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Review

Eph/ephrin signaling: Genetic, phosphoproteomic, and transcriptomic approaches

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ABSTRACT

The Eph receptor tyrosine kinases and their ephrin partners compose a large and complex family of signaling molecules involved in a wide variety of processes in development, homeostasis, and disease. The complexity inherent to Eph/ephrin signaling derives from several characteristics of the family. First, the large size and functional redundancy/compensation by family members presents a challenge in defining their *in vivo* roles. Second, the capacity for bidirectional signaling doubles the potential complexity, since every member has the ability to act both as a ligand and a receptor. Third, Ephs and ephrins can utilize a wide array of signal transduction pathways with a tremendous diversity of cell biological effect. The daunting complexity of Eph/ephrin signaling has increasingly prompted investigators to resort to multiple technological approaches to gain mechanistic insight. Here we review recent progress in the use of advanced mouse genetics in combination with proteomic and transcriptomic approaches to gain a more complete understanding of signaling mechanism *in vivo*. Integrating insights from such disparate approaches provides advantages in continuing to advance our understanding of how this multifarious group of signaling molecules functions in a diverse array of biological contexts.

Contents

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1. Introduction

The Ephs are the largest family of receptor tyrosine kinases (RTK) in vertebrates, and are composed by A- and B-subfamilies. Originally isolated from an Erythropoietin-Producing Hepatocellular carcinoma, Eph receptors are bound by EPh Receptor INteracting (ephrin) proteins of the A-type and B-type with varying intra and inter-subfamily affinities. A-type Ephs bind with ephrin-As, except for EphA4 which can also bind with all B-type ephrins; A-type ephrins bind with A-type Ephs, except for ephrin-A5 which can also bind with EphB2. Eph/ephrin signaling plays marquee roles in the nervous system where it controls numerous aspects of neuronal connectivity, but is also critical in a wide variety of other contexts, including angiogenesis, craniofacial development, intestinal homeostasis, cancer, and skeletal development/homeostasis. The cell biological effects of activation of Eph/ephrin signaling are abundant and often idiosyncratic; Eph/ephrin signaling initiates cell adhesion and cell repulsion, oncogenesis and tumor suppression, cell migration and mitogenesis. It is becoming increasingly clear that the molecular mechanisms utilized to achieve such varied outcomes are even more extensive. The Eph/ephrin family has the capacity for bidirectional signaling, such that either Ephs or ephrins can serve as receptors to transduce a signal into the cell in which they are expressed. In the forward direction, Eph/ephrin signaling can activate a large number of signal transduction pathways that are both kinase dependent and independent and at least two molecular mechanisms by which reverse signaling occurs have been identified.

One of the principles emerging from this signaling maze is that biological context is absolutely crucial when deciphering Eph/ephrin signaling function. For this reason, genetic approaches dissecting signaling mechanism *in vivo* constitute a keystone methodology. For example, mouse genetics has been heavily used to interrogate the relative requirements for forward and reverse signaling. Unfortunately, interpretation of these results is not always entirely straightforward, and these methods have been fraught with technical challenges. Further, genetic ablation is not sufficient on its own to comprehend extensive signaling networks and therefore needs to be buttressed by large scale phosphoproteomic and transcriptomic methodologies. So far, most attempts at utilizing these approaches have focused on signaling activated by B-type ephrins and as such, our emphasis in this review is on Eph/ephrin-B signaling. We focus on efforts to utilize mouse genetics to define signaling mechanism and discuss recent advances in proteomic and transcriptomic approaches to define signaling networks.

2. Utilizing mouse genetics to define signaling mechanism

2.1. Forward signaling

Signaling mutations in EphB2 provided the first evidence for bidirectional signaling, and since this time the EphB2 receptor signaling mechanism has been extensively interrogated by mouse genetics approaches. Null loss of function of EphB2 results in phenotypes affecting a wide variety of developmental processes (Table 1.), especially when compounded with mutations in semi-redundant family members EphB3 and EphB1. A subset of these phenotypes were observed when mutations disrupting only forward signaling were analyzed, indicating that EphB2 serves as a receptor in those contexts, and suggesting it functions as a ligand in others. Early studies indicated that EphB2/EphB3 signaling is critical for axon guidance of the anterior commissure, corpus callosum, and for formation of the secondary palate [1,2]. Since this time, genetic perturbations of ephrins have identified the signaling partners in each of these contexts (reviewed in [3]). For example, a mutation disrupting forward signaling in which the intracellular domain of EphB2 has been replaced with LacZ results in a cleft palate phenotype similar to the one observed in *EphB2*$^{-/-}$ null mutant embryos [4]. Null mutations of ephrin-B1 display a corresponding cleft palate phenotype, whereas point mutations affecting ephrin-B1's reverse signaling function do not [5–7]. These results therefore indicate that ephrin-B1 forward signaling through EphB2 and EphB3 controls palatogenesis. Approaches such as these have been used extensively to define the receptor/ligand and forward/reverse signaling relationships (Table 1).

Given the size and redundancy inherent to the Eph/ephrin signaling family, and the diversity of biochemical signaling response possible, it has been suggested that individual highly specific signaling effectors may be unlikely to exist. Rather a complex network of effectors with redundant functions could transduce signaling. EphA4 has been the subject of a series of genetic studies characterizing downstream signaling pathways therefore providing a strong understanding of forward signaling mechanism. Targeted null mutation of *EphA4* or its signaling partner *ephrin-B3* caused defects in axon guidance of the anterior commissure, corticospinal tract (CST), and spinal interneuron axons, and disrupts the central pattern generator (CPG) resulting in a rabbit-like hopping gait [8–10]. By generating a targeted knock-in mutation specifically disrupting kinase activity (*EphA4*$^{KD/KD}$), it was established that EphA4 forward signaling is required for CST formation and CPG activity, whereas kinase independent reverse signaling controls axon guidance of the anterior commissure [11]. Correspondingly, disrupting ephrin-B3 reverse signaling did not cause CST phenotypes, indicating a role for forward signaling and not reverse [12]. Interestingly, a mutation (*EphA4*$^{EE/EE}$) with constitutive activation of kinase activity behaved relatively normally with respect to CST formation, indicating that although kinase activity is fully activated in these mutants, ligand binding triggers other critical steps, such as receptor clustering, providing a multi-step activation model for Eph/ephrin signaling [13].

Compelling genetic and biochemical evidence has identified the RacGAP α2-Chimaerin as a critical signaling effector mediating EphA4 function in CST axon guidance and CPG function. Physical interaction between EphA4 and α2-Chimaerin was observed, and *α2-Chimaerin*$^{-/-}$ null mice displayed CST abnormalities and a rabbit-like hopping gait [14–16]. Compound mutations of the signaling adaptor proteins Nck1 and Nck2 within the nervous system, also led to abnormal CST axon guidance and defects in the CPG [17]. These proteins have long been known to bind and be phosphorylated by Eph receptors, can also interact with α2-Chimaerin, strongly implicating them in downstream signaling by EphA4. Although not described in detail, it was reported that *Nck1*$^{-/-}$; *Nck2*$^{+/-}$ mutants also display phenotypes outside the CNS, suggesting that Nck adaptor function may be relevant in mediating Eph receptor function elsewhere [17]. These findings support the existence of Eph signaling effectors with specific unique function. In addition these signaling partners appear to have receptor and context-specificity; whereas α2-Chimaerin is a critical effector of CST axon guidance, it was not reported to be broadly involved in other Eph-dependent processes.

2.2. PDZ-dependent reverse signaling

The c-terminus of B-type ephrins bind PDZ-domain containing proteins constitutively, and upon binding of EphB signaling partners activate a PDZ-dependent reverse signal (reviewed in [3,18]). The *in vivo* requirements for this signaling mechanism have been examined by generating targeted point mutations disrupting the C-terminal valine of B-type ephrins, a residue that has been shown to be required for PDZ-protein binding.