



Signalling in intestinal stem cells and 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, causing around 650,000 deaths worldwide per year. The development of a full-blown malignant colorectal tumour is a slow process that often takes more than a decade. Most CRCs originate from pre-neoplastic lesions called adenomas that initially are benign and occur frequently. The transformation of an intestinal adenoma into an aggressive cancer requires the accumulation of multiple genetic alterations. The most common alteration in CRC is the inactivation of the Adenomatous Polyposis Coli (APC) tumour suppressor gene, which is a central component of the Wnt signalling pathway. Loss of APC function results in activation of Wnt signalling via constitutive transcription mediated by the β -catenin/Tcf complex. Alterations in APC (and less frequently in other Wnt pathway components) affect up to 80% of all types of neoplastic lesions in the intestine. Even the earliest precursors of intestinal tumours, the so-called dysplastic crypts, show mutational activation of the Wnt pathway. Remarkably, most β -catenin/Tcf target genes induced after APC mutations in intestinal cells were also found to be physiologically expressed in crypt intestinal stem cells (ISCs) and/or in transient amplifying progenitor cells (van de Wetering et al, 2002). Mice engineered to lack physiological Wnt signalling in the intestinal epithelium lose the crypt progenitor/stem cell compartment (Korinek et al, 1998). Conversely, constitutive activation of the Wnt pathway results in massive expansion of crypt progenitor/stem cell numbers in vivo and the onset of CRC (Sansom et al, 2004).

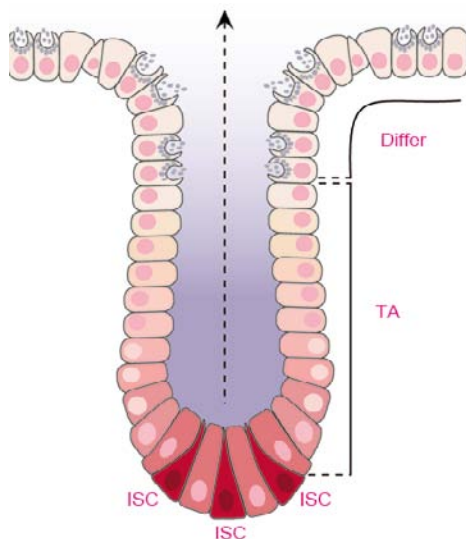


Figure 1. The mammalian intestinal epithelium. Organisation of a mammalian colon crypt. ISC; Intestinal stem cells, TA; Transient amplifying cells, and Differ; differentiated cells. Arrow indicates the direction on migration.

ISCs and CRC development

The columnar epithelium that lines the adult small intestine is shaped into glandular invaginations, called crypts, and finger-like protrusions known as villi. Hundreds of thousands of epithelial intestinal cells, generated daily at the base of the crypts, enter a migratory flow towards the tips of villi. Along their migratory path, intestinal cells differentiate into one of the four cell lineages characteristic of the intestine (enterocytes, enteroendocrine, goblet or Paneth cells). Differentiated cells exert their functions only for a few days as they are then shed into the lumen when they reach the villus tip. Self-renewing stem cells and Paneth cells reside at the crypt bottom and escape the upward migratory flow. The progenitors of differentiated cells, which arise from stem cell divisions, have limited self-renewing capacity and after a few rounds of mitosis, undergo lineage commitment and differentiation. While single crypts are fuelled by one stem cell clone and are monoclonal, each villus receives cells originating from different crypts, thereby explaining their polyclonal nature. The colonic epithelium has an overall structure similar to the small intestine but lacks villi. Differentiated cells are shed into the luminal space when they reach the surface epithelium. Colonic proliferative progenitor cells are lo-

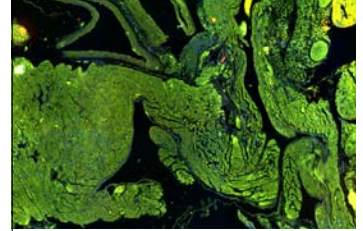
cated within the bottom two-thirds of the crypts, with stem cells residing at the bottom-most positions (Figure 1).

The location and the precise identity of mammalian ISCs are controversial issues because of the lack of specific marker genes and assays to study their properties (Batlle, 2008). To assess the gene programmes that operate in ISCs, we have recently developed a method to purify crypt cell populations. To this end, we used EphB2 as surface marker, a Wnt target gene that is expressed in gradient from the crypt base to the surface epithelium (Batlle *et al.*, 2002). FACS sorting of cells showing varying degrees of EphB2 expression has allowed us to obtain the expression profiles of ISCs, transient amplifying cells (TA) and differentiated cells. These profiles are instrumental tools to study the biology of crypt ISCs and also their participation in the initiation and progression of CRC. We are currently studying the role of several ISC-specific genes discovered in this screen (Figure 2). We have also performed extensive bioinformatic analysis of the expression profiles of our purified crypt cell populations versus several publicly available databases containing data from CRC patients. This has led to the discovery of an ISC gene signature that predicts poor outcome in CRC.

In addition to the work on mammalian ISCs, we are collaborating with Jordi Casanova and Andreu Casali (Developmental Biology Programme, IRB Barcelona) in the analysis of adult *Drosophila* ISCs. Recent studies have shown that in a similar fashion to the mammalian intestinal epithelium, Wnt and Notch signalling play essential roles in the specification and maintenance of midgut ISCs (reviewed in Casali and Batlle, 2009). We are currently investigating the extent to which the *Drosophila* midgut intestine represents a good model to study the role of ISCs in intestinal cancer.

***EphB* receptors as suppressors of CRC progression**

As target genes of the beta-catenin/TCF complex, EphB2 and EphB3 receptors are highly expressed in all early CRC lesions, such as dysplastic crypts and small adenomas. However, we observed that these receptors become down-regulated at the adenoma-carcinoma transition in virtually all patients analysed (>100). The level of EphB down-regulation strongly correlated with higher histological tumour grade, a parameter associated with malignancy. More importantly, by using transgenic and



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Elena Sancho's research group merged with Eduard Batlle's group in 2009.



knock-out animals, we engineered mice where APC mutations were placed in a background of low EphB activity in the intestine. These mice developed dozens of malignant tumours in the colon and the rectum, including invasive carcinomas. These findings thus demonstrate a causal role for EphB down-regulation in CRC progression. Thus, while progressing tumours maintain the beta-catenin/Tcf-imposed progenitor features as an essential component of their transformed phenotype, they are apparently selected to silence a subset of betacatenin/TCF target genes (*ie* EphB genes) to progress beyond the initial stages (Batlle *et al*, 2005).

Our research efforts address the mechanism of EphB-mediated tumour suppression in the intestine. We demonstrated that EphB signalling compartmentalises the growth of CRC cells *in vivo*. In *Apc*^{Min/+} mice, EphB⁺ tumour cells that form incipient adenomas are in continuous contact with normal intestinal epithelial cells expressing ephrinB ligands. Through the use of mice models deficient in EphB or ephrinB ligands, we demonstrated that *Apc* mutant tumour founder cells cannot colonise the regions of the normal epithelium that express high levels of ephrinB1, owing to EphB repulsive signals. We have proposed that tumour cell compartmentalisation may be a general mechanism of tumour suppression in tissues whose architecture is defined by Eph-ephrin interactions. Overall, our observations imply that fully malignant CRC cells bearing multiple mutations in oncogenes and tumour suppressors respect the boundaries imposed by EphB-ephrinB interactions (Cortina *et al*, 2007).

In this work we also generated *in vitro* models that mimic EphB/ephrinB interactions in CRC cell lines. We took advantage of CRC cell lines that do not express EphB receptors or ephrinB ligands to generate two populations of the same cell

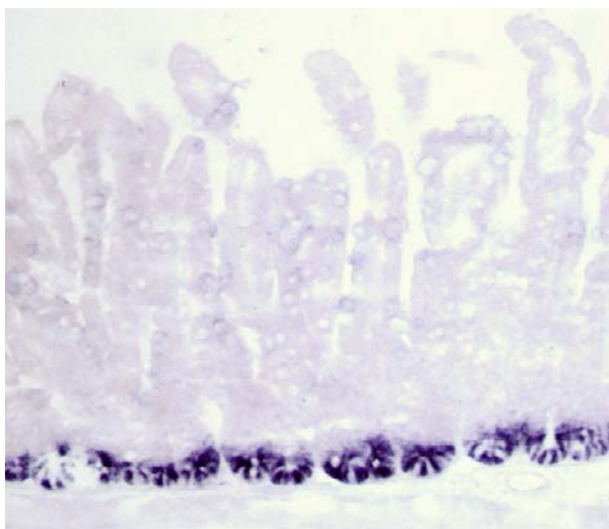


Figure 2. In situ hybridisation of a new ISC-specific gene, *OLFM4*. Cells detected by *OLFM4* probe at the crypt base (in blue) correspond to ISCs.

line expressing either EphB (plus GFP) or ephrinB (plus RFP) molecules. Co-culture of EphB- and ephrinB-expressing cells resulted in cell contact-mediated EphB-ephrinB bi-directional signalling. Analysis of cell dynamics in this *in vitro* model revealed that EphB signalling induces repulsion and compartmentalises the growth of CRC cells by enforcing E-cadherin adhesion (Cortina *et al*, 2007). We are currently exploring the mechanisms and cell dynamics downstream of EphB signalling by extensive usage of *in vitro* models and sophisticated microscopy methods. During 2009, we have identified the critical involvement of a metalloproteinase downstream of EphB-mediated cell sorting (manuscript in preparation).

TGF-beta signalling during CRC progression

One of the most prevalent mutations found during CRC progression are those that inactivate the TGF-beta signalling pathway (reviewed in Xu and Pasche, 2007). The TGF-beta pathway is involved in numerous processes in the 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 transphosphorylated 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 Massagué, 2008).

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%. 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*. However, when the onset of CRC is triggered by deficiency of the tumour suppressor APC, compound *Tgfr2* or *Smad4* 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 led to the general belief that TGF-beta acts as a tumour suppressor in the initial stages of carcinogenesis. However, several studies have proposed additional roles for TGF-beta in CRC progression (reviewed in Xu and Pasche, 2007). 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 non-epithelial component of solid tumours during disease progression. The tumour microenvironment is a complex mixture of cell types that includes fibroblasts, immune cells, blood ves-

sels and a multitude of factors. The knowledge and 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 worldwide. We are studying the transcriptional events controlled by TGF-beta in CRC epithelial cells and also in stromal cells. We have identified changes in approximately 500 genes in response to TGF-beta in CRC both in epithelial and stromal cells and have studied their modulation during CRC progression. Remarkably, the TGF-beta responding signature robustly classified benign and malignant colorectal tumours with 100% accuracy in unsupervised analysis. This finding implies that these genes may contain the information that drives the adenoma/carcinoma transition (Figure 3).

Our laboratory is dedicated to dissecting this information in order to identify TGF-beta genes that play an executive role in CRC progression. We are approaching this research from a multidisciplinary perspective that includes screening to identify TGF-beta regulated genes that are crucial for CRC progression, and the development of orthotopic models of colorectal tumours in nude mice that would be instrumental to investigate the role of the TGF-beta controlled gene signature.

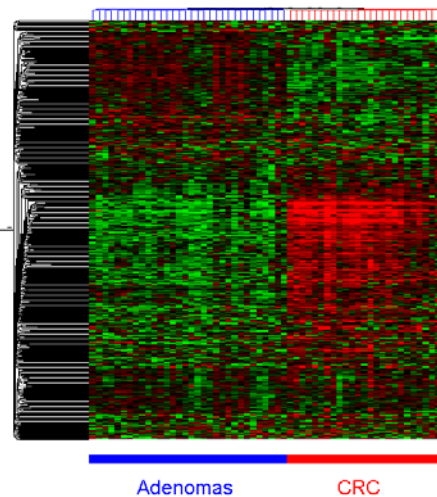


Figure 3. The TGF-beta signature discriminates between adenomas and carcinomas. Unsupervised clustering analysis of a collection of tumours of known transcriptomes, on the basis of target genes controlled by TGF-beta signalling, clearly classifies adenomas and carcinomas in two separate branches.

Scientific output

Publications

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Xu Y and Pasche B. TGF-beta signaling alterations and susceptibility to colorectal cancer. *Hum Mol Genet*, 16 Spec No 1, R14-20 (2007)

Research networks and grants

Biología del cáncer (ONCOBIO)

Spanish Ministry of Science and Innovation, CSD-2007-00017 (2007-2012)

Principal investigator: Eduard Batlle

Convenio de colaboración entre la Fundación Científica de la Asociación Española contra el Cáncer y la Fundación Privada Institut de Recerca Biomèdica en materia de ayudas para investigadores en el ámbito de la oncología

Spanish Association Against Cancer (2009-2010)

Principal investigator: Eduard Batlle

Dissecting the roles of the beta-catenin and Tcf genetic programmes during colorectal cancer progression

European Commission, ERC Starting Grant 208488 (2008-2013)

Principal investigator: Eduard Batlle

Estudio de la modulación del programa genético controlado por beta-catenina Tcf durante la progresión del cáncer colorrectal

'La Caixa' Foundation (2006-2009)

Principal investigator: Eduard Batlle

Señalización por Wnt, receptores Eph y cáncer de colon: un análisis funcional del inicio de la tumorigénesis intestinal

Spanish Ministry of Science and Innovation, SAF2008-01512 (2009-2011)

Principal investigator: Eduard Batlle

Collaborations

Common genes in pancreas cancer and CRC

Francisco X Real, Spanish National Cancer Research Center (Madrid, Spain)

Drosophila gut as a model for CRC development

Andreu Casali, IRB Barcelona (Barcelona, Spain)

Intestinal stem cells in CRC

Hans Clevers, Hubrecht Laboratory (Utrecht, The Netherlands)

Mediators of EMT in Drosophila and CRC

Jordi Casanova, IRB Barcelona (Barcelona, Spain)

Regulation of Wnt signalling pathway in CRC

Antonio García de Herreros, IMIM (Barcelona, Spain) and Mireia Duñach, Autonomous University of Barcelona (Barcelona, Spain)

Role of cdk6 in intestinal development

Mariano Barbacid and Marcos Malumbres, Spanish National Cancer Research Center (Madrid, Spain)

TGF-beta target genes in CRC

Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Awards and honours

Debiopharm Life Science award

École Polytechnique Fédérale de Lausanne (since 2006)

Awardee: Eduard Batlle