Exosomes expand the sphere of influence of Eph receptors and ephrins

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Membrane-anchored Eph receptors and ephrins represent a ubiquitous intercellular communication system that typically engages at sites of cell–cell contact to initiate bidirectional signaling. Gong et al. (2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201601085) show that cells can deploy the EphB2 receptor on exosomes to activate ephrinB signaling and collapse the growth cones of distant neurons.

The Eph receptors are the largest family of tyrosine kinases, including nine EphA and five EphB receptors in the human genome (Barquilla and Pasquale, 2015). They are transmembrane proteins and their extracellular region includes an N-terminal domain that binds the ephrin ligands. The ephrins are also immobilized on the cell surface, the five ephrinAs via a glycosylphosphatidylinositol moiety and the three ephrinBs via a transmembrane segment. The binding of an Eph receptor to an ephrin on a neighboring cell leads to the formation of Eph receptor–ephrin clusters that signal bidirectionally, generating "forward" signals in the Eph receptor–expressing cell and "reverse" signals in the ephrin-expressing cell. The Eph receptor/ephrin system is present in most, if not all, cell types and regulates a multitude of biological processes that play an important role in embryonic development, adult tissue homeostasis, and disease pathogenesis.

There is a lot of flexibility in the binding interactions of Eph receptors and ephrins, with high binding promiscuity between members of the same A or B subclass (Pasquale, 2005). In contrast, Eph receptor–ephrin binding is typically subject to strict spatial constraints, requiring close juxtaposition of two cells with appropriate localization of the Eph receptor and the ephrin on their respective plasma membranes. The fixed positioning on the cell surface and cell contact–dependent nature of the signaling are believed to be important for activities of the Eph system in axon guidance, topographic mapping, synaptic connectivity, and cell sorting (Poliakov et al., 2004; Pasquale, 2005). In a classical example, precise spatial gradients of Eph receptors in retinal neurons and ephrins in the visual centers of the brain contribute to the establishment of the topographic neuronal connections that enable transmission of visual images from the eye to the brain (Pasquale, 2005; Flanagan, 2006).

There are some exceptions to the contact dependence of Eph receptor/ephrin–mediated communication. Soluble forms of ephrinAs released from the cell surface by proteolytic cleavage can activate signaling by at least some EphA receptors in the absence of cell–cell contact through poorly understood mechanisms (Wykosky et al., 2008). The EphA, EphB, and ephrinB extracellular regions can also be released from the cell surface by proteases, but these molecules appear to inhibit rather than activate Eph receptor/ephrin signaling (Barquilla and Pasquale, 2015).

In this issue, the functional studies of Gong et al. now implicate Eph receptors and ephrins in a unique form of long-range intercellular communication that paradoxically still involves direct contact between two cell membranes. This form of communication takes advantage of extracellular vesicles such as exosomes, which are released by cells and capable of traveling to distant sites through tissue interstitial fluid and other body fluids including blood (György et al., 2015; Tkach and Théry, 2016).

Exosomes are nano-sized lipid bilayer–encapsulated particles that form as the internal vesicles of endosomal structures known as multivesicular bodies (Raposo and Stoorvogel, 2013; Tkach and Théry, 2016). They are secreted into the extracellular space upon fusion of the multivesicular bodies with the cell plasma membrane, often in a regulated manner. By transporting a variety of bioactive molecules, including specific proteins, nucleic acids, and lipids that become enriched in these organelles during their assembly inside the cell, exosomes mediate a unique and powerful form of intercellular communication by exerting a remarkable repertoire of effects on recipient cells. Their surface proteins can interact with binding partners on the surface of target cells, mimicking aspects of cell–cell communication. In addition, exosomes can fuse with the plasma membrane of recipient cells or undergo endocytosis, and the transfer of exosomal cargo can drastically influence the properties of recipient cells. For example, exosomes can mediate the spreading from one cell to another of oncogenic, metastatic, and immune regulatory biomolecules as well as of aggregation-prone proteins linked to neurodegeneration and other pathogenic molecules (Rajendran et al., 2014; György et al., 2015; Syn et al., 2016; Tkach and Théry, 2016).

Proteomics data obtained by Gong et al. (2016) revealed that the EphB2 receptor clustered on the cell surface is associated with multiple proteins characteristic of exosomes, suggesting that once endocytosed EphB2 may be sorted to multivesicular bodies destined to generate exosomes. Follow-up purification and analysis of extracellular vesicles including exosomes revealed that full-length EphB2 is indeed incorporated in

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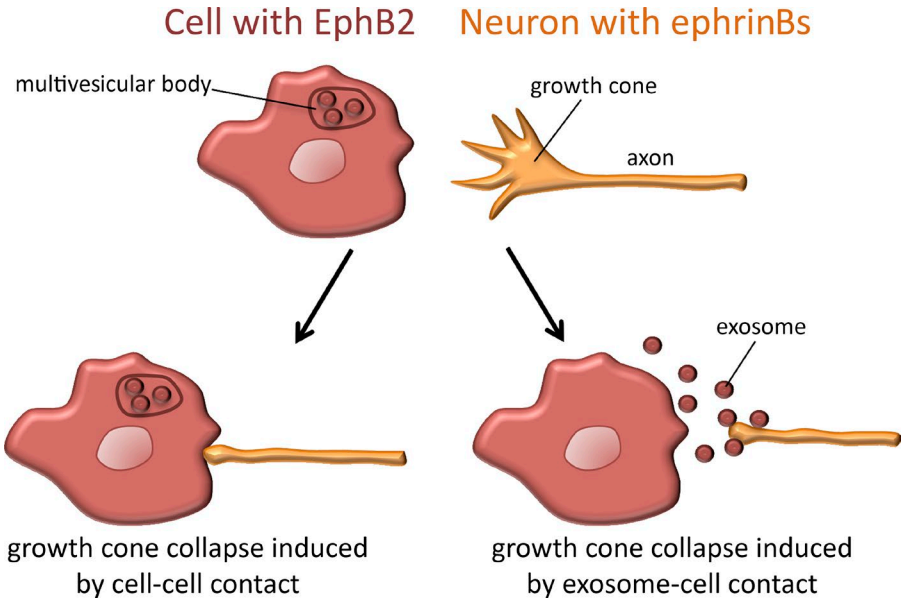

Figure 1. **EphB2-ephrinB reverse signaling can induce growth cone collapse through exosomes independently of cell–cell contact.** Through a previously well-known mechanism, EphB2 on the surface of a neuron or other cell type induces the collapse of a neuronal growth cone expressing ephrinBs through signals emanating from the cell–cell contact site (left). Novel findings have revealed that EphB2 can also be incorporated into exosomes (extracellular vesicles released from endocytic structures known as multivesicular bodies) and cause growth cone collapse independently of direct cell–cell contact (right).

these vesicles released from transfected HEK293 and HeLa cells overexpressing EphB2 but also from U251 glioma cells and cultured primary cortical neurons expressing endogenous EphB2. In fact, many endogenously expressed Eph receptors and also some ephrins were detected in the extracellular vesicle preparations from the glioma cells and cortical neurons. This is in line with previous proteomics studies performed to profile the components of exosomes and other extracellular vesicles isolated from a wide variety of sources. Databases such as ExoCarta (http://www.exocarta.org) and Vesiclepedia (http://www.microvesicles.org) show that all Eph receptor and ephrinB proteins have been detected in exosomes and/or extracellular vesicles purified from normal cells and body fluids as well as a wide variety of cancer cell types.

Despite the accumulating proteomics data demonstrating the presence of Eph receptors and ephrins in exosomes, no information has been available about the physiological significance of this localization. Now, Gong et al. (2016) demonstrate that EphB2 transported by exosomes/extracellular vesicles derived from neurons as well as nonneuronal cells is capable of binding ephrinBs on target neurons and of activating ephrinB reverse signaling. Like the cell–cell contact–dependent ephrinB reverse signaling induced by EphB2, the reverse signaling induced by exosome–cell contact can cause repulsive effects in neurons leading to growth cone collapse (Fig. 1). This suggests an important physiological role of Eph receptors/ephrins associated with exosomes, which expands their range of action beyond the immediate vicinity of the cell of origin. These data extend previous findings demonstrating the functional significance of exosome–cell contact in the signaling activities of other membrane-associated molecules. For example, the transmembrane ligand Dll4 carried by exosomes can also induce repulsive effects, causing the retraction of capillary sprouts by activating Notch receptor signaling (Sharghi-Namini et al., 2014). Furthermore, the lipid-modified, hydrophobic Wnt proteins can be transported on the surface of exosomes to overcome their poor solubility and retain their signal-inducing activity after traveling in the extracellular space (Gross et al., 2012).

Importantly, Eph receptor/ephrin communication occurring at the exosome–cell interface is likely subject to distinctive forms of regulation; for example, by factors that affect exosome release. Gong et al. (2016) indeed show that plasma membrane depolarization promotes the release of EphB2-positive exosomes from neurons, which suggests a link with synaptic activity. Moreover, growth cone collapse induced by exosomal EphB2 appears to occur with a slow time course (Gong et al., 2016), which may indicate quantitative or qualitative differences in Eph receptor/ephrin signaling induced by exosomes.

Gong et al. (2016) are the first to demonstrate functional effects of Eph receptors/ephrins through interaction of exosomes with target cells, which will inspire additional studies to further understand this new facet of Eph receptor/ephrin signaling and the extent of its physiological significance. For example, it will be interesting to elucidate whether ephrin-induced Eph receptor clustering, autophosphorylation, and/or ubiquitination are required for Eph receptor association with exosomal proteins. A previous study has highlighted the importance of these events for sorting of at least the EphA2 receptor to multivesicular bodies (Sabet et al., 2015), which is a prerequisite for incorporation into exosomes. Gong et al. (2016) showed that EphB2 ectopic expression is sufficient to promote the cell surface localization of several exosomal proteins, presumably as a result of their association with the receptor, but it is not known whether the overexpressed EphB2 may have undergone some level of constitutive clustering and activation.

Another intriguing question is whether EphB2 and other Eph receptors/ephrins are preferentially localized in exosomes rather than other types of extracellular vesicles or in a particular subtype of exosomes because extracellular vesicles can be heterogeneous in their size and cargo composition (Colombo et al., 2013; Willms et al., 2016). There is evidence for this in the case of EphA2, which was detected in a subpopulation of exosomes with a relatively small size isolated from B16F10 melanoma cells but not in a subpopulation with larger size or in other types of extracellular vesicles (Willms et al., 2016). This could explain why Gong et al. (2016) detected EphB2 and ephrinB1 in only a small fraction of the extracellular vesicles in their preparations. It will be interesting to investigate whether Eph receptors and ephrins may serve as markers of specific subtypes of exosomes and how their selective incorporation may be regulated.

Notably, none of the proteomics analyses have so far detected ephrinA proteins in any type of extracellular vesicles (Gong et al., 2016; ExoCarta and Vesiclepedia). This raises the intriguing possibility that long-range intercellular communication by the Eph system relies on distinct mechanisms depending on the family member involved. Activating forms of ephrinAs may be mainly shed by cells in soluble form, whereas full-length EphAs, EphBs, and ephrinBs may activate signaling in distant recipient cells through their association with exosomes. In addition, the exosome–plasma membrane interaction seems able to support clustering of Eph receptor–ephrin complexes (Gong et al., 2016) and thus likely more efficient and distinctive mechanisms of signal transduction (Salaita et al., 2010; Barquilla and Pasquale, 2015), although this remains to be further investigated.

An area of particular interest is that of cancer progression and metastasis. The Eph receptor/ephrin long-range, unidirectional signals generated by exosomes released from cancer cells and cells of the tumor microenvironment may lead to unique tumor-promoting or tumor-suppressing effects distinct from those of bidirectional signaling (Pasquale, 2010; Hood et al., 2011; Lazar et al., 2015). Once more information is available, it will be feasible to explore the therapeutic applications associated with modulating the deployment of exosomes carrying Eph receptors and/or ephrins associated with disease pathogenesis.

It will also be interesting to investigate whether Eph receptors and ephrins could affect exosome biology. Could Eph receptor–ephrin binding promote the formation of multivesicular bodies? Or affect the tropism of exosomes and their uptake in recipient cells? Exosomes show promise as cell-derived vehicles for delivery of therapeutic agents (György et al., 2015) and Gong et al. (2016) demonstrate preferential binding of EphB2-positive exosomes to cells expressing ephrinB1, followed by exosome internalization. Thus, it may be possible to take advantage of the Eph system for targeted delivery of therapeutic exosomes to cancer cells or other diseased cells overexpressing Eph receptors/ephrins as well as for promoting exosome uptake by recipient cells (Pasquale, 2010; Barquilla and Pasquale, 2015). The new functional data by Gong et al. (2016) open the way to a potentially fascinating exploration of the interplay between the Eph receptor/ephrin system and exosome-mediated intercellular communication.

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