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Location, Location, Location: EphB4:Ephrin-B2 Signaling Depends on Its Spatial Arrangement

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Cells have a challenge in crowded environments. They need to send specific messages to their nearest neighbors but not to cells two or three doorsteps away. This is a common problem in cell-to-cell communication that often arises during processes such as immune recognition and cellular development. To carry on this nearest-neighbor "conversation," cells resort to a mode of contact-dependent signaling dubbed as juxtacrine, which is in contrast to more conventional forms of cell-to-cell communication that involve the release of soluble molecules into the surrounding environment. For example, in paracrine, endocrine, and autocrine signaling, soluble factors are released from the cell and diffuse or are actively transported toward their targets.

To ensure high-fidelity, short-range communication between adjacent cells, ligand molecules are expressed on the surface of one cell, and the receptor is expressed on the surface of the proximal target cell. Because juxtacrine signaling requires the formation of a ligand-receptor complex precisely where two cells form an intimate physical touch, signal transmission is heavily influenced by physical processes that transcend conventional one-ligand one-receptor binding. For example, higher-order molecular processes such as oligomerization (1,2), spatial organization/confinement (3), and mechanotransduction (4) figure into juxtacrine signaling.

A prominent family of receptors that signal through a juxtacrine mechanism are the erythropoietin-producing human hepatocellular (Eph) receptors and their membrane-bound ligands, the ephrins. Eph receptors represent the largest family of receptor tyrosine kinases (RTKs) and play an integral role in neuronal development and patterning. As is the case for the vast majority of juxtacrine-signaling receptors, the Eph RTKs are particularly sensitive to oligomerization state (5), spatial localization (6), and confinement as well as mechanical interactions (3,7,8).

The EphB4 member of the Eph family, along with its membrane-associated ligand, ephrin-B2, regulates neural stem cell proliferation and survival (9). As neural-stem-cell-mediated adult neurogenesis plays important roles in learning and memory (10), elucidating the molecular pathways that drive neural stem cell self-renewal may lead to new therapeutic strategies to treat neurological disease. This underscores the importance of studying the regulation of EphB4 signaling in neural stem cells.

The work described by Dong, Groves, and co-workers (11) tested the hypothesis that neural stem cell development is sensitive to the clustering and mechanical state of the EphB4:ephrin-B2 receptor-ligand complex. They show that the quality of ephrin-B2-ligand signaling depends on its biophysical state, which controls the fate of neural stem cells. This type of regulation transcends the more conventional dogma of RTK signaling, in which ligand binding alone regulates signaling. Rather, this work suggests that physical processes need to be considered because of their distinct roles in regulating the signaling outputs of this class of receptors.

It was previously shown that to trigger EphB4 activation, the clustered soluble ectodomain of ephrin-B2 was required (9,12). However, this type of ligand departed from the native ligand, which is presented on the plasma membrane of an apposed cell. To better capture this geometry, Groves and co-workers employed supported lipid bilayers (SLBs) encoded with ligands as a "surrogate" astrocyte for neuronal stem cell binding (3,5–8,13) (Fig. 1). This artificial system mimics the signaling geometry between neural stem cells and astrocytes because the receptor-ligand complexes formed here are laterally mobile and thus allow for long-range rearrangements. To prevent rapid dissociation of the ligand from the SLB, the authors developed a versatile chemical conjugation strategy,

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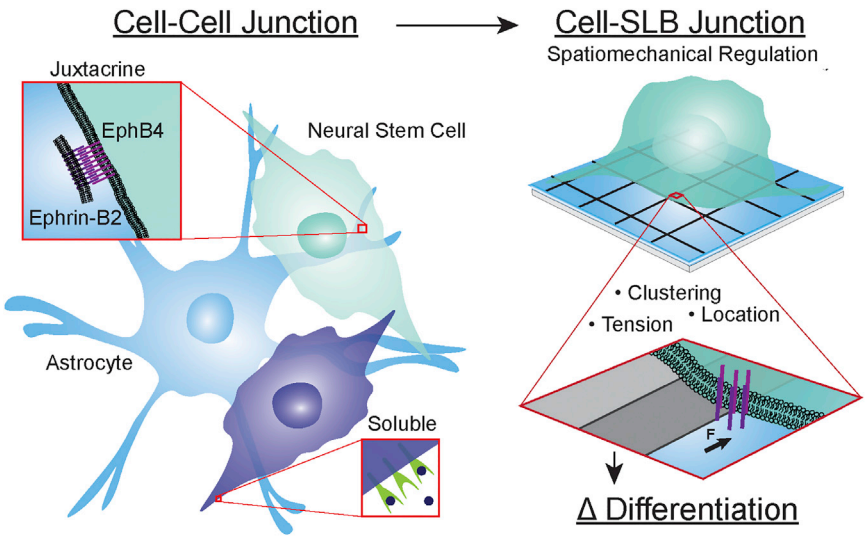

FIGURE 1 Supported lipid bilayers (SLBs) mimic astrocyte-neuronal stem cell interfaces to reveal spatial/mchanical regulation of EphB4 signaling.

DNA-SNAP-tag coupling, to tether the ephrin-B2 ligand to the synthetic SLB for long-term ligand display with significantly improved stability. In this approach, ephrin-B2 is genetically encoded with the SNAP-tag enzyme, which covalently links with benzylguanine-conjugated oligonucleotides at room temperature and with high yield. The DNA oligonucleotide can anchor the ephrin-B2 to the SLB, provided a complementary oligonucleotide is present on the SLB. For reference, the most common strategy to tether proteins onto phospholipid membranes is through nickel-NTA-polyhistidine chelation, which is simple to perform but suffers from limited stability in typical cell-culture media (13). Therefore, the DNA-SNAP-tag offers a highly efficient strategy to anchor proteins to phospholipid membranes for 12–24 h under cell-culture conditions.

Microscopic imaging allows the study of spatial reorganization of ephrin-B2 associated with EphB4 receptors upon neural stem cell seeding onto the ephrin-B2 SLB. Here, the authors show that ephrin-B2 colocalizes with EphB4 receptors on the SLB-cell interface and that EphB4 predominately governs neural stem cell adhesion onto the SLB.

Importantly, the ephrin-B2 displayed on the SLB was functional, and neural stem cells seeded on ephrin-B2 bilayers triggered neuronal differentiation at levels greater than that of treating the cells with a single dose of predimerized soluble ephrin-B2. Only when cells received a combination of serum and retinoic acid did these cells undergo similar levels of differentiation to that of the SLB-modified ephrin-B2 substrate. This result shows that the physiological membrane-bound context of ephrin-B2 is key to its full signaling functions.

Next, the authors decided to investigate the role of receptor clustering in its signaling functions. In a key experiment, the authors used "spatially mutated" SLBs (3). This is an interesting strategy in which the SLB is assembled onto a glass slide that is nanofabricated with a grid of 100-nm-wide metal lines (1). The metal grid is flush with the surrounding SLB but functions as a barrier to the diffusion of the phospholipids, and it therefore confines the ephrin-B2 ligand into specific boxes. Lateral clustering of the EphB4:ephrin-B2 complex is, in turn, limited because of the geometry of the metal grid. Using these spatially mutated EphB4 complexes, the authors found that the activation of the receptor and its downstream signaling targets were not impacted by the diffusion barriers that impaired clustering. Specifically, Western blotting showed that phosphor-extracellular-signal-regulated-kinase and active β-catenin levels were not changed on nanopatterned substrates that limit lateral clustering of the EphB4 receptor. However, hindering receptor clustering with smaller grid spacing showed a significant reduction in neural stem cell differentiation 5 days post seeding. This result suggests that although immediate downstream signaling (phosphorylation of targets) was not directly altered with receptor clustering, the "quality" of the ephrin-B2 signal was modulated by its spatial organization and potentially by the magnitude of the mechanical resistance imposed on the ligand-receptor complex.

The importance of the work is twofold. First, the work points to the potential general principles of how biophysical processes are intimately linked with the signaling functions for juxtacrine receptor-ligand interactions. Thus, this particular ligand-receptor pair may represent the tip of the iceberg, and many more and different receptor-ligand pairs that partake in juxtacrine signaling may be regulated by physical mechanisms of spatial organization, confinement, clustering, and mechanical tension. Second, the article provides a set of tools that can be broadly adopted by the community to investigate these biophysical mechanisms of signal regulation. The integration of chemical biology approaches for linking proteins to supported lipid membranes along with the nanolithography and microscopy offer an important toolset to elucidate the role of physical perturbations in Eph signaling, in particular, and more broadly defined juxtacrine signaling. The integration of newly developed probes to quantify molecular forces (14,15) onto the EphB4 complex will ultimately reveal the contribution

of mechanics and clustering to this type of signal regulation.

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