Wnt signalling, Eph/ephrin receptors and colorectal cancer

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Colorectal Cancer (CRC) is one of the leading causes of death by cancer worldwide, killing around 400,000 people each year. Most colorectal tumours develop as benign lesions but a small proportion progress to more malignant stages when the appropriate alterations in oncogenes and tumour suppressor genes occur. The final and deadliest step in CRC progression is the metastatic dissemination of colorectal cancer cells to other organs, mainly the liver. Our lab studies the initiation of CRC and its progression from the early stages to the formation of aggressive tumours. We develop and examine cell and animal models that mimic this devastating disease. The ultimate goal is to obtain information that allows us to design new therapeutic and diagnostic tools.

Colorectal Cancer initiation and Wnt signalling
Around 70 % of all CRCs show homozygous inactivation of the Adenomatous Polyposi Coli (APC) tumour suppressor gene. This genetic alteration results in the activation of the Wnt signalling pathway and the constitutive transcription by the β-catenin/Tcf complex. Loss of APC function is present throughout the sequence of intestinal carcinogenesis (*ie*, from benign adenomas to fully malignant CRC and metastasis). Even the earliest precursors of colorectal tumours, the so-called dysplastic crypts, show mutational activation of the Wnt pathway. In mice, activating mutations in Wnt signalling pathway components lead to the formation of dysplastic crypts and benign adenomas similar to the preneoplastic lesions developed by humans. Together, these observations have led to the notion that constitutive transcription by the β-catenin/Tcf complex is the key step for initiation of CRC (reviewed in Sancho *et al*, 2003; Sancho *et al*, 2004)

Given the relevance of Wnt signalling for CRC cancer, we undertook the identification of the gene programme targeted by β-catenin and Tcf in the intestine. We identified a set of 100 genes which expression was driven by the β-catenin/Tcf complex in CRC cells. We showed that this genetic programme is also expressed by normal progenitor and stem cells of the intestinal epithelium, which receive physiological Wnt signals. Since this seminal finding, a main objective of our research has been to decipher the instructions given by β-catenin/Tcf to intestinal progenitors and CRC cells. Our and other laboratories have shown that β-catenin/Tcf appears to dictate three different sets of instructions that collectively regulate the biology of normal and transformed intestinal cells.

The core module of instructions enforces the undifferentiated-proliferative phenotype of progenitor crypt cells. Mice genetically manipulated to block β-catenin/Tcf activity in the intestine lack proliferative progenitors (Korinek *et al*, 1998; Pinto *et al*, 2003). Conversely, APC deficiency in the intestinal epithelium leads to an enormous amplification of the progenitor compartment at expenses of the differentiated compartment (Sansom *et al*, 2004). This core set of instructions also determines the proliferative-undifferentiated phenotype of CRC cells.

The second module of the β-catenin/Tcf programme is necessary for Paneth cell maturation (van Es *et al*, 2005; Andreu *et al*, 2005). Paneth cells are a secretory cell type localised close to the bottom of the crypts in the small intestine that display prominent nuclear β–catenin localisation.

EphB receptors and cell positioning in the crypts
The third module of instructions controls the compartmentalisation of epithelial cells along the crypt axis and regulates their ordered migration (Batlle *et al*, 2002). The main effectors of this function are the β-catenin/Tcf targets EphB2 and EphB3, two members of the Eph family of receptors. Eph receptors comprise the largest subgroup of receptor tyrosine kinases characterised for binding to membrane tethered ligands known as ephrins (Pasquale, 2005). Eph receptors act as a pathfinding clues for migrating cells or in the establishment of boundaries between different cell populations during embryonic development (Pasquale, 2005). This function has been associ-

ated with their ability to provoke cell repulsion upon activation by ephrin ligands. In the intestine, β-catenin/Tcf drives the expression of EphB2 and EphB3 in epithelial cells located at the crypt base, *ie*, stem cells and their immediate descendants as well as Paneth cells. We demonstrated that EphB2/EphB3 double mutant mice show defects in cell positioning in the crypts that include loss of the boundary between the proliferative and differentiated compartments, abnormal migration of precursor cells along the crypt axis and lack of compartmentalisation of Paneth cells at the bottom of the crypts (Batlle *et al*, 2002).

In our laboratory at the IRB we are trying to understand the molecular basis of cell positioning mediated by EphB receptors in the intestinal epithelium. We have recently generated an *in vitro* model that mimics EphB-ephrinB interactions by epithelial cells. We took advantage of the fact that several cell lines do not express EphB or ephrinB molecules to generate two populations of the same cell line that express either EphB receptors (plus GFP) or ephrinB ligands (plus RFP). Co-culture of EphB and ephrinB-expressing cells resulted in cell-cell contact activation of EphB-ephrinB bi-directional signalling. Analysis of cell dynamics in this *in vitro* model has revealed that EphB signalling induces repulsion, restricts cell intermingling and compartmentalises the growth of epithelial intestinal cells (Figure 1, Palomo S, Cortina S, Gallego L, Humà M, Jonkheer S, Soriano P, Sancho E and Batlle E. EphB receptors suppress colorectal cancer by compartmentalising tumour cells, submitted). We are currently using this model to identify and dissect the functions of EphB downstream signalling.

EphB receptors and colorectal cancer progression

We initially suspected the role of EphB receptors as suppressors of CRC progression after analysing the β-catenin/Tcf target gene programme in a collection of human CRC samples at different stages of malignancy (Batlle *et al*, 2005). Dysplastic crypts and small adenomas retained expression of most β-catenin/Tcf targets present in crypt progenitors pinpointing a common tumour initiation mechanism through mutational activation of the Wnt signalling pathway. These initial lesions showed homogenous EphB2, EphB3 and EphB4 expression in all cells at equivalent levels to that of normal crypt progenitors. Strikingly, the majority of colorectal carcinomas contained more than 50% EphB receptor negative cells despite evident nuclear β-catenin localisation. (See Figure 2.)

Does loss of EphB expression confer any advantages to CRC cells? The results of our genetic experiments were unequivocal. We engineered mice where the APCmin mutation was placed in a genetic background with low EphB activity. $APC^{min/+}$ mice develop benign intestinal lesions such as dysplastic crypts and adenomas as a result of constitutive activation of the Wnt signalling pathway. In the absence of EphB activity, tumour progression in the large intestine of $APC^{min/+}$ mice is strongly accelerated resulting in the development of aggressive colorectal adenocarcinomas. Therefore, while constitutive activation of the Wnt signalling pathway is required for the initiation of tumourigenesis (transition from normal epithelium to early adenoma stage), not all the instructions codified within the β-catenin/Tcf crypt progenitor programme promote tumourigenesis. Rather, the module that specifies cell positioning seems to block tumour progression beyond the earlier stages.

Tumour compartmentalisation: a new mechanism of tumour suppression?

At the onset of tumourigenesis, APC mutant-cells are confined to the epithelium within the so-called dysplastic crypts. These tumour-founder cells expand laterally and repopulate the surrounding crypts with their mutant descendants. It is during this initial phase that $EphB_+$ tumour cells are continuously in contact with normal epithelial cells expressing ephrinB ligands. An attractive hypothesis that we are currently testing is that tumour cells are forced to respect the boundaries imposed by EphB-ephrinB interactions much like normal progenitors and paneth cells are compartmentalised in the healthy tissue. Silencing of EphB receptors would generate a subset of tumour cells with unrestricted capacity for repopulating the epithelium. Alternatively, EphB receptors could also suppress tumour progression in more advanced stages. Thus, a fundamental issue is to identify the $ephrinB^+$ territories that restrict the colonisation of $EphB^+$ tumour cells during CRC progression. We have analysed the expression patterns of all ephrin ligands in a panel of intestinal tumour samples from patients. We have identified four potential sites of ephrinB-EphB interactions; the normal epithelium, mesenchyme, blood vessels and

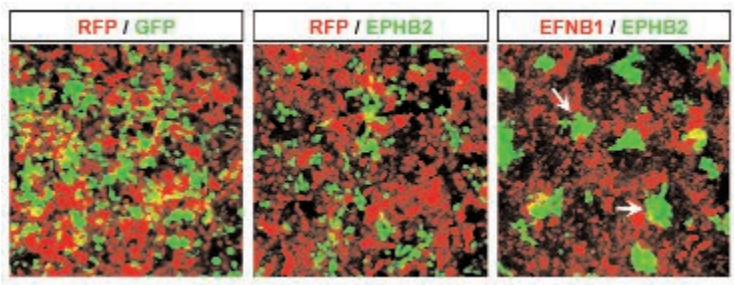

***Figure 1**. Examples of co-cultures of epithelial cells infected with lentivirus bearing GFP, EphB2-GFP or ephrinB1-RFP cDNAs. A dramatic cell sorting and compartmentalisation occurs in EphB-GFP/ephrinB1-RFP co-cultures (arrows).*

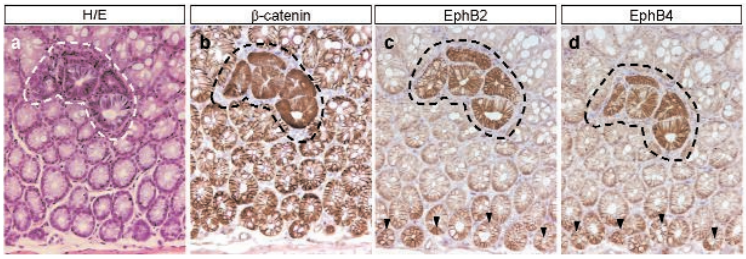

Figure 2. An early colorectal adenoma (dashed line) showing prominent nuclear β-catenin (b) accumulation and expression of EphB receptors (c and d). The arrows indicate normal crypt progenitor cells which also stain positive for EphB receptors.

lymph nodes. We are studying the relevance of the different EphB-ephrinB interactions for tumour progression by generating *in vitro* and *in vivo* models that mimic these scenarios.

The mechanism of EphB silencing during tumour progression

While genetic alterations or epigenetic silencing in individual EphB genes have been identified in a fraction of CRCs, our observations indicate that EphB2, EphB3 and EphB4 are co-ordinately silenced in the majority of CRC samples (Batlle *et al*, 2005). Thus, it is unlikely that mutations, LOH or methylation account for the coordinated silencing of all three EphB genes during CRC progression. In the great majority of cancers downregulation of EphB receptors occurs at the mRNA level (Batlle *et al*, 2005). EphB expression is fully dependent on β-catenin/Tcf activity yet many CRCs and cell lines downregulate EphB levels despite constitutive activation of the Wnt signalling pathway. Together, these observations point to a common mechanism of transcriptional silencing of EphB genes that acts in a dominant fashion over β-catenin/Tcf activation. By profiling $EphB^+$ and $EphB^-$ CRC cells, we have identified several genes that are bona-fide regulators of EphB expression during CRC progression. We have obtained evidences that these genes represent a complex genetic network regulating the adenoma-carcinoma transition. We are currently addressing the specific roles of these candidate genes in the acquisition of malignancy.

EphB receptors as markers of malignancy

Epithelial tumour cells that compose benign colorectal lesions such as small adenomas stain homogenously positive for EphB2, EphB3 and EphB4 receptors. Lesions at the adenoma-carcinoma transition (*ie*, large adenomas) contain clusters of tumour cells negative for EphB receptors expression. In carcinomas, the $EphB6^-$ population occupies the majority (>50%) of the tumour mass (Batlle *et al*, 2005). The increase in frequency of $EphB^-$ tumour cells is strongly associated with malignancy. $EphB^-$ cells are preferentially associated with poorly differentiated areas and invasion fronts of carcinomas while $EphB^+$ cells are organised in well-differentiated regions. As adenomas represent the benign precursors of carcinomas and tumours of higher grade often behave more aggressively than low grade ones, our observations implied that silencing of EphB expression has occurred in a subset of tumour cells concomitantly with the acquisition of malignancy. Our laboratory is currently testing whether the ratio of $EphB^+$ and $EphB^-$ cells in a tumour predicts clinical outcome of CRC patients. Also, we are using the gene expression signature obtained from $EphB^+$ and $EphB^-$ CRC cells to classify tumour samples from patients.

PUBLICATIONS

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RESEARCH NETWORKS AND GRANTS

Characterization of the mechanism controlling Intestinal Stem Cell specification and positioning. Use of intestinal Stem cells for tissue regeneration.
European Union FP6 - MERG-CT-2004-006329:2005
Principal Investigator: Eduard Batlle

Papel de los Receptores EPHB en el posicionamiento de las células epiteliales y en el cancer colorectal (The role of EphB receptors in intestinal cell positioning and colorectal cancer)
Ministerio de Eduación y Ciencia (SAF2005-04981):2006-2008
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Agencia de Gestió d'Ajuts Universitaris i de Recerca (Catalan Government) (2005SGR 00775): 2006-2009
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AWARDS

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