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Research article

Eph receptor interclass cooperation is required for the regulation of cell proliferation

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ABSTRACT

Cancer often arises by the constitutive activation of mitogenic pathways by mutations in stem cells. Eph receptors are unusual in that although they regulate the proliferation of stem/progenitor cells in many adult organs, they typically fail to transform cells. Multiple ephrins and Eph receptors are often co-expressed and are thought to be redundant, but we here describe an unexpected dichotomy with two homologous ligands, ephrin-B1 and ephrin-B2, regulating specifically migration or proliferation in the intestinal stem cell niche. We demonstrate that the combined activity of two different coexpressed Eph receptors of the A and B class assembled into common signaling clusters in response to ephrin-B2 is required for mitogenic signaling. The requirement of two different Eph receptors to convey mitogenic signals identifies a new type of cooperation within this receptor family and helps explain why constitutive activation of a single receptor fails to transform cells.

1. Introduction

Eph receptors, the largest family of tyrosine kinase receptors, regulate the proliferation and positioning of stem and progenitor cells in many adult organs and are implicated in several aspects of tumorigenesis, making them attractive therapeutic targets in both regenerative medicine and cancer [8,24]. Eph receptors interact with membrane bound ephrin ligands at direct cell contacts. In the absence of cell-cell interactions, Eph receptors and ephrins are loosely pre clustered in lipid rafts and form low-affinity Eph-Eph and ephrin-ephrin complexes [29], which become more compact and well-organized when Eph-ephrin heterotetramers and oligomers assemble to form signaling centers [25]. Eph receptors and ephrins can each be divided into two subclasses, A and B, based on sequence homology and binding preference. A class ephrins bind to EphA receptors and B class ephrins engage EphB receptors, with the exceptions that EphA4 in addition binds ephrin-B2 and-B3, and EphB2 binds ephrin-A5 [7,12].

Eph receptors and ephrins are expressed in counter gradients along the crypt-villus axis in the intestine, with high levels of EphB2 and EphB3 in stem and progenitor cells at the bottom of the crypt and high ephrin-B1 and -B2 expression in differentiating cells [1,13]. Mice lacking EphB2 and EphB3 display reduced progenitor cell proliferation [13] and distorted cell positioning [1,13]. Proliferation is mediated by an Eph receptor kinase dependent signaling cascade, via Abl and cyclin D1, whereas EphB mediated cell positioning is kinase independent and mediated via PI3K [9].

Tyrosine kinase receptors typically convey mitogenic signals and are often proto oncogenes. Eph receptors are atypical in that they convey mitogenic signals in some situations, but not in most, and they are seldom transforming. Moreover, even in situations where Eph receptors have strong mitogenic activity they may not be transforming. For example, Eph receptor signaling accounts for approximately one third of the mitogenic activity in the intestinal epithelium, as its blocking (acutely or in knock out mice) causes 30% reduction of cell proliferation (with Wnt signaling unaffected; [13]). Despite this, activating Eph receptor mutations have not been identified in intestinal tumorigenesis and mice engineered to carry activating EphB mutations only display a modest increase in

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cell proliferation [13]. This is in stark contrast to the Wnt/ß-catenin signaling pathway, a key mitogenic pathway in the normal epithelium, which activity is increased by activating mutations in almost all intestinal cancers [5]. It has been unclear why Eph receptors, in contrast to most tyrosine kinase receptors, only convey mitogenic signals in some situations and why they typically are not transforming.

Multiple ephrins and Eph receptors are typically coexpressed in tissues and are often redundant. We have explored the role of individual ephrins and Eph receptors in the intestinal stem cell niche to gain better understanding of their regulation of cellular homeostasis. Surprisingly, we found that ephrin-B1 and ephrin-B2 have largely non-overlapping and unique functions. Ephrin-B2 regulates cell proliferation whereas ephrin-B1 alone controls cell positioning within the stem cell niche. We report that EphA/B receptor interclass cooperation in response to ephrin-B2 binding is obligatory to convey mitogenic signals. The need for cooperation by the two different Eph receptor subclasses help explain why these receptors do not convey mitogenic signals in most situations and why individual Eph receptors fail to transform cells.

2. Results

2.1. Ephrin-B1 and ephrin-B2 have unique functions in the intestinal stem cell niche

We first examined the expression of ephrin ligands in the mouse intestine. RT-PCR analysis demonstrated a high expression of ephrin-A1, ephrin-B1 and ephrin-B2 in both small intestine and colon (Appendix Fig. S1). We focused our study on ephrin-B1 and -B2 as they bind and activate the EphB2 and EphB3 [7], the two receptors that have been shown previously to regulate cell migration and proliferation in the intestinal stem cell niche [1,13]. In the small intestine, high expression of both ephrins can be detected at the crypt-villus junctions and their levels decrease gradually towards the bottom of the crypts [1]. Similarly, ephrin-B1 and -B2 are expressed by differentiating cells in the upper parts of the colon crypts, with decreasing levels in the lower parts of the crypts [13].

To assess the role of ephrin-B1 and ephrin-B2 in regulating cellular dynamics in the intestinal crypt, we first injected soluble non-clustered ephrin-B1-Fc, ephrin-B2-Fc, the two together or PBS intravenously in adult wild type mice. Non-clustered recombinant ephrin-Fc proteins can be used as dominantly blocking agents because they interfere with endogenous ephrin-Eph interactions [17]. Particularly, ephrin-B2-Fc binds Eph receptors, but fails to activate signaling, and thus acts as an inhibitor [13]. Ephrin-B1-Fc can induce Eph receptor phosphorylation [26]. However, in contrast to the pre-clustered form, it is unable to evoke cellular responses such as endothelial capillary-like assembly in a two-dimensional in vitro assay [19], cell attachment to fibronectin-coated surfaces [26] and neuronal repulsion [28].

Paneth cells are positioned exclusively at the bottom of the crypts in the small intestine, but are mislocalized in double mutant mice lacking EphB2 and EphB3 [1]. Quantitative analysis of the distribution of Paneth cells is a sensitive marker of EphB2/EphB3 mediated cell positioning [9,13] and we used it to decipher the roles of ephrin-B1 and -B2 in the regulation of cell migration in the small intestine. The absence of Paneth cells from the colon, precluded analysis of ephrin-mediated cell distribution in this part of the intestine. Injection of ephrin-B1-Fc resulted in a drastic cell displacement phenotype (Fig. 1A and B), quantitatively comparable to complete loss of EphB signaling in receptor mutant mice [13]. In contrast, injection of ephrin-B2-Fc did not result in significant Paneth cell displacement. Injection of ephrin-B1-Fc and ephrin-B2-Fc together did not give further cell mislocalization compared to ephrin-B1-Fc alone (Fig. 1A and B).

In the small intestine, cell proliferation cannot be assessed independently of cell positioning: redistribution of proliferative cells in the crypts due to the displacement of postmitotic Paneth cells from the bottom of the crypts, results in changes in proliferation [13]. Thus, we measured cell proliferation in the colon, which lacks Paneth cells, enabling assessment of cell proliferation independently of cell positioning. The number of BrdU incorporating proliferating cells was decreased after an ephrin-B2-Fc injection compared to PBS (Fig. 1C, D, and Appendix Fig. S2A). In contrast, injection of ephrin-B1-Fc did not affect cell proliferation in the colon crypts. The combined administration of both ephrin-B1-Fc and ephrin-B2-Fc resulted in a decrease in proliferation comparable to injecting ephrin-B2-Fc alone (Fig. 1C, D, and Appendix Fig. S2A). Thus, injection of recombinant proteins, which compete with endogenous ephrins for binding to Eph receptors, suggests that ephrin-B1 preferentially coordinates cell positioning and ephrin-B2 regulates cell proliferation.

We next turned to mutant mice to further explore the relative role of the different ligands. Mice lacking ephrin expression were complete knock out animals, except for ephrin-B2 mutants. Mice lacking ephrin-B2 die shortly after birth, precluding analysis of adult animals. Therefore, mice carrying loxP flanked *ephrin-B2* alleles were crossed with Villin-Cre or Villin-CreER mice, to delete ephrin-B2 specifically in the intestinal epithelium (referred to as ephrin-B2 $-/-$). We also analyzed ephrin-B2 lacZ/6YFdV mice, which carry one allele where the intracellular domain is replaced by lacZ and the second allele contains point mutations in the intracellular domain rendering it unable to convey reverse signaling [27], but still able to stimulate EphB forward signaling [31]. The third B class ligand, ephrin-B3, is not expressed or only at very low levels in the intestine (Appendix Fig. S1). Paneth cells were mislocalized in ephrin-B1 $-/-$ mice (Fig. 1E), to a degree quantitatively comparable to mice lacking both EphB2 and EphB3 [13]. Neither of the ephrin-B2 mutants (Fig. 1F) nor ephrin-B3 $-/-$ mice (Fig. 1E) showed any Paneth cell displacement phenotype. A slightly higher binding affinity of ephrin-B2 to EphB2 compared to ephrin-B1 [23] and thus ability to compete with endogenous ephrin-B1 for the receptor likely explains why we observed a slight Paneth cell displacement in ephrin-B2-Fc injected mice but not in ephrin-B2 $-/-$ mutants (Fig. 1B and F).

While there was no difference in the number of proliferating cells in ephrin-B1 $-/-$, ephrin-B3 $-/-$, ephrin-B1 $-/-$; ephrin-B3 $-/-$ or ephrin-B2 lacZ/6YFdV mice (Fig. 1G and Appendix Fig. S2B), the number of proliferating cells in ephrin-B2 $-/-$ mice was significantly reduced (Fig. 1H and Appendix Fig. S2C). The result with the ephrin-B2 lacZ/6YFdV mutant in particular corroborates that mitogenic signaling is controlled by ephrin-B2 activating EphB [9].

We also asked whether the lack of ephrin ligands affects the small intestine villi. Analysis of villi length showed no differences between mutant mice and control animals (Appendix Fig. S3).

Thus, interfering with endogenous ephrin-Eph interactions by infusion of soluble ligands and analysis of mutant mice establish a surprising dichotomy with ephrin-B1 regulating cell positioning and ephrin-B2 regulating cell proliferation in the intestinal stem cell niche.

2.2. Redundant EphB receptors regulate proliferation and migration

How can ephrin-B1 and ephrin-B2, two homologous and often redundant ligands, have unique functions? One possibility may be that they engage different EphB receptors. Mice lacking both EphB2 and EphB3 have mislocalized cells and reduced cell proliferation in intestinal crypt, but it is not clear to what degree these