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Signalling networks in focus

Interdependent epidermal growth factor receptor signalling and trafficking

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ABSTRACT

Epidermal growth factor (EGF) receptor (EGFR) signalling regulates diverse cellular functions, promoting cell proliferation, differentiation, migration, cell growth and survival. EGFR signalling is critical during embryogenesis, in particular in epithelial development, and disruption of the *EGFR* gene results in epithelial immaturity and perinatal death. EGFR signalling also functions during wound healing responses through accelerating wound re-epithelialisation, inducing cell migration, proliferation and angiogenesis. Upregulation of EGFR signalling is often observed in carcinomas and has been shown to promote uncontrolled cell proliferation and metastasis. Therefore aberrant EGFR signalling is a common target for anticancer therapies. Various reports indicate that EGFR signalling primarily occurs at the plasma membrane and EGFR degradation following endocytosis greatly attenuates signalling. Other studies argue that EGFR internalisation is essential for complete activation of downstream signalling cascades and that endosomes can serve as signalling platforms. The aim of this review is to discuss current understanding of intersection between EGFR signalling and trafficking.

Signalling network facts

- EGFR has multiple ligands and EGFR signalling and trafficking following ligand stimulation have been proposed to depend on ligand type, concentration and duration of stimulation.
- Certain signalling cascades activated by EGFR may originate at the plasma membrane, whereas others may require EGFR internalisation for full activation.
- Phosphorylation of EGFR and EGFR substrate 15 (Eps15) has been shown to be required for EGFR endocytosis, whereas EGFR ubiquitylation for EGF-induced formation of intraluminal vesicles (ILVs) of late endosomes and EGFR degradation.
- Upregulation of EGFR signalling due to EGFR overexpression, activating mutations and *EGFR* gene amplification has been associated with tumourigenesis and cancer cell metastasis, and thus is commonly targeted in anticancer therapies.

1. Introduction

Epidermal growth factor (EGF) was first discovered in 1962 by Stanley Cohen (Cohen, 1962). It was isolated from mouse salivary glands and identified to promote development of incisors and eyelids. Further observation that EGF binds in a specific manner to cell membranes led to the identification of the EGF receptor (EGFR) in the mid-1970 (Okeefe et al, 1974; Carpenter et al., 1975). Beginning with these discoveries, functions of EGFR signalling have been extensively studied and multiple signalling cascades activated downstream of EGFR have been characterised. These studies have led to the identification of critical roles of EGFR signalling network in embryogenesis, angiogenesis and cancer development (Yewale et al., 2013; Zeng and Harris, in press).

EGFR (ErbB1) belongs to the family of ErbB receptors, which also comprises ErbB2, ErbB3 and ErbB4. They consist of the extracellular domain, single transmembrane domain and the intracellular domain. ErbB2 is unique among other ErbB receptors because its extracellular domain does not bind any known ErbB receptor ligand (Klapper et al., 1999) and thus ErbB2 receptors do not homodimerise in physiological conditions; further structural studies also revealed that ErbB2 is in an active conformation in an unbound state (Garrett et al., 2003). In contrast, ErbB3 is capable of ligand binding, but displays weak tyrosine kinase activity (Shi

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et al., 2010). In addition to the membrane-bound receptors, soluble forms also exist, which lack the transmembrane and intracellular domains. These soluble receptors are generated either *via* alternative mRNA splicing or through proteolytic cleavage of the cell surface receptors, known as ectodomain shedding. In the case of EGFR, soluble isoforms have been found to be generated by both pathways and have a potential to become useful as diagnostic and/or prognostic cancer biomarkers (Adamczyk et al., 2011; Maramotti et al., 2012; Wilken et al., 2013).

Following stimulation with a ligand, ErbB receptors at the plasma membrane can homo- or heterodimerise, a property that depends on the nature of the ligand and on the complement of receptors expressed by a particular cell type (Tzahar et al., 1996; Olayioye et al., 2000). Heterodimerisation brings additional diversity to signalling properties; additionally, heterodimers may acquire new signalling characteristics, which do not necessarily simply reflect the sum of properties of the two monomers (Olayioye et al., 2000).

Several EGFR ligands have been described, which include EGF, heparin-binding (HB)-EGF, transforming growth factor α (TGF-α), amphiregulin, betacellulin, epiregulin and epigen (Schneider and Wolf, 2009). Each of these ligands may regulate EGFR signalling and trafficking in different manners. For example, stimulation of human laryngeal epithelial carcinoma cells Hep-2 with HB-EGF or betacellulin triggered persistent EGFR phosphorylation and ubiquitylation, and ultimately degradation; in contrast, stimulation with TGF-α resulted in only subtle EGFR phosphorylation and ubiquitylation, and complete EGFR recycling (Roepstorff et al., 2009). The effect of TGF-α may be explained by high pH sensitivity of the TGF-α-EGFR binding, which causes dissociation of TGF-α within acidic endosomes, leading to recycling of unbound EGFR; accordingly the binding between EGFR and betacellulin is highly acid-resistant and thus is preserved within the acidic environment of endosomes. Betacellulin also promotes persistent recruitment of Cbl ubiquitin ligase and strong EGFR ubiquitylation. The effects of some of the ligands have yet to be explained. For example HB-EGF causes only transient Cbl recruitment (Roepstorff et al., 2009). A recent study also found that knockdown of clathrin, a protein involved in EGFR endocytosis, completely inhibited EGFR internalisation following stimulation with EGF, but only partially with HB-EGF and betacellulin, raising the possibility that the type of the ligand may dictate the entry route of EGFR (Henriksen et al., 2013). Because elevated levels of EGFR ligands are associated with tumour growth and can be upregulated in cancer (Derynck et al., 1987; Schneider and Wolf, 2009; Vlaicu et al., 2013), these differences have potential significant implications for cancer progression.

Current understanding suggests that decreased EGFR degradation and/or increased recycling prolong activation of downstream signalling cascades (Tomas et al., 2014); therefore, it is likely that the governing of EGFR trafficking by ligand type, concentration and duration of stimulation may have major consequences for signalling outcomes. Indeed, studies employing a range of concentrations of EGF have shown that both EGFR signalling and trafficking largely depend on ligand concentration (Sigismund et al., 2005, 2008). In particular at higher ligand concentrations (20 ng/ml), EGFR became ubiquitylated and internalised *via* clathrin-independent endocytosis (CIE), whereas at lower concentrations (1–2 ng/ml) EGFR was not ubiquitylated and internalised *via* CME (Sigismund et al., 2005). Therefore it was proposed that following CME, EGFR is preferentially recycled and thus downstream signalling is prolonged; in contrast following CIE, EGFR trafficking is shifted towards canonical lysosomal degradation, or potentially non-canonical degradation (Jones et al., 2014), leading to signal attenuation (Sigismund et al., 2008). From these (and other) studies an interesting pattern of concentration-dependent consequences not only for EGFR trafficking, but also for signal transduction has emerged; however, little is known about the functions of ligands other than EGF in this context. It may be proposed that unique signalling and trafficking patterns could be attributed to EGFR activation following stimulation with a particular ligand.

As mentioned above, ligand binding leads to EGFR dimerization and activation of intracellular tyrosine kinase domains. The crystal structure of the extracellular domain of EGFR bound to TGF-α in a 2:2 complex revealed that each ligand binds only one EGFR molecule, and that dimerization is mediated by the receptors themselves (Garrett et al., 2002). Following ligand binding, the EGFR kinase domains form an asymmetric dimer and undergo activation by an allosteric mechanism. In this model, the C-lobe of one kinase domain ('*activator*') interacts with and activates the N-lobe of the other ('*receiver*') leading to activation of the kinase domain of the '*receiver*', which in turn phosphorylates tyrosine residues within its C-terminus. The '*receiver*' then becomes an '*activator*' and so both EGFR molecules within a dimer become activated (Zhang et al., 2006). In contrast to the majority of cell surface receptors (Hubbard, 2004), EGFR activation does not require *trans*-phosphorylation of the conserved tyrosine residue within the activation loop, as its mutation was shown not to inhibit EGFR activation on the surface of lipid vesicles (Zhang et al., 2006).

Following ligand stimulation, EGFR activates multiple signalling cascades, ultimately promoting cell proliferation, migration and anti-apoptotic activities (Schneider and Wolf, 2009; Yewale et al., 2013; Zeng and Harris, in press). Simultaneously EGFR is internalised through clathrin-dependent and independent mechanisms (Huang et al., 2004; Sigismund et al., 2008; Rappoport and Simon, 2009; Goh et al., 2010; Henriksen et al., 2013). Numerous reports indicate that receptor endocytosis and trafficking through the endocytic system regulate signalling output, and *vice versa*, signalling downstream of activated EGFR controls its trafficking. This review highlights the current understanding of an intersection between EGFR signalling and trafficking.

2. Functions of signalling in trafficking and *vice versa*

EGFR signalling downstream of activated EGFR largely regulates cell fate through activation of multiple signalling pathways (Schneider and Wolf, 2009; Yewale et al., 2013; Zeng and Harris, in press). At the same time ligand-bound EGFR undergoes regulated endocytosis from the cell surface followed by trafficking through the endocytic system (Sigismund et al., 2005; Rappoport and Simon, 2009; Roepstorff et al., 2009). Multiple lines of evidence reveal that EGFR signalling is governed by receptor compartmentalisation, and that EGFR trafficking through the endocytic system relies on signalling outcomes. Several examples of this interdependent modulation of EGFR signalling and trafficking are shown in Fig. 1.

2.1. Regulation of EGFR signalling by endocytic trafficking

Although EGFR endocytosis was originally perceived solely as a signal attenuator (Carpenter and Cohen, 1979), isolation of EGF along with activated EGFR from intracellular vesicles of squamous carcinoma cells shed light on the possible importance of endocytic trafficking for signal propagation (Cohen and Fava, 1985). Following on from this, various approaches have been undertaken to inhibit endocytosis and study the consequences of its inhibition for EGFR signalling.

One of the first reports on the potential function of endocytosis in EGFR signalling comes from a study of a dominant-negative