EDUARD BATLLE

Wnt signalling and EphB-ephrin interactions in intestinal stem cells and CRC progression

A wealth of data has revealed an essential role for Wnt signalling in the maintenance of mammalian intestinal stem cells (ISCs) as well as in colorectal cancer (CRC; reviewed in Van der Flier and Clevers, 2008). Mice engineered to lack Wnt signalling in the intestinal epithelium lose the crypt progenitor compartment. Conversely, constitutive activation of the Wnt pathway results in a massive expansion of crypt progenitor/stem cell numbers and the onset of intestinal tumorigenesis. Most human CRCs are initiated by mutations in the tumour suppressor gene APC, which switches on the Wnt pathway in a constitutive fashion. Remarkably, most Wnt target genes induced by APC mutations in intestinal tumours are physiologically expressed in crypt ISCs and/or in transient amplifying progenitor cells. Recently, the tumour-initiating potential of several crypt cell populations has been assessed (Barker *et al*, 2008). These studies have revealed that deletion of the APC gene in mouse ISCs triggers tumour formation with high efficiency whereas transient amplifying progenitor cells or differentiated cells are relatively resistant to Wnt-driven transformation. Thus, it appears that some specific features of ISCs are required to initiate CRC. Overall, these data have led to the notion that Wnt signalling sustains the expression of the crypt ISC gene programme, which, upon mutational activation of the Wnt pathway, is constitutively imposed on tumour-initiating cells (*ie*, on stem cells and perhaps also on early progenitor cells; Van de Wetering *et al*, 2002; Barker *et al*, 2007; Van der Flier and Clevers, 2008).

The functional analysis of Wnt target genes identified in CRC cell lines revealed that beta-catenin/Tcf dictate different sets of instructions that collectively regulate the biology of crypt progenitor/stem cells and also intestinal tumours. So far, three independent gene modules have been identified (reviewed in Batlle and Clevers, 2006):

- The core module enforces the undifferentiated-proliferative phenotype of crypt progenitor/stem cells and of CRC cells. Blockage of beta catenin/Tcf-mediated transcription in fully malignant CRC cell lines results in cell cycle arrest and differentiation. Strong evidence suggests that c-Myc acts as master regulator within this gene module.

- The second module of the beta-catenin/Tcf programme is required for Paneth cell maturation. Paneth cells are a secretory cell type localised close to the bottom of the crypts that receive physiological Wnt signals.

- The third module controls the positioning of epithelial cells along the crypt axis and regulates their ordered migration. The main effectors of this function are the beta-catenin/Tcf targets EphB2 and EphB3, two members of the Eph family of receptor tyrosine kinases. Eph receptors and their cognate ligands, ephrins, are well-known mediators of cell repulsion during embryo development. We demonstrated that EphB2/-B3-deficient mice show a range of defects in intestinal cell compartmentalisation, including the loss of the tight boundary between the differentiated and proliferative cell compartments, abnormal migration of progenitor cells along the crypt axis and mispositioning of Paneth cells (Batlle *et al*, 2002).

EphB receptors as suppressors of CRC progression

Most early CRC lesions in humans showed homogenous EphB2, EphB3 and EphB4 expression as a result of mutational activation of the Wnt pathway. Strikingly, we observed that the expression of all three EphB receptors is silenced around the adenoma-carcinoma transition in most intestinal tumours. We proved a causal role for EphB silencing in CRC progression by engineering mice in which the *Apc*^{min} mutation was placed in

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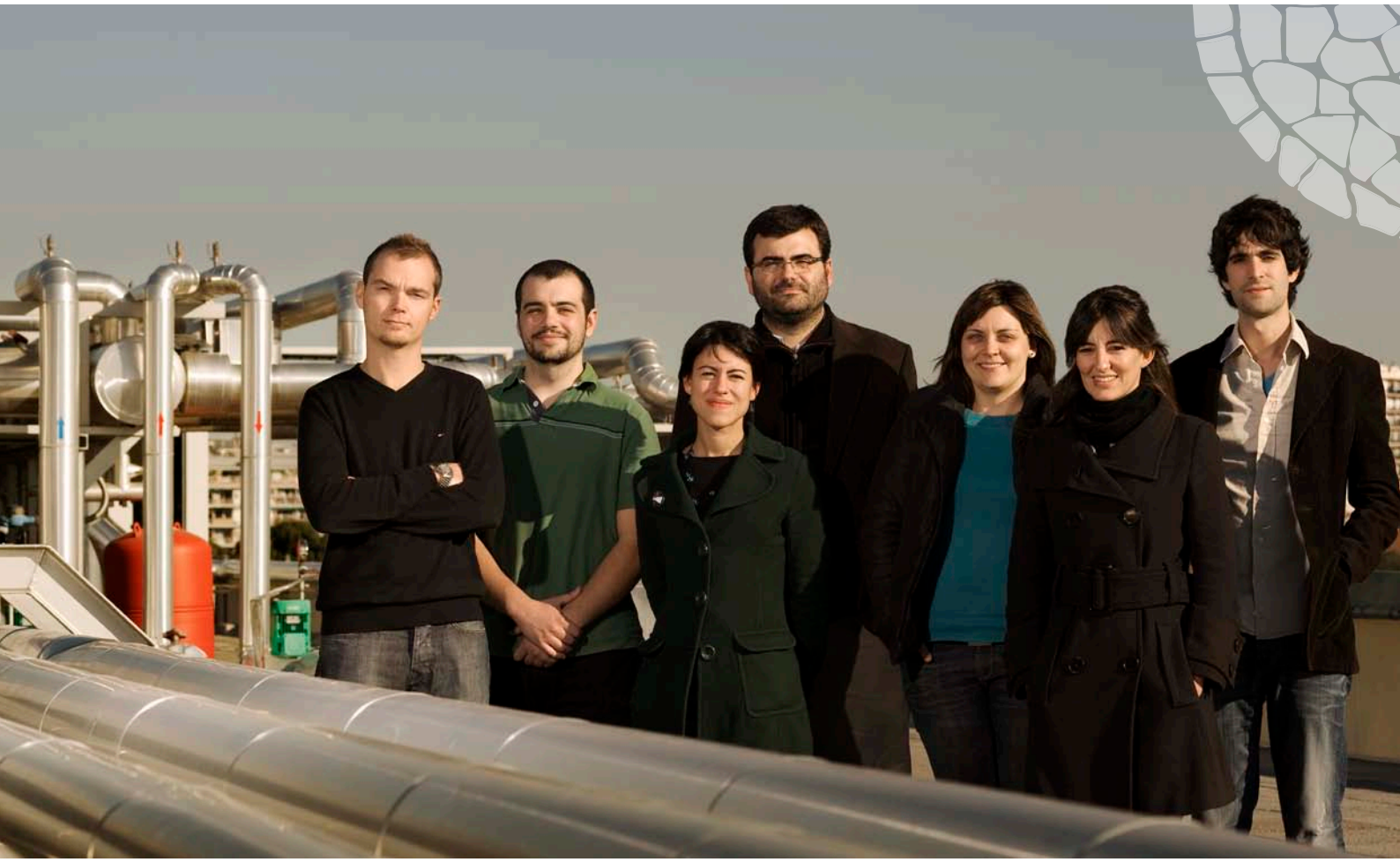

a genetic background with low EphB activity. In the absence of EphB activity, tumour progression in the large intestine of *Apc*$^{min/+}$ mice is strongly accelerated, resulting in the development of aggressive colorectal adenocarcinoma (Batlle *et al*, 2005). Thus, over several years of malignization, the original tumour-initiating gene programme is refined. While CRC cells retain certain characteristics of progenitor/stem cells, not all the instructions codified by the beta-catenin/Tcf4 programme promote tumorigenesis. Instead, some of these instructions, such as the positional information imposed by EphB receptors, block the acquisition of malignancy beyond the earlier stages.

Over the last three years, our lab has studied the mechanism of EphB-mediated tumour suppression in the intestine. We have generated *in vitro* models that mimic EphB/ephrinB interactions in CRC. We have taken advantage of CRC cell lines that do not express EphB receptors or ephrinB ligands to generate two populations of the same cell line that express 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.

We also demonstrated that tumour compartmentalisation occurs *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 mouse models deficient in EphB or ephrinB ligands, we showed that *Apc* mutant tumour founder cells cannot colonise the regions of the normal epithelium that express high levels of ephrinB1 because of EphB repulsive signals. We have proposed

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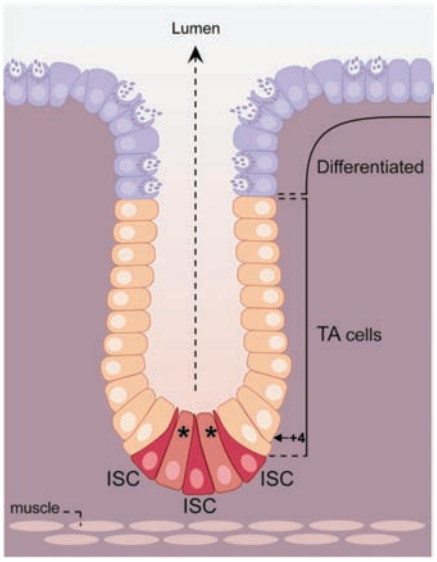

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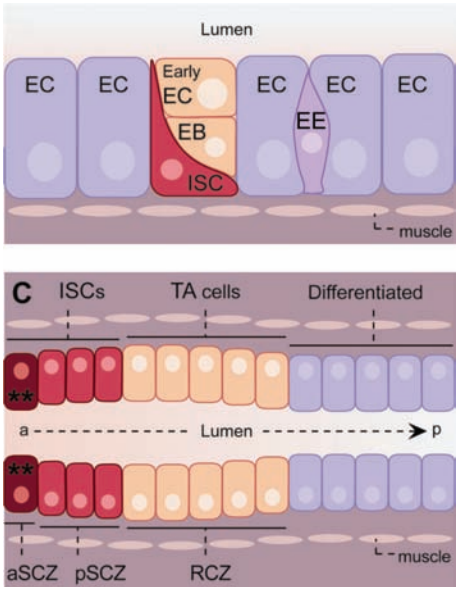

Figure 1. *The mammalian and Drosophila intestinal epithelium. Organisation of a mammalian colon crypt (a), the Drosophila midgut (b) and hindgut (c) epithelium. The colour code is kept in the three schemes to indicate identity between cell types, ISCs being shown in red, progenitor cells in light orange and differentiated cells in blue. Cells intermingled between ISCs in A (labelled with asterisks) represent Paneth cells in the small intestine or other secretory types in the colon which localise at the crypt base. In the fly hindgut, ISCs marked with two asterisks are Wg-secreting cells that localise in the anterior region of the Spindle Zone. Arrow indicates the direction of migration and cell renewal, ie, from the base towards the lumen in crypts, or in an anterior (a) posterior (p) fashion in the hindgut. ISC; intestinal stem cells. TA; transient amplifying. EB; enteroblast. EC; enterocyte. EE; enteroendocrine cells. aSCZ and pSCZ; anterior or posterior Spindle Zone respectively. RCZ; Round Cell Zone.*

that tumour cell compartmentalisation is 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).

Intestinal stem cells

In mammals, the inner lining of the intestinal tube is a monostratified epithelium folded into millions of invaginations known as crypts (Figure 1a). Each crypt represents a cell production factory that contributes to the constant renewal of the epithelial layer. The tremendous regenerative power of the mammalian intestinal epithelium is reflected by the magnitude of cell production in the crypts: the small intestine of an adult mouse contains roughly a million crypts, each producing around 300 cells per day, which together generate an estimated 300 million cells every day of their life.

This remarkable process is ultimately sustained by a small population of stem cells (4-6 cells) that reside at the base of each crypt. The progeny of mammalian ISCs does not differentiate immediately but rather it is amplified by cell division during a process of continuous upward migration along the crypt axis. Around 150 undifferentiated cycling progenitor cells or Transient Amplifying (TA) cells occupy the crypt length. Progenitor cells divide with fast kinetics (about 1 division every 12 hours). Cell cycle arrest and functional differentiation occur as migrating TA cells reach the upper part of the crypt. Three differentiated cell types populate the intestinal tract; mucosecreting, enteroendocrine and absorptive cells. The small intestine contains an additional secretory cell type, Paneth cells, which localise at the bottom-most positions of the crypt (Figure 1).

The location and the precise identity of mammalian ISCs are controversial issues due to the lack of specific marker genes and assays to study their properties (reviewed in Batlle *et al*, 2008). In a pioneering study, Hans Clevers and colleagues have recently identified bona-fide gastrointestinal stem cells at the bottom-most positions of the stomach, small intestine and colon crypts (Barker *et al*, 2007). ISCs in the intestinal tract can be specifically recognised by the expression of Lgr5, a Wnt-target gene that codifies for an orphan G-protein coupled receptor of unknown function. Lgr5+ ISCs are multipotent, divide approximately once every day and are capable of regenerating the intestinal epithelium for long periods (>12 months).

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 a surface marker, a Wnt target gene expressed in gradient from the crypt base to the surface epithelium (Batlle *et al*, 2002). FACS sorting of cells expressing different levels of EphB2 has allowed us to obtain the expression profiles of ISCs (Figure 2), TA cells and differentiated cells. These expression profiles are instrumental tools to understand the biology of the crypt ISCs as well as their role in the initiation and progression of CRC.

In addition to the work on mammalian ISCs, we have recently

started a collaboration project with Jordi Casanovas and Andreu Casali (IRB Barcelona) to analyse adult ISCs in *Drosophila*. The stem cells of the *Drosophila* midgut lie in a basal position relative to the rest of the epithelial cell types and show a wedge-like morphology that resembles that of mouse Lgr5+ cells (Figure 1b). Unlike in mammals, midgut ISCs are the only known cell type in the posterior midgut that proliferates, as their progeny is not further amplified. Upon cell division, the descendants of midgut ISCs regenerate the stem cell pool and/or become quiescent progenitor cells (known as enteroblasts or EB cells), which ultimately differentiate to absorptive (AC) or enteroendocrine (EE) cells (reviewed in Casali and Batlle, 2009).

Recent work has shown that, in a similar fashion to that of the mammalian intestinal epithelium, Wnt and Notch signalling play essential roles in the specification and maintenance of midgut ISCs in *Drosophila* (reviewed in Casali and Batlle, 2009). We are currently examining the extent to which the *Drosophila* midgut intestine represents a good model to study the role of ISCs in intestinal cancer.

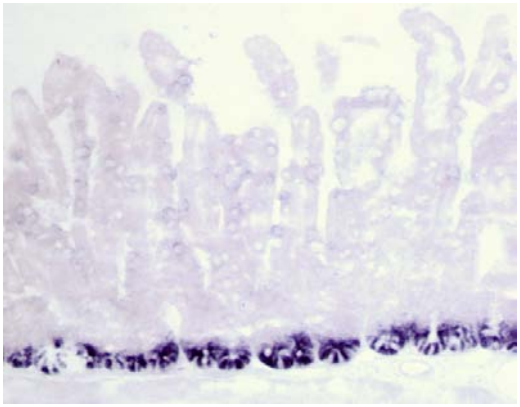

Figure 2. *In situ hybridisation of an ISC marker gene, OLFM4. Cells detected by OLFM4 probe at the crypt base (in blue) correspond to ISCs.*

SCIENTIFIC OUTPUT

Publications

Batlle E. A new identity for the elusive intestinal stem cell. *Nat Genet*, **40**(7), 818-19 (2008)

Casali A and Batlle E. Intestinal stem cells in mammals and *Drosophila*. *Cell Stem Cell,* in press (2008)

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