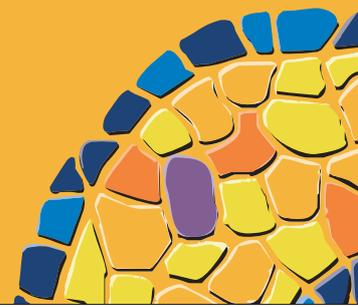

Our results, together with those obtained from several animal models in which Wnt signalling was genetically manipulated (Pinto *et al*, 2003; Korineck *et al*, 1998), implied that the stem cell and progenitor compartments were controlled by Wnt signalling. These findings led us to propose that the first step towards malignancy in CRC consists of the acquisition of a crypt progenitor-like phenotype (van de Wetering *et al*, 2002). Our hypothesis has marked a milestone in the field and has completely changed the view on the initiation of CRC. We are currently developing animal models that will formally prove this concept and help to shed light on the mechanisms behind why Wnt signalling mutations are an important pre-requisite for the development of CRC.

Our studies are now oriented towards the identification of the nature of the founding CRC cells and the mechanisms by which they escape cell renewal. During 2008, we have generated several DNA constructs to allow the development of animal models that will be used for this purpose. These include the conditional expression in the intestine of oncogenes involved in CRC combined with colour markers that will help to identify mutant cells. These studies may shed additional light on specific pathways that can be targeted to block CRC progression.

Having identified that the initial event triggering transformation is the blockage of founder tumour cells into a progenitor phenotype, our lab now seeks to identify differences between the true physiological progenitors and initial founder mutant cells. To this end, during 2008, in collaboration with Eduard Batlle's lab (IRB Barcelona), we have developed a protocol which allows the isolation of epithelial cells from the bottom of colonic crypts (*ie*, stem cells and early progenitors) from fresh tissue. This protocol is also applicable to the isolation of tumour cells from early adenomas or displastic crypts. We are currently comparing the genetic profile of physiological progenitors with that of tumour cells from adenomas. We aim to identify tumour-specific molecular targets susceptible to being targeted by the pharmaceutical industry. These will be useful, particularly for patients suffering Familial Adenomatous Polyposis (FAP). These patients inherit a mutation in APC, and as a result of loss of heterozygosity (LOH) they develop hundreds of polyps in the intestinal tract and are therefore predisposed to the development of malignant CRC.

TGF-beta signalling during CRC progression

Our lab also addresses how the acquisition of mutations in other signalling pathways may modulate the initial progenitor phenotype imposed by Wnt signalling to overcome the bottle-
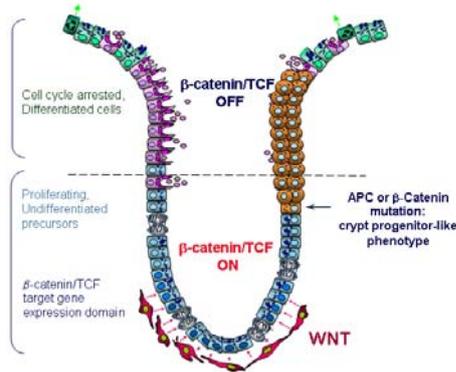
Figure 2. Schematic representation of a colon crypt in relation to wnt signalling and proposed model for the initiation of CRC. The intestinal epithelium is organised in a series of invaginations called crypts of Lieberkühn. Epithelial cells within these crypts are in constant renewal. This is achieved by a small group of stem cells that reside at the bottom of the intestinal crypts. Stem cells divide asymmetrically to give rise to early progenitors that rapidly divide whilst migrating towards the intestinal lumen. As they do so, they become predetermined towards the differentiation into one of the functional cell types present in the intestine (adsorptive, mucosercreting or entereoendocrine cells). Differentiation takes place in the top part of the crypts. The proliferative compartment of intestinal crypts is maintained by the target gene programme directed by beta-catenin/ Tcf in response to wnt signals. When these cells physiologically down-modulate beta-catenin/Tcf activity, they cease to proliferate and differentiate. Cells mutant in components of wnt signalling (APC, beta-catenin, axin) become independent of these signals, exhibit constitutive activation by beta-catenin/Tcf and are blocked in a progenitor proliferative phenotype.
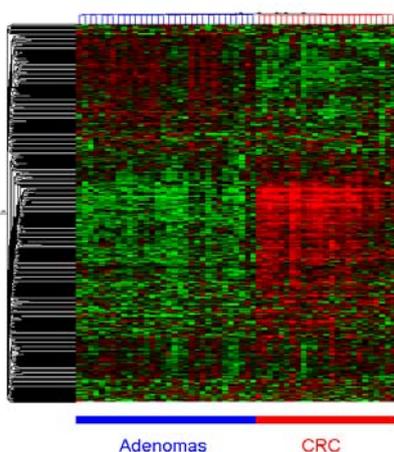
Figure 3. The TGF-beta responding signature (TBRS) is differentially expressed between adenomas and adenocarcinomas. Unsupervised clustering analysis of a collection of tumours of known transcriptomes on the basis of target genes controlled by TGF-beta signalling.

necks associated with CRC progression. One of the most prevalent mutations found during CRC progression are those inactivating the TGF-beta signalling pathway (reviewed in Grady and Markowitz, 2003; Figure 1). The TGF-beta pathway is involved in numerous processes in development and homeostasis of adult tissues. TGF-beta ligands activate the signalling pathway by binding to TGF-beta receptor type II homodimers. Ligand-bound receptor II recruits TGF-beta receptor I homodimers, which are subsequently transphoshorylated and thus activated by receptor type II. Phosphorylation of the intracellular mediators smads by activated receptor I allows dimer formation with smad-4 and translocation to the nucleus, where the specific outcome of the signalling will depend on the cell type and the context of the cell itself (reviewed in Shi and Massague, 2003).

Around 80% of all microsatellite instable CRCs contain mutations in type-II TGF-beta receptor (TGFBR2) that impair signalling. In addition, inactivation of downstream TGF-beta pathway effectors, in particular SMAD4 and SMAD2, have also been found in a significant fraction of microsatellite stable CRCs. Overall, the incidence of TGF-beta resistance in CRCs appears to be around 30% (reviewed in Grady and Markowitz, 2003). In addition, virtually all CRC cell lines have lost their TGF-beta response. Modelling CRC progression in mice has revealed that disruption of TGF-beta signalling in the intestinal epithelium does not initiate intestinal tumorigenesis per se (Biswas *et al*, 2004; Munoz *et al*, 2006). However, when the onset of CRC is triggered by deficiency of the tumour suppressor APC, compound Tgfbr2 (Munoz *et al*, 2006) or Smad4 (Takaku *et al*, 2004), null alleles accelerate adenoma to carcinoma progression in the intestinal tract. Collectively, the data described above strongly support the notion that TGF-beta signalling suppresses CRC. This is in accordance with data obtained for solid tumours, such as breast cancer, prostate cancer and skin tumours, among others, which have lead to the general belief that TGF-beta acts as a tumour suppressor in the initial stages of carcinogenesis. However, several studies have suggested additional roles for TGF-beta in CRC progression. The expression of TGF-beta increases in late stage CRCs (Tsushima *et al*, 1996), and TGF-beta serum levels are associated with disease progression and predict recurrence and metastasis in CRC patients (Robson *et al*, 1996; Tsushima *et al*, 2001).

Our lab currently focuses on the role of TGF-beta signalling in CRC progression. For many years, tumorigenesis was studied from the perspective of tumour cells alone. Recently, much attention has been given to the contribution of the stromal component of solid tumours during disease progression. The tumour microenvironment is a complex mixture of cell types that includes fibroblasts, immune cells, blood vessels and a multitude of factors. The control of stromal changes within a developing tumour has become a major topic of research in oncology that has drawn the attention of some of the leading groups in cancer. We are studying the transcriptional events controlled by TGF-beta in CRC cells as well as in stromal cells. We have identified changes in approximately 500 genes in response to TGF-beta in intestinal fibroblasts and have studied the modulation of the stromal TGF-beta-controlled gene programme during CRC progression. Remarkably, the TGF-beta responding signature (TBRS) obtained from fibroblasts is differentially expressed between adenomas and adenocarcinomas, thereby implying that these

genes may contain the information that drives the adenoma/carcinoma transition.

Our lab is devoted to dissecting this information in order to identify TGF-beta genes that play an executive role in the adenoma/carcinoma transition. Overall, we are performing detailed analysis of the observed gain of function in TGF-beta signalling during CRC progression, particularly regarding the stromal component of the tumour (Figure 1). We are currently characterising TGF-beta target genes that show strong classifying capacity between adenomas and carcinomas present within the f-TBRS

that could have a potential role in tumorigenesis and metastatic dissemination of CRC. We are approaching this from a multi-disciplinary perspective, which includes the development of orthotopic models of colorectal tumours in nude mice to test the role of the TGF-beta-controlled gene signature, and screening for TGF-beta-regulated genes that are relevant for CRC by performing systematic shRNA-mediated down-regulation of genes contained in this signature. Moreover, we are developing animal models that will mimic the initial loss of TGF-beta signalling in CRC epithelial cells as well as a gain of function at later stages of the disease.

SCIENTIFIC OUTPUT

References

Biswas S, Chytil A, Washington K, Romero-Gallo J, Gorska AE, Wirth PS, Gautam S, Moses HL and Grady WM. Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. *Cancer Res,* 64(14), 4687-92 (2004)

Grady WM and Markowitz SD. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet*, 3, 101-28 (2002)

Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ and Clevers H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature Genet,* 19(4), 379-83 (1998)

Munoz NM, Upton M, Rojas A, Washington MK, Lin L, Chytil A, Sozmen EG, Madison BB, Pozzi A, Moon RT, Moses HL and Grady WM. Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. *Cancer Res,* 66(20), 9837-44 (2006)

Pinto D, Gregorieff A, Begthel H and Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev*, 17(14), 1709-13 (2003)

Robson H, Anderson E, James RD and Schofield PF. Transforming growth factor beta 1 expression in human colorectal tumours: an independent prognostic marker in a subgroup of poor prognosis patients. *Br J Cancer,* 74(5), 753-58 (1996)

Sancho E, Batlle E and Clevers H. Signalling pathways in intestinal cancer and development. *Annu Rev Cell Dev Biol*, 20, 695-23 (2004)

Shi Y and Massague J. Mechanisms of TGF-beta signalling from cell membrane to the nucleus. *Cell*, 113(6), 685-00 (2003)

Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF, and Taketo MM. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell*, 92(5), 645-56 (1998)

Tsushima H, Kawata S, Tamura S, Ito N, Shirai Y, Kiso S, Imai Y, Shimomukai H, Nomura Y, Matsuda Y and Matsuzawa Y. High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology*, 110(2), 375-82 (1996)

Tsushima H, Ito N, Tamura S, Matsuda Y, Inada M, Yabuuchi I, Imai Y, Nagashima R, Misawa H, Takeda H, Matsuzawa Y and Kawata S. Circulating transforming growth factor beta 1 as a predictor of liver metastasis after resection in colorectal cancer. *Clin Cancer Res*, 7(5), 1258-62 (2001)

Van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der HK, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den BM, Soete G, Pals S, Eilers M, Medema R and Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell*, 111(2), 241-50 (2002)

Collaborations

TGF-beta target genes in CRC
Giancarlo Marra, Institute of Molecular Cancer Research (Zurich, Switzerland)

TGF-beta target genes in CRC metastasis
Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Wnt signalling in CRC
Hans Clevers, Hubrecht Laboratory (Utrecht, The Netherlands)