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

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Eph receptor tyrosine kinases and their ligands, ephrins, play key roles in the regulation of migration and cell adhesion during development, thereby influencing cell fate, morphogenesis and organogenesis. Recent findings suggest that Eph signalling also controls the architecture and physiology of several tissues in the adult body under normal and pathological conditions, such as cancer. Major research efforts in our laboratory in recent years have focused on elucidating the role of Eph-ephrin signalling in the intestinal epithelium and colorectal cancer (CRC).

Eph receptors in intestinal cell positioning

Eph receptors constitute the largest subfamily of transmembrane tyrosine kinase receptors described to date, with 14 members identified in mammals. Their ligands, the ephrins, are membrane-anchored proteins which are grouped into two subclasses: type-A ephrins (ephrinA1-ephrinA6), which are attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor, and type-B ephrins (ephrinB1-ephrinB3), which contain transmembrane and intracellular domains (Figure 1). Depending on their sequence similarity and on their affinity for ephrins, Eph receptors are also classified into two groups. In general, EphA receptors (EphA1-EphA10) bind ephrinAs and EphB receptors (EphB1-EphB6) bind ephrinBs, yet promiscuity in their binding specificities has been described for some members. Upon cell-to-cell contact and ligand-receptor engagement, intracellular signalling is induced in a bidirectional fashion: 'forward signalling' starts in receptor-expressing cells, while 'reverse signalling' initiates in cells expressing the corresponding ligand (Figure 1). Eph-ephrin signalling regulates a number of cellular events during embryonic development such as cell migration, repulsion vs adhesion, and cell-to-cell communication. Most of these responses are achieved through the capacity of Eph-ephrin signalling to regulate actin cytoskeleton dynamics (reviewed in Pasquale, 2005).

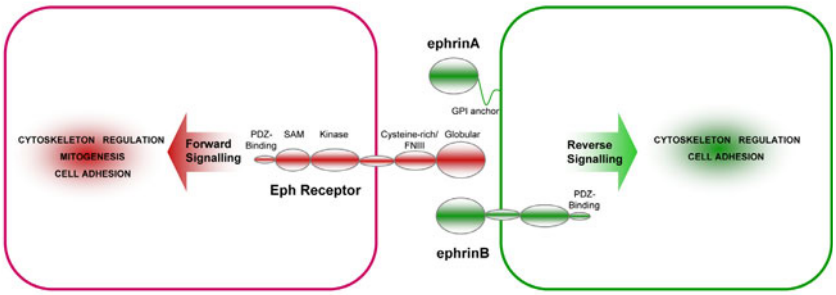

***Figure 1**. Eph receptors and ephrins. Eph receptors are tyrosine kinase transmembrane molecules. Their extracellular domain consists of a ligand-binding globular region, a cysteine-rich domain and two fibronectin type III (FNIII) repeats. The tyrosine kinase domain, the sterile-alpha motif (SAM) and the PDZ-binding motif are located in the intracellular region and are responsible for the interaction with downstream effector molecules, which regulate events such as cytoskeleton dynamics, cell proliferation or cell adhesion. Ephrins are also plasma membrane-anchored molecules with an extracellular Eph-binding domain. EphrinAs are tethered to the cell surface through a glycosylphosphatidylinositol (GPI) anchor, while ephrinBs have an intracellular domain, containing a PDZ-binding motif which allows interaction with proteins affecting cytoskeleton organisation and cell adhesion.*

Our laboratory pioneers the study of Eph signalling in the intestinal epithelium. The innermost layer of the intestinal tube is a mono-stratified epithelium which is folded into millions of bag-shaped invaginations called crypts. At the base of each crypt reside a handful of exceptionally active stem cells that constantly regenerate the epithelium (Barker *et al*, 2007). Cell renewal is accomplished in a bottom-up fashion. Intestinal Stem Cells (ISCs) continuously regenerate three cell lineages that coexist in the small intestine and colon (ie, mucosecreting, absorptive and enteroendocrine cells). These cell types migrate towards the lumen as they undergo terminal differentiation. The small intestine contains a fourth epithelial cell type, Paneth cells (PCs), that escape from this migratory flow and remain at the base of the crypts as mature cells. The main driving force behind intestinal cell renewal is Wnt signalling. A still uncharacterised source of Wnt factors present at the base of each

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crypt promotes beta catenin/Tcf-driven transcription in cells localised within this niche, ie, in ISCs and PCs. Blockage of Wnt signalling in the gut results in loss of the progenitor compartment and defects in PC maturation whereas constitutive activation of the Wnt pathway causes the expansion of the crypt progenitor compartment and the onset of tumorigenesis (reviewed in Clevers and Batlle, 2006). We originally showed that the expression of EphB2 and EphB3 in crypts is driven by Wnt signalling (Batlle *et al*, 2002). EphB2 is present at highest levels in ISCs and its expression gradually decreases in progenitor cells as they migrate towards the lumen (Figure 2). EphB3 displays a restricted localisation in cells present at the bottom-most positions of the crypts (ie, PCs, ISCs and probably early progenitors). Conversely, the expression of ephrinB1 and ephrinB2 is negatively controlled by beta-catenin/Tcf activity and these isoforms show the highest expression levels in differentiated cells (Batlle *et al*, 2002). In the intestine of EphB3 null mice, localisation of PCs is no longer restricted to the crypt base, instead this cell type migrates upwards and is found dispersed throughout the epithelium (Batlle *et al*, 2002). We have recently demonstrated that this phenotype is also observed in intestine specific conditional ephrinB1 knockout mice (Cortina *et al*, 2007). In double EphB2-/-; EphB3-/- mice, the boundary between the proliferative and differentiated cell compartments is lost and crypt cells intermingle instead of undergoing unidirectional upward migration (Batlle *et al*, 2002). Thus, EphB/ephrinB signalling is required to establish cell compartments and to organise ordered migration of

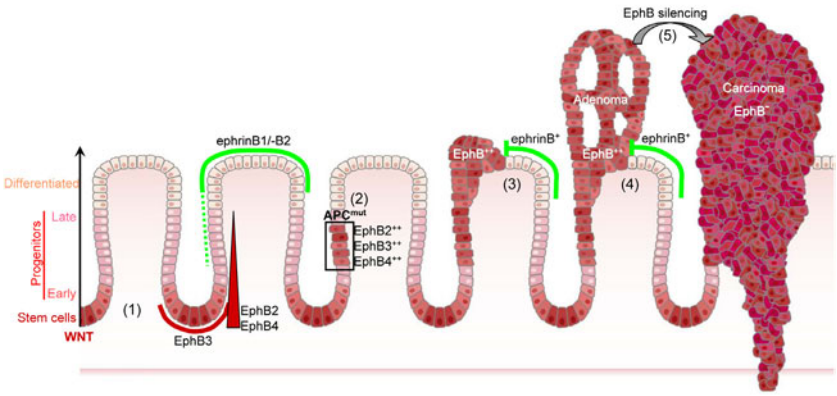

Figure 2. *EphB-ephrinB interactions during CRC progression. (1) Expression domains of EphB and ephrinB genes in the colon. Wnt signalling occurs at the bottom-most positions of the crypts. (2) Mutations in the tumour suppressor gene APC activates the Wnt pathway and transforms intestinal epithelial cells into tumour-initiating cells (cells within the square). As a result of constitutive beta-catenin/Tcf activity, Apc mutant cells express high levels of EphB2, EphB3 and EphB4 receptors. (3) Tumour-initiating cells acquire stem cell properties and repopulate the crypts with their mutant descendants until they reach the surface epithelium. There, tumour cells accumulate and form benign polyp-like outgrowths known as adenomas. Contact of tumour cells with normal differentiated cells that express high levels of ephrinB ligands results in the activation of EphB signalling. (4) Expansion of adenomas is blocked by EphB repulsive signals that limit the spread of tumour.*

epithelial cells along the crypt axis. Furthermore, EphB2 and EphB3 receptors are also expressed in ISCs. As a follow up of this work, our laboratory is currently attempting to provide evidence that EphB signals may also be required for stem cell retention within the crypt niche.

Eph signalling in CRC progression

Most colorectal tumours are initiated by mutations in the Wnt pathway which lead to the activation of the beta-catenin/Tcf complex in intestinal epithelial cells (reviewed in Clevers and Batlle, 2006; Sancho *et al*, 2004). We originally proposed that mutational activation of the Wnt pathway imposes a constitutive crypt progenitor phenotype on early tumour cells (van de Wetering *et al*, 2002). As a result, intestinal adenomas, the precursors of CRC, are characterised by high expression of crypt Wnt target genes, including EphB2 and EphB3 receptors (Batlle *et al*, 2005). However, as adenomas become aggressive, the expression of EphB receptors is silenced despite the persistence of Wnt pathway mutations (Batlle *et al*, 2005; Figure 2). We demonstrated through genetic analysis in mouse models that the combination of Apc mutations with blockage of EphB activity promotes the formation of aggressive colorectal tumours (Batlle *et al*, 2005; Cortina *et al*, 2007). Thus, EphB signalling blocks the acquisition of malignancy during CRC progression. The mechanism responsible for EphB silencing in malignant colorectal tumours remains unknown; however, promoter methylation as well as point mutations in EphB2 and EphB4 genes have been found in some cases. Our group performs intensive research into the regulation of the EphB3 gene. We have identified an enhancer region which directs EphB3 expression in CRC cells. We are currently screening for transcription factors and signalling pathways that regulate the activity of this promoter.

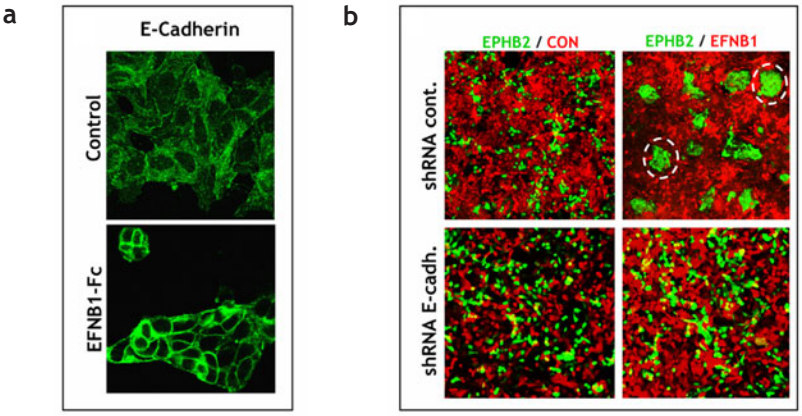

***Figure 3**. E-cadherin adhesion is essential for EphB-mediated tumour cell compartmentalisation. (a) EphB activation results in fast cell contraction, aggregation and re-localisation of E-cadherin to the membrane of DLD-1 EphB2-expressing cells. Activation of EphB signalling was achieved by treatment with ephrinB1/Fc fusion protein (EfnB1-Fc) for 1 h. (b) Co-culture of EphB2-GFP-expressing cells with control-RFP results in cell intermingling between both populations (top left). However, ephrinB1-RFP cells restrict the growth of EphB2-GFP cells to compact clusters (top-right panel; dashed circles). Down-regulation of E-cadherin levels by shRNA inhibits EPHB-mediated cell compartmentalisation (bottom-right).*

A major achievement in this period has been the identification of the mechanism by which EphB suppresses CRC progression (Cortina *et al*, 2007). We generated *in vitro* models that mimic EphB/ephrinB interactions in CRC. We took advantage of fully malignant 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 (Figure 3). More importantly, *in vivo*, EphB+ tumour-initiating cells become compartmentalised upon contact with normal intestinal cells that express high levels of ephrinB1, thus limiting the space available for adenoma expansion (Figure 2). Overall, our experiments also demonstrate that even fully malignant CRC cells bearing multiple mutations in oncogenes and tumour suppressors respect the boundaries imposed by EphB-ephrinB interactions. We proposed that tumour cell compartmentalisation is a general mechanism of cancer suppression in tissues whose architecture is defined by Eph-ephrin interactions.

Publications

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Research Networks and Grants

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Research Directors: Eduard Batlle and Elena Sancho

Papel de los receptores EPHB en el posicionamiento de las células epiteliales y en el cáncer colorectal
Ministerio de Educación y Ciencia, SAF2005-04981: 2005-2007
Research Director: Eduard Batlle

The modulation of the beta-catenin/Tcf genetic programme during CRC progression
Fundació La Caixa, Biomedical Research Projects — Oncology: 2007-2009
Research Directors: Eduard Batlle and Elena Sancho

Collaborations

A role for TGF-beta in CRC progression
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Control of intestinal stem cell positioning
Hans Clevers, Hubrecht Laboratorium (Utrecht, The Netherlands)

Eph signalling in pancreas development
Francisco X Real, Institut d'Investigació Mèdica (Barcelona, Spain)

Isolation of colorectal cancer stem cells using Wnt target genes
Gabriel Capellà, Institut Català d'Oncologia (L'Hospitalet de Llobregat, Spain)

Stem cell gene expression signatures in the prediction of CRC outcome
José Baselga, Vall d'Hebrón Hospital (Barcelona, Spain)

The genetic programmes linked to EphB down-regulation during CRC progression
Giancarlo Marra, Institute of Molecular Cancer Research, University of Zurich (Zurich, Switzerland)

The role of c/EBP isoforms in intestinal development and cancer
Claus Nerlov, European Molecular Biology Laboratory, Monterotondo (Rome, Italy)