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Endocytic Trafficking of the Epidermal Growth Factor Receptor in Transformed Cells

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1. Introduction

The epidermal growth factor receptor (EGFR) is the prototypical receptor tyrosine kinase. It is localized to the plasma membranes of cells with an extracellular ligand binding domain and an intracellular kinase domain. Through the binding of extracellular ligands, the receptor undergoes a conformational change that alters the biochemical properties of proteins (effectors) within the cell. Ultimately, these changes result in modulation of the rate of cell growth, protein and DNA synthesis, cell motility, and cell proliferation. The EGFR is necessary for the proper development of organisms, as indicated by the fact that genetic knockout of the receptor results in animals that are embryonic lethal or die shortly after birth. This developmental role is also observed in adult animals, as pharmacological inhibition of the EGFR disrupts tissue homeostasis.

In addition to these developmental roles, there is a strong association between overexpression and/or hyperactivation of the EGFR and cancer. Currently, there are several small molecule inhibitors and neutralizing, humanized antibodies against the EGFR that successfully treat EGFR-positive cancers (*i.e.* non-small cell lung carcinomas, colon, and head and neck cancers). Breast cancer is among those cancers that are characterized by enhanced EGFR levels and activity. However, the aforementioned pharmacologic agents have been of little success in the treatment of breast cancers. Therefore, a more detailed understanding of the cellular and molecular biology of EGFR function is required in order to successfully attenuate the growth and metastasis of EGFR-positive cells.

Cells have numerous endogenous mechanisms to that regulate the specificity and duration of EGFR signaling. Endogenous regulatory mechanisms are logical pharmacological targets to inhibit EGFR activity because of their intrinsic ability to modulate signaling. One of the most important regulators of EGFR signaling is the endocytic pathway. The endocytic pathway can control both the duration of signaling and the spatial placement of the receptor. Historically, endocytosis has been considered a mechanism for the negative regulation of EGFR, as it decreases the number of cell surface receptors and inactivates the ligand:receptor complex by targeting it for lysosomal degradation. More recently, it has been appreciated that signaling of the EGFR varies based on the subcellular localization of the receptor. Specifically, in a given cell, the liganded EGFR can promote cell proliferation at some cellular locations (*i.e.* plasma membrane), whereas at others (*i.e.* the limiting membranes of endosomes) the receptor can induce apoptosis. Thus, a potential molecular etiology of EGFR overexpression in transformed cells is disrupted normal endocytic

trafficking of the EGFR. Slowed kinetics of receptor degradation will increase the steady-state levels of the EGFRs over time. If this hypothesis holds true, the endocytic pathway is a logical target for pharmacological manipulation to prevent the progression of such cells.
This chapter will explore the basic cell biology of the EGFR and the endocytic mechanisms that regulate its signaling. To be discussed are the workings of the endocytic pathway, strategies used to understand the relationship between endocytic trafficking and EGFR signaling, and data indicating how trafficking regulates signaling. These mechanisms will be dissected and potential points for attenuating the enhanced EGFR activity will be discussed.

2. The EGFR

The EGFR is an approximately 180 kDa transmembrane protein that is oriented on the plasma membrane such that the amino terminus half of the protein is extracellular and the carboxyl terminus portion is intracellular. Thus, by virtue of its orientation, the amino terminus of the EGFR detects and binds ligands; the carboxyl terminal kinase domain converts the conformational change induced through ligand binding into intracellular, biochemical signals. The EGFR is also known as ErbB1, and is part of the larger ErbB family of receptor tyrosine kinases that is comprised of ErbB1, ErbB2, ErbB3 and ErbB4. This family shares many features such as membrane topology, mechanism of activation and signaling, and downstream effectors. However, each member is unique in its tissue expression, its activating ligands, magnitude and duration of effector signaling, and membrane trafficking (Hynes and MacDonald, 2009).
There have been seven ligands that have been reported to be able to activate the EGFR – epidermal growth factor (EGF), heparin binding epidermal growth factor (HB-EGF), betacellulin (BTC), transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin, and epigen (Harris *et al.*, 2003). The regulated secretion of these ligands is an important determinant of when a receptor gets activated. Further, overexpression of certain ligands, namely TGF-α, has been associated with an increase in receptor activity and enhanced cell proliferation (Matsui et al., 1990; Sandgren et al., 1993).
Based on the available data, it is commonly held that all ligands initiate signaling in the same fundamental manner (Figure 1). Briefly, ligand binding to an EGFR monomer induces a conformation change in the receptor that exposes a dimerization motif (cysteine rich domain). Through association with another EGFR or related family member (ErbB2, ErbB3, or ErbB4), a dimeric receptor forms, and initiates activation of the intracellular kinase domain (Dawson et al., 2005). In turn, the kinase domain phosphorylates tyrosine residues on the very carboxyl terminus of the protein. These newly formed phosphotyrosines serve as docking site for downstream signaling molecules (effectors). The EGFR-stimulated effector activity alters the intracellular biochemistry and results in cellular changes, such as cell proliferation, differentiation, and migration.
There is a long list of effector molecules reported to be downstream of the activated EGFR. Two of the major questions for scientists that study EGFR biology and pathology are 1) to identify which receptor:effector interactions occur under physiological and pathological conditions and which ones are an artifact of cell culture models and 2) to determine which effectors are necessary and sufficient for a specific cell physiology/pathology. Asking these questions is confounded by the fact that receptor:effector interactions can arise as an unintended consequence of receptor overexpression. This is true in model systems that are generated by the scientist as well as naturally occurring cell transformation. As discussed

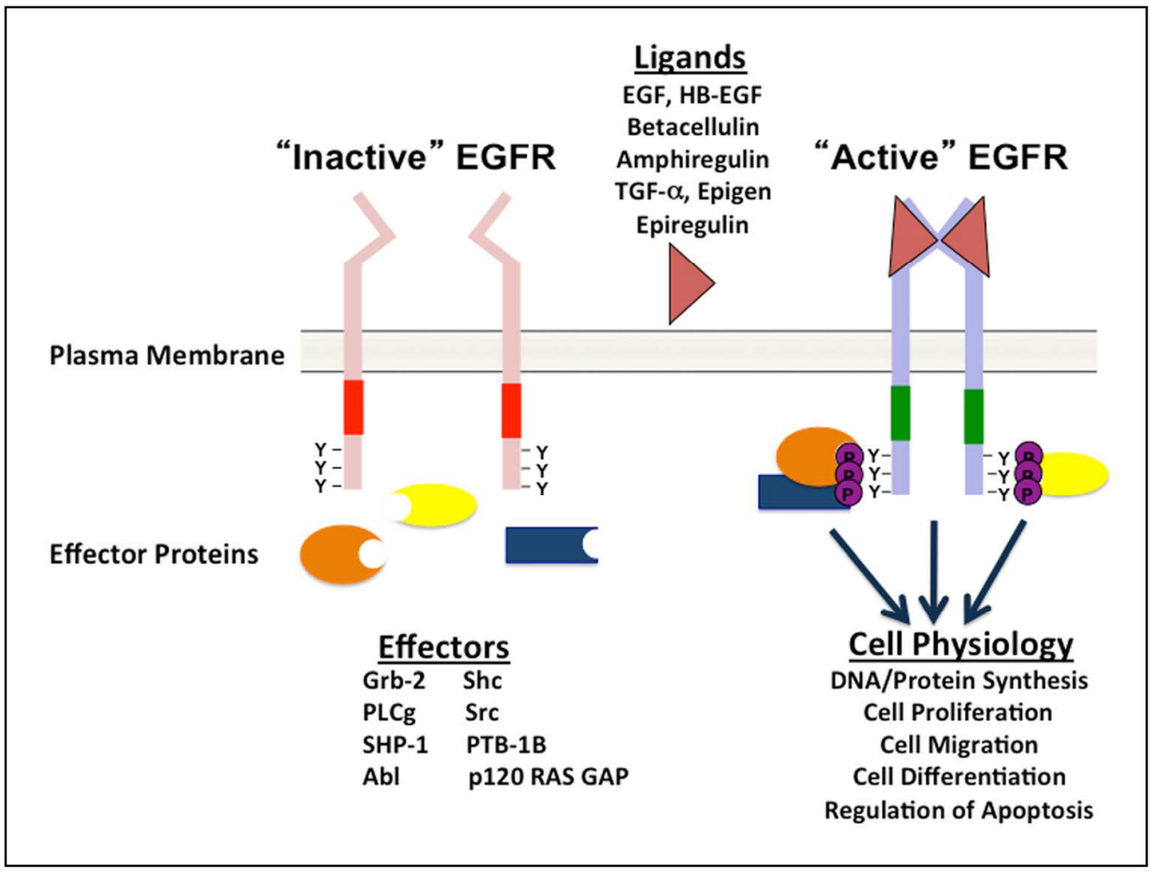

Fig. 1. Schematic of EGFR Activation. Binding of one of the EGFRs endogenous ligands induces a conformational change in the receptor that permits dimerization and kinase activity. Kinase active receptor than transphosphorylates the receptor with which it is associating. The resulting phosphotyrosines serve as docking sites for down stream signaling molecules.

below, many cancers are characterized by overexpression of the EGFR. Therefore, interactions that do not occur in a biological setting may be very relevant in a pathological condition.

There are numerous lines of evidence that indicate the EGFR plays important physiological roles in tissue development and homeostasis (Jorissen *et al.*, 2003). It is expressed on the surface of virtually every cell in the body. Mice that have been engineered to knock out the EGFR gene are either embryonic lethal or die shortly after birth, indicating its role in pre-natal development (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). Patients taking EGFR inhibitors as part of an anti-cancer chemotherapeutic regimen, experience complications such as colitis, dermatitis, and corneal abrasions, indicating the receptor contributes to the homeostasis of those tissues (Tullo et al., 2005; Zhang et al., 2007). This is supported by the genetically engineered deletion of EGFR ligands (*i.e.* EGF, transforming growth factor-α (TGF-α), or amphiregulin) have revealed roles in maintaining development of tissue, such as mammary glands, eyes, hair, and epidermis (Luetteke *et al.*, 1999) and provide evidence of the receptors' role in tissue homeostasis.

3. EGFRs in cancer

In addition to roles in tissue development and homeostasis, the driving force behind the study of the EGFR has been its role in cancer. The EGFR is overexpressed and/or hyperactivated in many cancers including, but not limited to, cancers of the breast, ovary, colon, lung, head and neck, pancreas, and brain (Rowinsky, 2004). Further, overexpression of the EGFR in many cancers correlates with poor patient prognosis (Nicholson *et al.*, 2001).

There are a number of events that can cause the increase in EGFR activity that is associated with cancer. These include gene amplification, activating mutations of the receptor's kinase domain, and deletions of the extracellular domain that regulate the receptor's ligand-binding dependent activity (Hynes and MacDonald, 2009; Uberall et al., 2008). Despite this strong association, it remains unclear whether overexpression/hyperactivation of the EGFR is the root cause of cell transformation or a cell becoming transformed is what leads to the overexpression/hyperactivation of the EGFR.

Regardless of the cause of increased EGFR activity, there are several anti-cancer therapies that specifically target the EGFR that have been approved by the United States Food and Drug Administration (FDA). These drugs fall in two classes. First, humanized monoclonal antibodies target the extracellular portion of the receptor and antagonize the binding of endogenous EGFR ligands. These antibodies are 528 mouse IgG_{2a}, the 225 mouse IgG_1, and the C225 humanized monoclonal antibody. The second class of drugs are small molecule inhibitors of the EGFR kinase domain [Iressa (gefitinib) and Tarceva (erlotinib)]. Both classes of drugs are able to prevent the progression of cancer by inducing apoptosis specifically in those cells with increased EGFR activity. Therapeutic use of these drugs has been approved for non-small-cell lung cancer, squamous cell carcinoma of the head and neck, and colorectal cancers (Baselga and Arteaga, 2005).

One issue that remains unresolved is the limited therapeutic efficacy of EGFR inhibitors in the treatment of breast cancers. This is true, despite the fact that EGFR overexpression is associated with some breast cancers. To date, only Tykerb (lapatinib) is approved for the treatment of breast cancer. However, it should be noted that Tykerb inhibits both the EGFR and its receptor tyrosine kinase family member, ErbB2, and is only approved for use in conjunction with the aromatase inhibitor Femara (letrozole) (Cameron et al., 2010). Despite the limited success of EGFR inhibitors in treating breast cancers, the evidence from other cancers indicates the receptor still has potential as a therapeutic target. In order to develop better anti-cancer chemotherapeutic agents that target the EGFR in breast cancer, a more detailed understanding of EGFR signaling is required.

4. EGFR endocytic pathway

Not only are the effectors with which the EGFR interacts important, but also the quality of interaction. Both the duration and magnitude of effector activity are what determine how cell physiology is modified. A tremendous body of literature supports the idea that the regulation of signaling is the critical determinant in how receptor specific signals are produced. This is the most logical explanation for how a number of different cell surface receptor (*i.e.* EGFR, insulin receptor, and platelet derived growth factor receptor) can share an overlapping set of effector molecules, yet each produces a receptor-specific change in cell physiology.

One of the principle mechanisms by which EGFR signaling is regulated is ligand-mediated endocytosis. Internalization of the EGF:EGFR complex can be either clathrin-dependent or

clathrin-independent. Clathrin-dependent endocytosis is the most well studied as it is the predominant route of receptor internalization in response to physiological concentrations of ligand.

By all accounts, all of the endogenous EGFR ligands can promote internalization of the EGFR, through a series of well-defined steps (Figure 2). Following binding of ligand, the ligand:receptor complex translocates along the plasma membrane to a domain that is enriched on the intracellular face with clathrin. The clathrin lattice invaginates to form a clathrin-coated pit that pinches off generating an intracellular clathrin-coated vesicle. Once inside the cell, clathrin is shed from the vesicle, giving rise to an intermediate vesicle. The intermediate vesicle fuses with and delivers the ligand:receptor complex to the early endosome. This compartment is where sorting of the cargo occurs. The predominant route of trafficking of the EGF:EGFR complex is into a late endosome/multivesicular body, by way of vesicle maturation. Alternative routes include recycling to the plasma membrane, trafficking to the nucleus, and delivery to the endoplasmic reticulum (Liao and Carpenter, 2007; Masui et al., 1993; Wang et al., 2010). For those receptors that get delivered to the late endosome/multivesicular body, that compartment fuses with the lysosome that degrades the ligand and receptor in the protease rich environment.

Each endocytic compartment has a number of distinguishing features. First, as the compartments get further away from the plasma membrane, they become more acidic and increase in density. In addition, each endocytic location has a unique protein composition. These three features have proven to be important in understanding ligand:receptor interactions, receptor trafficking, and receptor signaling. Further, researchers continue to utilize these features to distinguish one compartment from another. For instance, the different densities of the endosomes can be use to separate compartments by sedimentation centrifugation (Vanlandingham and Ceresa, 2009). Endosome specific proteins can be used as markers to identify which intracellular compartment the receptor resides (Vanlandingham and Ceresa, 2009).

It is important to note that following endocytosis, not all ligands target the EGFR for lysosomal degradation. A recent, comprehensive study of the trafficking of EGFR ligands was performed using human laryngeal carcinoma (Hep2) cells as a model (Roepstorff et al., 2009). In this study, six of the endogenous EGFR ligands were analyzed for their ability to induce receptor internalization, lysosomal degradation, and recycling. Following ligand treatment, the authors used indirect immunofluorence to monitor receptor co-localization with early and late endosome markers [early endosome autoantigen 1 (EEA1) and Lysosome associated membrane protein 1 (Lamp1)]. In addition, biochemical assays were used to assess the kinetics of ligand stimulated EGFR degradation and recycling. The data from the manuscript indicate that two ligands TGF-α and epiregulin lead to receptor recycling. HB-EGF and betacellulin, like EGF, target the receptor for degradation. Interestingly, amphiregulin treatment yields a phenotype that is somewhere in between – recycling with slower kinetics.

While the molecular basis for these differences in receptor trafficking are not entirely clear for all ligands, one ligand has been particularly well studied, particularly in the context of cancer. As stated above, treatment with TGF-α induces EGFR internalization and recycling to the plasma membrane. Once back at the plasma membrane, the receptor can be re-stimulated with available ligand and another round or signaling can occur (McClintock and Ceresa, 2010). The question becomes: what are the properties of TGF-α that promote

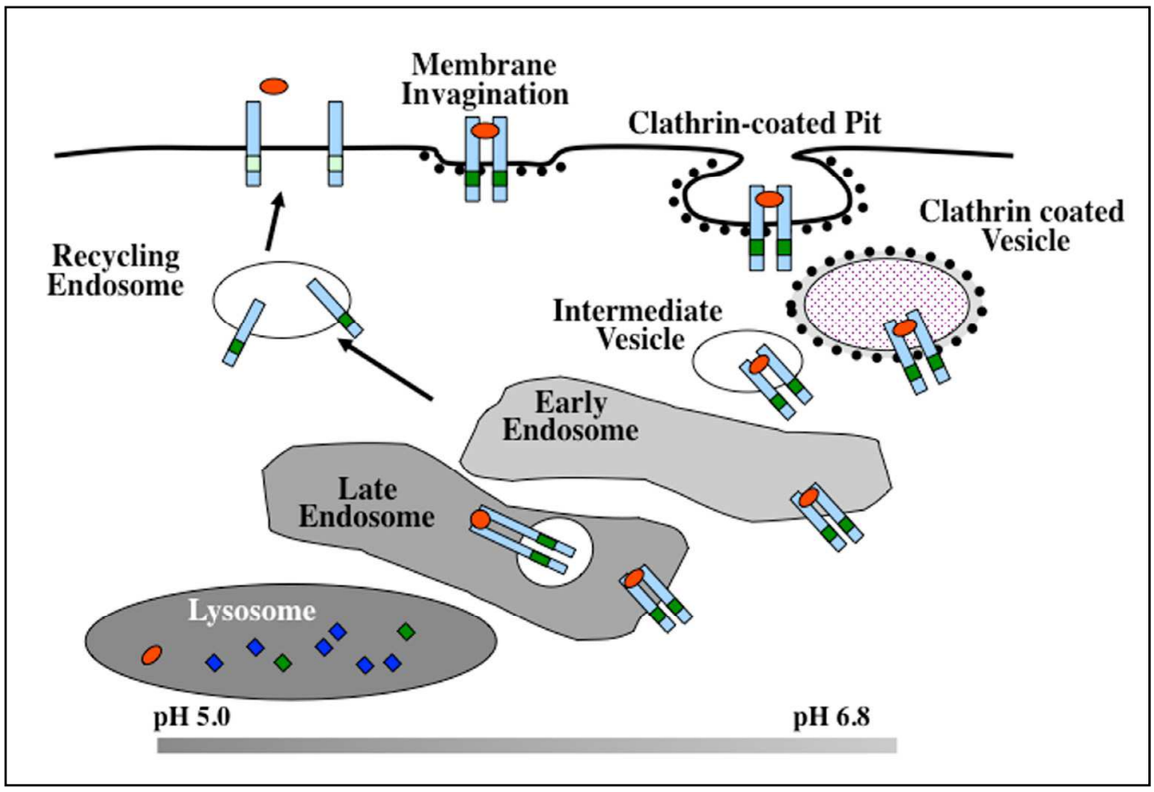

Fig. 2. Schematic of EGFR Endocytic Trafficking.

receptor recycling, whereas EGF (and others) does not? It is widely held that the difference lies in the pH sensitivity of ligand binding. Both EGF and TGF-α bind the EGFR with comparable affinity at neutral, physiological pH (pH 7.3-7.4). At pH 6.8, 50% of TGF-α will dissociate from the receptor. EGF requires a pH of 5.9 for 50% of ligand dissociation (Rutten et al., 1996). This is biologically important because the pH of the early endosome is ~6.8 (Gruenberg and Maxfield, 1995), therefore significant dissociation occurs. The loss of ligand likely converts the receptor back into a kinase-inactive conformation and is accompanied by dephosphorylation of the receptor. This would prevent association with the c-Cbl ubiquitin ligase that ubiquitylates the receptor, thereby targeting it to the lysosome for degradation.

Since EGFR endocytosis frequently culminates in the targeted destruction of the ligand:receptor complex in the lysosome, it is logical to assume this route of membrane trafficking would negatively regulate signaling. However, the role of endocytosis is more complex. Data exist that support the endocytic pathway positively regulating EGFR signaling, as well. It is clear that endocytic trafficking provides temporal regulation of receptor signaling.

4.1 Endocytosis as a negative regulator of EGFR signaling

Formal proof that endocytosis negatively regulates EGFR signaling came from the laboratory of Michael Rosenfeld (Wells *et al.*, 1990). In order to understand the role of endocytosis in signaling, Wells et al. generated mutant EGFRs that were deficient in the ability to internalize. They stably expressed these receptors in NR6 cells (derived from NIH3T3 cells) that are devoid of endogenous EGFRs. Once they established that ligand mediated endocytosis of these receptors was blocked, they examined EGF-mediated cell

transformation using a colony formation assay. A dose response curve of EGF-mediated cell transformation revealed the EGFRs defective in the ability to internalize required lower doses of EGF to undergo transformation and produced more transformed cells. From these data, it was generally accepted that endocytosis is a negative regulator of EGFR signaling.

There have been numerous of additional lines of evidence to support this model, particular in cancer models. Importantly, several come from naturally occurring cancers. For instance, one common EGFR mutation, EGFRvIII, demonstrates the relationship between receptor internalization and signaling. EGFRvIII is associated with cancers of the brain, lung, prostate, and ovary. The receptor itself is a truncation mutant that is devoid of amino acids 6-273, which encompasses the ligand binding domain and dimerization arm (Wikstrand et al., 1995). Grandal et al. compared the levels of internalization and receptor phosphorylation in NR6 cells expressing either EGFRvIII or wild type EGFR (Grandal et al., 2007). While EGFRvIII can be internalized, its rate of degradation is much slower than the EGFR. These delayed kinetics correspond to prolonged signaling. While the authors do not perform a detailed analysis of signaling, there is clearly enhanced signaling based on their association with cancer.

A second example demonstrates how other ErbB family members can both delay EGFR degradation and enhance its signaling. Like the EGFR, ErbB2 is frequently overexpressed in cancers, notably breast cancers (Guerin et al., 1988). Several groups have examine how ErbB2 (and other ErbB family members) impinge on EGFR trafficking. Worthylake et al. performed a detailed biochemical characterization of EGFR ligand binding, cell surface expression, recycling and degradation with and without ErbB2 present (Worthylake et al., 1999). Using mammary epithelial cells (MTSV1 and derivative lines) as a model, they examined the EGFR and ErbB2 when the two receptors were expressed at a 9:1 and 1:1 ratio. One of their major findings was that ErbB2 inhibits the downregulation of the EGFR.

Similarly, Offterdinger and Bastiaens examined if expression of ErbB2 affected the signaling of the EGFR (Offterdinger and Bastiaens, 2008). Using Chinese Hamster Ovary (CHO) cells that express EGFRs, but low levels of ErbB2, the authors stably transfected the cells with ErbB2. Using single cell assays, the two cells line were transiently transfected with an EGFR that had been tagged with green fluorescent protein (GFP). With this model, they observed expression of ErbB2 attenuated the rate of EGFR-GFP internalization, it also prolonged the phosphorylation of the EGFR. Since kinase dead ErbB2 had similar affects on trafficking and signaling, the authors concluded that the increased phosphorylation was not due to ErbB2 kinase activity, but rather decreased EGFR degradation/phosphorylation in the presence of ErbB2.

Additionally, there are a number of cancers that have been associated with defects in the endocytic trafficking of the EGFR and other cell surface receptors. Mutations and deletions of proteins involved in trafficking have been reported to be associated with acute myeloid leukemia (AML), hepatocellular carcinomas (HCC), breast and prostate cancer (Mosesson et al., 2008). It general, these protein aberrations result in a delay in the kinetics of receptor degradation and yield a higher level of receptor signaling, consistent with endocytosis negatively regulating signaling.

4.2 Endocytosis as a positive regulator of EGFR signaling

In the last fifteen years, a second role for the endocytic pathway to regulate EGFR signaling has come to light. This secondary mechanism is the spatial regulation conferred by placing the active receptor in the same microenvironment as downstream signaling molecules (or

effectors). The first evidence for this came from the laboratory of Sandra Schmid. Vieira et al, expressed a dominant negative mutant of the large guanine nucleotide-binding protein, dynamin, in HeLa cells, which prevents endocytosis of the EGFR via clathrin-coated pits. EGFR stimulation of cells expressing this dominant negative protein retains the liganded EGFRs at the plasma membrane (Vieira *et al.*, 1996). This approach removes the potential confounding effects of receptor mutagenesis or differences in expression levels in stable cell lines. The data from this study revealed that the activity from some effectors was enhanced when endocytosis blocked (EGFR phosphorylation, Shc, Grb2). Conversely, other effectors required endocytosis for maximal activity, namely, mitogen activating protein kinase (MAPK) and phosphatidyl inositol 3'-kinase (PI3K). This finding introduced the idea that endocytosis could be a positive regulator of signaling.

This finding was not without controversy. Subsequent groups reported that the expression of dominant negative dynamin inhibited MAPK activity. Thus, it was suggested that the attenuated MAPK activity was not due to a failure of the active EGFR to reach the endosome and stimulate MAPK, but rather a dynamin-dependent activation of MAPK (Johanessen et al., 2000). In addition, it has been suggested that blocking normal endocytic trafficking may affect the ligand binding properties to the EGFR (Ringerike et al., 1998).

However, since the initial report, there have been several additional lines of evidence that endocytosis provides spatial regulation to EGFR signaling. Two separate studies from the Lauffenburger's group provide compelling support. To examine the spatial regulation of EGFR:effector interactions, the authors exploited the different trafficking itineraries used by TGF-α as compared to EGF. By comparing the effects of the two ligands, they were able to alter the ratio of active EGFR at the plasma membrane and within the cell. They discovered that EGFRs at the plasma membrane preferentially activated phospholipase Cγ1 (Haugh et al., 1999b). Although these data indicate that endocytosis negatively regulates PLCγ1 activity, it also supports the idea that receptor:effector interactions are spatially regulated . Evidence that the spatial regulation of signaling is a positively regulator came from the follow-up publication. Using a similar approach, the authors were able to demonstrate that p21ras was actively activated from within the cell (Haugh et al., 1999a).

The detailed kinetic analysis performed in these studies is not trivial. One of the strengths of this work is that the signaling is studied in the context of wild type EGFRs that are expressed at the physiological concentrations receptors (Chen et al., 1996). Further, there was not perturbation of the endocytic pathway. Thus, these data likely provide the most accurate snapshot of what occurs physiologically.

Regardless of exactly how endocytic trafficking affects EGFR signaling, it is clearly an important mechanism. Understanding this process will undoubtedly generate important insights regarding the etiology of cancer and, potentially, the treatment of cancers.

4.3 Deciphering the role of endocytosis in EGFR signaling

There is plenty of direct and indirect evidence to indicate the endocytic trafficking regulates EGFR signaling, but a deeper understanding of the molecular mechanism is necessary. For instance, elucidating the molecular mechanism(s) will provide insight into how to specifically manipulate the pathway and modulate signaling as desired. There are a number of fundamental questions that need to be answered. Does endocytosis regulate EGFR signaling spatially, temporally, or both? Are the regulatory processes unique for individual cells and tissues? Do variations in receptor expression (as often occur in cancers) affect the regulation?

Temporal regulation of signaling refers to the duration of signaling, and would be consistent with endocytosis serving strictly as a negative regulator. How this might occur is not entirely clear. There has been much speculation in the literature whether this occurs by: 1) promoting receptor dephosphorylation, thereby eliminating its ability to interact with downstream effectors, 2) sequestration into the intraluminal vesicles of the late endosome/multivesicular body which prevents interactions with cytosolic effector proteins, or 3) degradation of the receptor. Alternatively, it may be that a combination of the three events (or possibly others) may contribute to signal attenuation.

In contrast, if endocytosis provides spatial regulation to signaling, it could either positively or negatively regulate signaling, depending of the effector protein and its subcellular location. Spatial regulation suggests the co-localization of the receptor and effector. Since this could occur at any point along the pathway (*i.e.* plasma membrane, early endosome, late endosome), its effect on signaling could be positive or negative. If receptor:effector communication is occurring at the plasma membrane, then endocytosis negatively regulate those interactions; conversely, receptor:effector interaction at intracellular locales, such as the early endosome, would be positively regulated by endocytosis.

It is important to note that the model of spatial regulation of EGFR signaling is predicated on the fact that there is compartmentalization of effector proteins. Only if an effector is concentrated at a given subcellular locale will endocytosis be able to confer specificity. If an effector is ubiquitously expressed throughout the cell, either in normal or pathological conditions, then spatial regulation cannot be achieved. This is an important consideration when examining cancer cells with aberrant protein expression. The unregulated signaling may be a consequence of lost spatial regulation of signaling.

As mentioned above, it cannot be ruled out that the endocytic pathway plays both positive and negative roles, depending on the effector. In fact, such a model may help explain the diversity in the cell biology that can be mediated by the receptor. For instance, signals that promote cell migration may be negatively regulated by endocytosis whereas those that control cell proliferation may require endocytosis.

Understanding the contribution of the endocytic pathway to EGFR signaling has important implications for a number of reasons. First, it will reveal the fundamental mechanisms of cell surface receptor signaling. It is likely this regulatory process is not unique to the EGFR or receptor tyrosine kinases. Thus, understanding this regulatory process has implications for other cell surface receptors and may guide the development of novel therapeutic agents for diseases other than cancer. Second, understanding the molecular mechanisms that regulate EGFR signaling will provide potentially useful clues to how the EGFR contributes to the pathology that underlies many cancers. These findings may provide new insights into the causes and potential prevention of cancer. Finally, the intricacies of signaling need to be appreciated in order to develop novel therapeutics that target the EGFR. Currently, the EGFR inhibitors (antibodies and tyrosine kinase inhibitors) are only effective against a subset of EGFR-positive cancers. A better of understanding of EGFR signaling will aid in the targeted design of therapeutics for a broader spectrum of cancers.

5. Strategies for deciphering the role of endocytic trafficking in EGFR signaling

Membrane trafficking and signal transduction are two very dynamic processes. Studying these events in real time has proven difficult. As a result, most investigators use a strategy of

disrupting endocytic trafficking or at least dramatically slowing it, and determining how signaling is affected. To this end, investigators have relied heavily on two fundamentally different strategies: mutagenesis of the receptor itself or perturbation of the endocytic pathway. Here we discuss the basic components of these strategies and the strengths and weaknesses of each approach.

5.1 Receptor mutagenesis to inhibit EGFR endocytic trafficking

Endocytosis-deficient, mutant EGFRs allows one to examine the EGFR directly, without disrupting proteins that are also used by other receptors that share the endocytic pathway. Therefore, the effects seen in response to EGF treatment are truly from the EGFR and do not represent a change in the fundamental properties of the cell. One technical limitation to this approach is that in order to accurately study two different receptor populations (*i.e.* wild type and an endocytosis–deficient mutant), the two receptors need to be stably expressed in cells that are devoid of the EGFRs. First, all cell line are not amenable to stable transfection, therefore the repertoire of cell lines can be limited. Second, in making stable cell lines, it is difficult to control the level of protein expression. Differences in receptor expression will affect the total level of activated receptor in response to ligand as well as number of effectors that are activated downstream. Thus, it could be hard to determine if the difference in signaling reflect changes in endocytic trafficking or receptor expression. Finally, as with all mutagenesis strategies, there is the potential that mutation of the receptor inhibits one function (such as endocytosis), there will an unexpected change in another (for instance, signaling).

In addition to the technical limitations to using mutant EGFRs, there is an even greater biological limitation. The molecular mechanisms the regulate EGFR endocytic trafficking are complex and multi-faceted. Despite the EGFR being arguably the most studied receptor in the field of endocytic trafficking, there are constantly new mechanisms of internalization being identified. Often newly discovered mechanisms are redundant with a previously identified mechanisms. The multiple mechanisms for regulating internalization are consistent with the notion EGFR endocytosis is critical to the proper function of the cell.

The complexity of EGFR endocytosis is illustrated by a recent study by Goh et al. (Goh et al., 2010). In this study, the authors used a combination of receptor mutagenesis and RNA interference (RNAi) to completely block EGFR endocytosis in porcine aortic endothelial (PAE) cells. PAE cells were chosen because they do not express the EGFR or any of its ErbB family members. Ultimately, the authors demonstrate that there are at least four completely redundant or partially interrelated mechanisms for EGFR endocytosis. When all four processes were targeted, the result was a signaling-capable EGFR with normal kinetics of kinase activation and tyrosine phosphorylation.

5.2 Disruption of endocytic trafficking by inhibition of stage-specific regulatory proteins

An alternative approach for understanding how the endocytic pathway affects EGFR signaling is to disrupt the machinery that guides the ligand:receptor complex through the endocytic pathway. Entry and exit from each endocytic location is highly regulated by a set of proteins that are unique for that endocytic stage. Knock down or expression of dominant negative forms of those proteins can be used to alter EGFR trafficking. For instance, as described above, internalization of the receptor is regulated by the large GTPase dynamin. Expression of dominant negative dynamin (Damke et al., 1994) or knock down of dynamin expression by RNAi (Huang et al., 2004) prevents EGFR endocytosis.

It should be noted that there are several proteins that are required for the internalization of the EGFR. Expression of dominant negative mutants or knock down of many of these proteins of proteins will impair the kinetics of EGFR endocytosis. For instance, work by the Sorkin laboratory has shown that knock down of clathrin heavy chain, Grb2, and dynamin II will all cause a decrease in EGFR endocytosis to some extent (Huang *et al.*, 2004). Further, since each step along the endocytic pathway is regulated by a variety of small molecular weight G-proteins, endosomal sorting complex required for transport (ESCRT), and adaptor proteins, the activity and expression of these proteins can also be targeted to inhibit intracellular trafficking. Considerable work has been done analyzing how the RAB family of proteins contributes to EGFR trafficking; therefore modulation of RAB expression or activity can alter movement of the receptor into and out of various endocytic compartments (Ceresa, 2006).

While this approach eliminates the complications like variable levels of receptor expression and potential conformational changes in the receptor, there are other caveats. First, many of the proteins used to regulate EGFR endocytic trafficking are shared with other receptors. Therefore, knocking down at protein to prevent EGFR endocytosis, will likely affect the internalization of other receptors. This likely will not be a problem if the focus is on receptor:effector communication, because the basal activity can be established by examining cells in the absence of an EGFR ligand. However, the analysis of whole cell physiology may be more complex, depending on what receptors are present in the cell, their basal activity, and the cell physiology that is being examined. Second, one must keep in mind that disrupting endocytic trafficking, only inhibits the kinetics of the pathway. It is not an absolute block. Therefore, receptors may accumulate at one endocytic stage, but in all likelihood, still be able to proceed to the next compartment, albeit at a dramatically slowed rate.

The third issue is more likely to be a problem. Disrupting endocytic trafficking can alter the steady-state distribution of the receptor . It is often under appreciated that the EGFR internalizes in the absence of ligand, albeit at a much slower rate than in the presence of ligand. Approximately, 2-3% of the EGFR constitutively internalizes every minute. Following internalization, these unliganded receptors recycle back to the plasma membrane (Herbst *et al.*, 1994).

The cause of unliganded EGFR internalization has not been determined experimentally, but may reflect receptors that spontaneously form an "active" conformation in the absence of ligand or receptors that randomly localize to a clathrin enriched membrane domain as the clathrin-coated pit forms. While the molecular determinants guiding unliganded receptors to internalize is not fully understood, it is clear that unliganded and liganded EGFRs trafficking through the same endocytic machinery. Therefore, when the endocytic trafficking is disrupted, receptors that constitutively internalize will accumulate within the endocytic pathway where the block is in place. This may, or may not, affect the total number of EGFRs in the cell. However, it has been shown for mutants of RAB5 to change the amount of cell surface EGFR (Dinneen and Ceresa, 2004).

This caveat is particularly important when using strategies that block receptor internalization (*i.e.* knock down of dynamin, clathrin heavy chain, Grb2), as an increase in cell surface receptors may result. In this case, one would need to be cautious when enhanced signaling is observed to determine whether it is the result of the block in endocytosis or increased number of ligand:receptor complexes. Similarly, if reagents were used to block

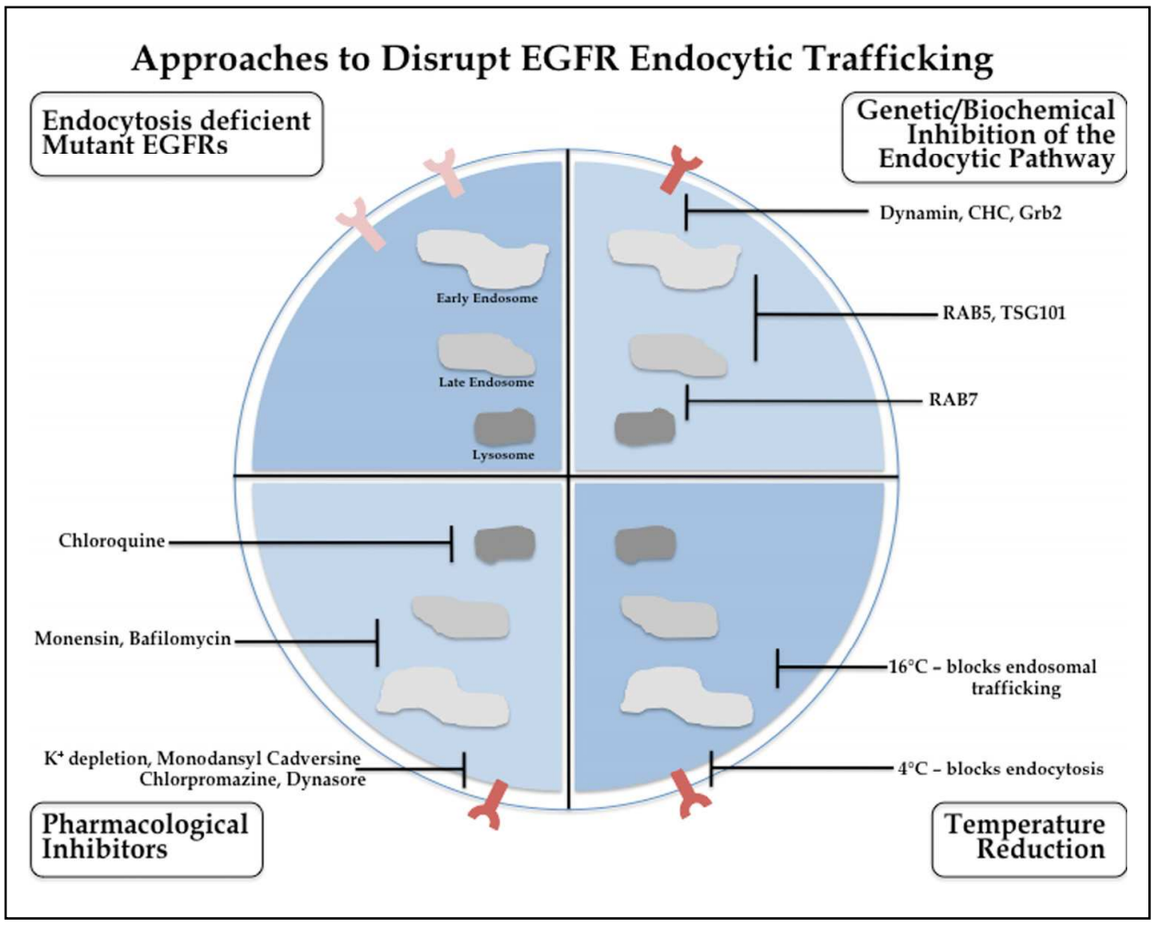

Fig. 3. Summary of Different Strategies Used to Study the Relationship between EGFR Endocytic Trafficking and Signaling.

endocytic trafficking within the cell (*i.e.* knock down of RAB5, RAB7, or TSG101), there may be an intracellular accumulation of receptors and a decrease in cell surface receptors. Under this scenario, the challenge is to understand whether decreases in signaling are due to the endocytic trafficking or decreased ligand:receptor complexes.

5.3 Pharmacological inhibitors of the endocytic machinery

Pharmacological inhibitors of the endocytic pathway offer an alternative strategy for studying EGFR endocytic trafficking. There are a limited number of such agents available. To date, these compounds have been primarily used for studying endocytic trafficking. Only recently have they been used to study how trafficking affects signal transduction.

As with most aspects of the endocytic pathway, the knowledge and reagents are greatest at the plasma membrane and decrease as one progresses to the lysosome. This is true for pharmacological inhibitors as well. At the plasma membrane, there are a number of pharmacological options available. One of the earliest strategies was intracellular potassium depletion. Work by Larkin et al. demonstrated that depletion of intracellular potassium reduced the formation of clathrin-coated pits and dramatically reduce low density

lipoprotein internalization (Larkin et al., 1983). This strategy is particularly attractive because the technique is relatively easy to perform and the endocytic inhibition is readily reversible. Since that report, other agents have been used including, chlorpromazine (inhibitor of clathrin assembly/disassembly) (Feugaing et al., 2007), monodansyl cadversine (Ray and Samanta, 1996), and most recently the dynamin inhibitor, dynasore (Hill et al., 2009).

One of the most well described inhibitors of intracellular trafficking is the ionophore, monensin. Monensin functions by inhibiting early endosome acidification. When monensin-treated cells are incubated with EGF, the receptor accumulates in the early endosome, and rates of receptor degradation and recycling are dramatically reduced (King, 1984). A similar drug, bafilomycin, disrupts trafficking in a similar manner (Presly et al., 1997). Intracellular endocytic trafficking can also be inhibited by the anti-malarial drug, chloroquine. Due to its properties as a weak base, it accumulates in the lysosome and inhibits trafficking to that compartment (Anderson et al., 1984).

5.4 Temperature reduction to inhibit endocytic trafficking

Membrane trafficking is temperature dependent. Exploiting this property can be a useful, non-invasive strategy for disrupting endocytic trafficking. Carrying out experiments at 4°C will effectively reduce all endocytosis, whereas a 16°C incubation prevents movement out of the early endosome. Both inhibitions are reversible by restoring cells to physiological temperatures (37°C). Further, temperature reduction is readily applicable to all cells.

Many of the same advantages of pharmacological inhibitors are seen with temperature reduction. The effects on membrane trafficking are rapid and reversible, which minimizes the changes in the steady-state distribution of the receptor. The biggest disadvantage is in studying EGFR-signaling. Most receptor:effector communication is temperature dependent. While receptor:effector interactions would likely still occur at lower temperature (although it would take longer to reach steady-state), the kinetics of activation would be altered, thereby making the interpretation of signaling difficult.

As with most of cell biology, the best approach is multiple approaches. Complementing receptor mutagenesis and endocytic inhibitors will provide the strongest case that differences in signaling are due to changes in trafficking.

6. Ligand based strategies for studying EGFR endocytosis

Given the limitations of using a receptor based and a cell-based strategies, an alternative approach is to use a modified ligand to disrupt trafficking. An emerging strategy is to conjugate EGF (or another EGFR ligand) to an immobilized matrix. For instance, EGF can be covalently bound to polystyrene beads that are too large to internalize or a tissue culture dish. This strategy allows the ligand to bind and activate the receptor, but the conjugated matrix (polystyrene bead or tissue culture dish) physically impedes receptor internalization.

There are several advantages to this approach. First, there is no limitation to what EGFR-expressing cell type can be studied. This is particularly important since often times cell lines that are good models for studying EGFR endocytic trafficking are not good models for studying receptor signaling. In addition, previous methods favored the used of cell lines that are receptive to the introduction of cDNA, siRNA, and viral transduction. Second, since the endocytic inhibitor affects only liganded receptors, a homogenous population of

Approach	Advantages	Disadvantages
Endocytosis Deficient, Mutant EGFR	•Specific for the EGFR	•Requires stable cell line •Difficulty getting comparable receptor numbers •Numerous endocytic processes
Biochemical/Genetic Disruption of Endocytic Machinery	•Can examine endogenous receptors at physiological levels •Can examine an array of endocytic locations	•Other cell surface receptors are affected •Can change the steady-state distribution of EGFRs • Difficult to define endocytic compartments
Pharmacological Inhibitors	•Act Quickly •Can Use in a range of cell lines	•Questions of specificity on endocytic location •Disrupt trafficking of multiple receptors
Temperature Reduction	•Act Quickly •Can Use in a range of cell lines	•Disrupt trafficking of multiple receptors •Decreases Receptor and Effector enzyme activity
Tethered Ligands	•Can Use in a range of cell lines •Specific for activated EGFR	•May select for high affinity receptors

Table 1. Advantages and Disadvantages to Various Methods for Studying EGFR Endocytic Trafficking.

receptors is being examined, and there are no concerns that some receptors have escaped the endocytic inhibition. Finally, since the endocytic inhibitor is the ligand, there are no changes in the steady-state distribution of the receptor or compensatory mechanisms developed by the cell.

The use of EGF-beads has some limitations as well. First, by design, the EGF-beads have multiple molecules of EGF bound to them. Therefore, there could be multiple ligand:receptor interactions with one EGF-bead. While this will not likely affect the kinetics of ligand association, the increased stability may affect the kinetics of ligand dissociation. This may affect the duration of ligand binding. Second, it is unknown how the conjugation process affects ligand binding and subsequent biological activity. As illustrated in Figure 4, the EGF molecule is conjugated to a carboxylate modified polystyrene bead via zero length crosslinker. Depending on which amino group is the site of conjugation that may affect the ability of the ligand to bind to the receptor. Although all studies using modified EGF clearly demonstrate at least some of the immobilized ligand retains its biological activity, it is not always clear to what extent. Finally, the bead itself has unintended consequences. While the large physical size of the bead provides a means for blocking entry through clathrin-coated pits, it may also sterically hinder the formation of EGF:EGFR complexes within a subdomain of the cell.

At this point it is difficult to discern which issues are theoretical and which are practical. From a biochemical perspective, we attempted to circumvent these issues by comparing

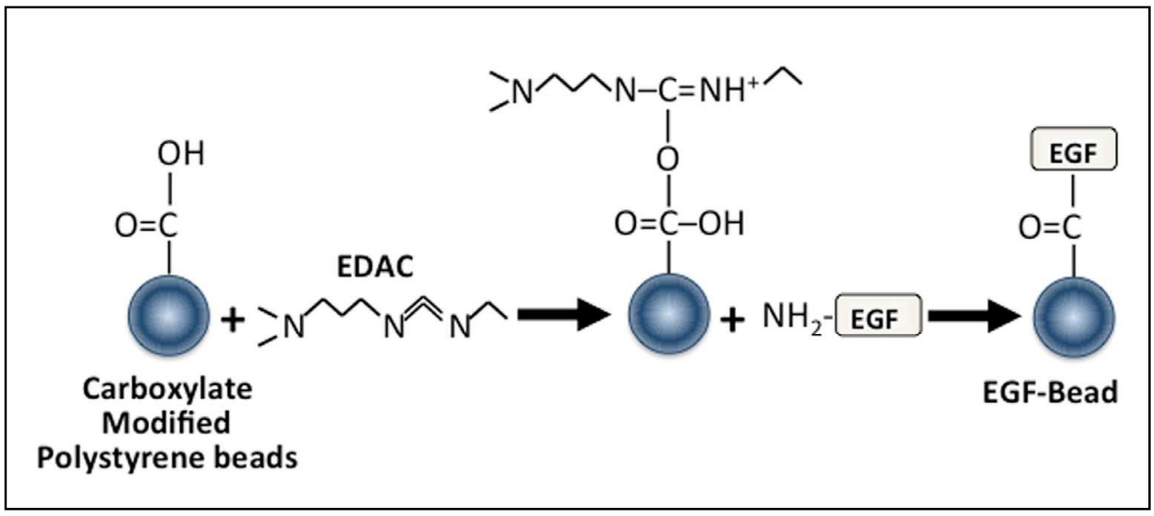

Fig. 4. Schematic of the Synthesis of EGF-beads. 900 nm polystyrene beads modified with carboxylate groups are reacted with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) to form an active O-acylisourea intermediate that readily couples to the terminal amino group in EGF (Verveer *et al.*, 2000).

cells that had comparable levels of EGFR phosphorylation. Therefore, the basis for comparison was activated receptors at the cell surface (stimulated by EGF-beads) or within the cell (EGF stimulated).

6.1 Studying of EGFR-mediated cell signaling with immobilized EGF

The initial studies with immobilized EGF were not designed to study differences in EGFR signaling from the plasma membrane as compared to inside the cell. They were designed to understand how signaling was propagated at the plasma membrane. A study by Verveer et al. demonstrated that a liganded EGFR could propagate phosphorylation along the plasma membrane to other EGFRs that were not bound to a ligand (Verveer *et al.*, 2000).

The authors of this study used a microscopy-based assay that allowed the measure of receptor phosphorylation in real time. Briefly, cells expressing a green fluorescence protein (GFP)-tagged EGFR were microinjected with a Cyanine 3 (Cy3) labeled anti-phosphotyrosine antibody. When the receptor is phosphorylated, the anti-phosphotyrosine antibody binds and places the Cy3 in close enough proximity (nanometers) to the GFP conjugated to EGF for fluorescent energy transfer (FRET). From these studies, it was concluded that both soluble EGF and EGF-beads could propagate signaling along the plasma membrane of cells.

Secondary to the authors intent, this study demonstrated that EGF-beads could: 1) bind to the EGFR and 2) be retained at the plasma membrane. In addition to the insights about EGFR signal propagation provided by this study, it also provided an important foundation for future studies analyzing the temporal and spatial basis of EGFR signaling.

A second study using EGF-beads, also took a single cell approach (Kempiak *et al.*, 2003). The goal of this study was to assess the localized signaling of the EGFR using chemotaxis as the physiological readout. The authors demonstrate that EGF beads, like EGF, can induce a chemotactic response as visualized by video microscopy. Further, cell surface receptors could initiate the cell migration by triggering cytoskeletal reorganization as measured by

staining with rhodamine phalloidin. The authors examined selected signaling pathways and found that phosphorylation of Akt was about three time greater following treatment with EGF-beads as compared to EGF.

It is worth re-stating that the goal of these two studies was not to distinguish cell surface versus intracellular EGFR signaling. Rather, both studies set up out to examine localized EGFR signals initiated from the plasma membrane. Therefore, they provide important information regarding cell surface receptor signaling, but are limited in what they reveal about what occurs within the cell.

6.2 Using EGF immobilized to a solid support to study EGFR-mediated cell migration

Although the EGF-beads are a useful way to study EGFR from the plasma membrane, variations on this theme have proven useful as well. Most notably, EGF that has been immobilized to the bottom of tissue culture dishes has proven to be an effective tool in studying cell migration. Bioengineering groups seeking to develop new methods for drug delivery performed these studies. Although EGFR is closely associated with the development of cancer, it has also been shown to be instrumental in wound healing and tissue homeostasis as well. Thus, cancer biologists should take notice of these engineering studies, as the tools developed for *in vitro* models of wound healing may provide important mechanistic insights into EGFR-mediated changes in cell biology and well as useful tools for studying the fundamental cell biology of the EGFR.

Early studies by Chen et al. used covalently linked EGF to a polystyrene dish using photo irradiation. Briefly, EGF was coupled with N-(4-azidobenzoyloxy)succinimide and the resulting solution was coated on a polystyrene dish, and the water was allowed to evaporate. Ultraviolet light was used to crosslink the modified EGF to the dish (Chen *et al.*, 1997).

To test the new ligand, Chinese Hamster Ovary (CHO) cells or mouse fibroblasts were plated on top of the immobilized EGF and monitored for cell growth by ^{3}H-thymidine incorporation. The rate of cell growth was approximately three fold higher with the immobilized EGF as compared to soluble EGF.

A modification of this approach was used by Stefonek and Masters (Stefonek and Masters, 2007). While these authors used the same basic model of immobilizing EGF to the bottom of a tissue culture dish, they did so with a concentration gradient. Using photo-patterning, precise control of the spatial localization of the EGF was achieved. Human keratinocytes (HaCaT cells) were plated on this EGF matrix and monitored for cell migration over the course of 16 days. These studies demonstrated that cells migrated toward the higher concentrations of immobilized EGF. Migration was approximately five fold greater than the control conditions. Both groups considered their findings to support the model that endocytosis negatively regulates EGFR signaling. Restricting endocytosis, prevents receptor degradation, and prolongs signaling, thereby enhancing cell migration.

Our understanding of EGFR signaling is enhanced by these studies for several reasons. First, in both studies with immobilized EGF are looking at EGFR-mediated responses in large populations of cells, rather than events in one cell. In doing so, these studies lay the groundwork for providing a biochemical analysis of changes in signaling. Second, they examine changes in cell biology that are of interest to a cancer biologist. Cell migration and proliferation are two key EGFR-mediated signaling events that need to be attenuated in the successful treatment of cancer. Finally, as bioengineering groups, the authors introduce the biologists to ways of modifying ligands in a manner that does not compromise their biologic activity.

However, in terms of understanding the spatial and temporal regulation of signaling, these studies are limited in their scope. These studies provide evidence, at least in these physiological assays that indicate endocytosis is a negative regulator of signaling. This finding supports the original report by Wells et al, in which mutant, endocytosis resistant EGF were more potent and efficacious activator of cell transformation (Wells *et al.*, 1990). However, it is not clear from these experiments whether all signaling pathways are enhanced or just a subset. Further, since all physiological responses being analyzed are enhanced, it is unclear whether some responses are positively regulated by endocytosis.

6.3 A biochemical analysis of EGFR signaling using EGF-beads

Work done by Hyatt and Ceresa combined the flexibility of EGF-beads with the biochemical analysis provided by immobilized EGF, to analyze EGFR signaling (Hyatt and Ceresa, 2008). This study, in contrast to the previous ones, specifically seeks to understand 1) whether EGFR signaling was spatially regulated, and 2) if so, how? Thus, in this work, the authors used EGF-beads to stimulate cells and examined cell physiology in response to activated EGFRs at the plasma membrane and within the cell.

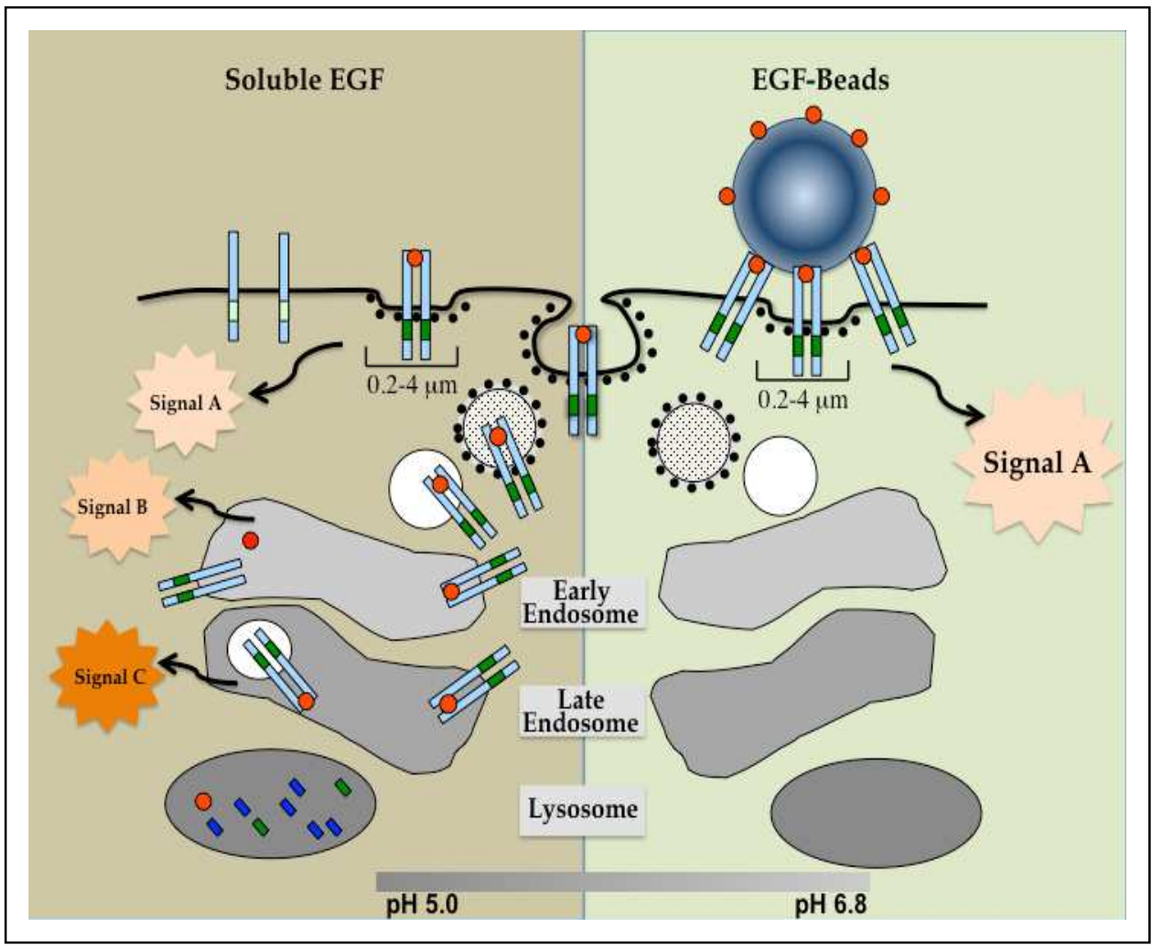

Fig. 5. Depiction of How Soluble EGF and EGF-beads elucidate Differences in EGFR Signaling.

MDA-MB-468 cells were used to study signaling by cell surface and intracellular receptors. MDA-MB-468 cells were first described by Cailleau et al, as part of a series of metastatic breast cancer cell lines derived at the MD Anderson Cancer Center in Houston, Texas (Cailleau *et al.*, 1978). Cell lines derived from metastatic breast cancer tumors were examined for common features as well as for developing model systems for studying the cell biology of transformed cells. Despite a number of similarities, each cell line has some unique features. In the case of the MDA-MB-468 cells, one of its distinguishing features is the overexpression of EGFR. It is reported that MDA-MB-468 cells express ~ 1.3 x 10^6 EGFRs/cell (Filmus *et al.*, 1985). This is about 15-25 times what is regarded as a physiological level of receptors (50-100,000 EGFRs/cell).

The high level of EGFRs makes it amenable for studying the spatial regulation of signaling for a number of reasons. First, the high levels of receptors provide a dynamic range of response following stimulation with ligand. This increases the signal-to-noise ratio. Second, cell lines with high levels of EGFR have slowed kinetics of endocytic trafficking. This has been demonstrated both in cell lines that naturally overexpress EGFR (*i.e.* cancer cell lines), as well as cells that have by stably transfected with receptors to increase their expression (French et al., 1994; Stoscheck and Carpenter, 1984). It is commonly held that the delay in trafficking is the result of saturation of the machinery that moves the ligand:receptor complex through the endocytic pathway. Regardless of the cause, the absence of appreciable receptor degradation facilitates analyzing the spatial regulation of signaling. In MDA-MB-468 cells, any changes signaling that accompany retaining the activated EGFR at the plasma membrane must be due to the receptor's location.

In this paper, the first apparent difference in signaling by EGF and EGF-beads was a change in cell morphology. Cells were incubated with either ligand and examined by light microscopy. The differences in the cells were readily apparent. As had been reported by numerous other groups, treatment with EGF caused the induction of apoptosis that was characterized by the transition of cells from spread-out cobblestone morphology to one that was rounded up. In contrast, cell treated with EGF-beads, maintained a morphology that was indistinguishable from untreated cells.

It is worth noting that EGF-mediated apoptosis is not is something that is commonly associated with EGFR signaling in the context of developmental biology, maintenance of healthy tissue, or cancer biology. However, among cell lines that overexpress the EGFR, such as MDA-MB-468 cells and A431 cells, EGF-mediated apoptosis has been well described (Armstrong et al., 1994; Gill and Lazar, 1981; Kottke et al., 1999; Tikhomirov and Carpenter, 2004). Further, indirect evidence for the role of apoptosis in cancer biology has been suggested by Tikhomirov and Carpenter. They note that in the literature, more moderate levels of ErbB receptor tyrosine kinases are associated with more invasive carcinomas. They suggest that the induction of apoptosis in cell lines with higher levels of ErbB receptor expression generates a cancer cell whose growth and metastasis is self limiting (Tikhomirov and Carpenter, 2004).

Of course, the easiest explanation for the differences in cell survival following EGF and EGF-bead treatment would be that the EGF-beads were not adequately stimulating the receptor. This was tested using both single cell and biochemical approaches. A time course of EGF-bead stimulation resulted in cells with anti-phosphoEGFR staining along the plasma membrane of the cell. This is evidence that 1) the EGFR was activated and 2) the EGFR was retained at the plasma membrane, two key features of the EGF-beads if a meaningful

analysis of signaling is to be made. Further, when cell lysates were prepared from EGF-beads treated dishes of cells, a comparable level of EGFR phosphorylation was observed as seen with treatment with soluble EGF.

These data provide evidence that endocytosis is required for the induction of apoptosis, however, it does not determine whether distinct signals are occurring at the plasma membrane. To answer this question, the authors measured cell proliferation as a function of ^{3}H-thymidine incorporation. In cells treated with soluble EGF, there was a dose dependent decrease in ^{3}H-thymidine incorporation, which likely reflects the fact that the cells are undergoing apoptosis in response to EGF. Cells treated with EGF-beads had an approximately three-fold increase in ^{3}H-thymidine incorporation.

In addition, the authors demonstrate that intracellular, but not cell surface, EGFRs activate the caspase-3, an executioner caspase in the apoptotic pathway. Further, inhibition of caspase-3 prevents the induction of apoptosis, but does not enhance the proliferative effects of the cell surface EGFRs.

Together, these data provide evidence that cell surface and intracellular EGFRs, not only signal differently, but the induce reciprocal effects. Often such reciprocal effects indicate an important regulatory mechanism in biology. This work stimulates a number of important questions. Can the induction of the EGFR-mediated apoptosis be engineered into a therapeutic treatment for cancer? What are the effector proteins being activated at the cell surface versus inside the cell? How are these differences in signaling manifest in cell lines with physiological levels of receptors?

7. Future studies in the temporal and spatial regulation of EGFR signaling

Over the last approximately 25 year, the complex relationship between EGFR signaling and membrane trafficking has been increasingly exposed. While it is widely accepted that endocytic trafficking can both positively and negatively regulate EGFR signaling, it remains unclear exactly to what extent. The field has faced technical limitations that accompany studying two dynamic processes. Further, since both processes branch out into multiple directions, the complexity increases. Through normal, physiological membrane trafficking, the EGFR can be found on the plasma membrane, in endosomes, lysosomes, the nucleus, and endoplasmic reticulum. Similarly, the activated EGFR can signal to multiple downstream effectors. Therefore, identifying which signaling events occur at which subcellular locations is anything but a trivial undertaking.

Thus far, the most fruitful approaches have been ones that have attempted to disrupt or at least dramatically slow down endocytic trafficking. The myriad approaches have been discussed in this chapter. Essentially, these approaches allow the investigator to take a snapshot of signaling. Despite the caveats of such approaches, to date, it has proven to be the most effective strategy.

Although tremendous progress has been made, it appears that we have only begun to understand how these processes come together much more needs to be done. For example, a more comprehensive map of EGFR:effector communication at the cell surface and within the cell. Definitively proving that a receptor:effector interaction occurs is a major undertaking, and requires substantial experimentation to establish such a model. To date, there are only a limited number of effectors whose activity has been studied. Although many of these effectors have well-established roles in EGFR signaling, this does not preclude the need to study other effectors. Further, a systematic analysis of plasma membrane versus

intracellular signaling for a large cohort of known EGFR effectors would be helpful for developing a model that predicts how receptor:effector communication occurs.

Once cell surface versus intracellular EGFR signaling is established, signaling from various locations within the cell needs to be understood. At this point, the most effective strategy is to disrupt membrane trafficking at discrete endocytic stages, despite the aforementioned caveats. Geographically, it does not seem likely that receptors in early endosomes and on the limiting membrane of the late endosome, would have access to different subsets of cytosolic effectors. However, the effectors may differentially associate with the various endocytic compartments either directly or through adaptor proteins. Together this analysis will generate a temporal and spatial map of EGFR:effector interactions that may be useful for anticipating how cell physiology is regulated.

In addition to delineating the receptor:effector interactions, another important question is whether the temporal/spatial regulation of EGFR:effector interaction is universal among all cell lines. This is a critical question because the EGFR mediates such a diverse array of physiological responses, endocytic regulation of EGFR signaling may provide important insight to explain the EGFR-dependent cell responses that are unique to a given cell line. This is particularly likely to be important in cancer cells that overexpress the EGFR. It is established that overexpression of the EGFR alters the endocytic trafficking of the EGFR and decreases the rate of receptor degradation. Since this effects both the duration and spatial placement of the activated receptor, it is reasonable to predict that there would be changes in signaling as well.

Ultimately, our goal for understanding the molecular basis of EGFR signaling is rooted in developing strategies for the pharmacological manipulation of signalling pathways. Knowing how the signaling pathways are regulated has a number of uses. First, understanding how each effector is activated will provide a better understanding of its contribution to a give cell physiology. Thus, the effector activity can be modulated downstream of the effector. Second, the endocytic pathway itself may be a useful target. Disrupting or accelerating endocytic trafficking may be sufficient to achieve desired changes in cell biology. Finally, an effort to study this regulation of signaling *in vivo* is necessary. Dissecting the *in vivo* regulation will not only help in develop anti-cancer therapeutics but also in other EGFR-mediated physiologies, such as wound healing.

8. Conclusion

Overexpression and hyperactivation of the EGFR as associated with many cancers. While inhibitors against the EGFR are effective for some EGFR-positive cancers, they are not effective in the treatment of breast cancers. In order to develop therapeutic agents to treat EGFR-positive breast cancers, a more thorough analysis of EGFR is needed, in particular, insight into how its signaling is regulated. Discussed here is the role of the endocytic pathway in controlling the duration and specificity of EGFR signaling. This field has made grade strides in understanding the relationship between the two processes. The basic science that underlies these studies has important implications in developing new tools for the detection, diagnosis, and treatment of breast cancers with elevated levels of the EGFR.

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10. References

Anderson, R.G., J.R. Falck, J.L. Goldstein, and M.S. Brown. 1984. Visualization of acidic organelles in intact cells by electron microscopy. *Proc Natl Acad Sci U S A*. 81:4838-4842.

Armstrong, D.K., S.H. Kaufmann, Y.L. Ottaviano, Y. Furuya, J.A. Buckley, J.T. Isaacs, and N.E. Davidson. 1994. Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. *Cancer Res*. 54:5280-5283.

Baselga, J., and C.L. Arteaga. 2005. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol*. 23:2445-2459.

Cailleau, R., M. Olive, and Q.V. Cruciger. 1978. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro*. 14:911-915.

Cameron, D., M. Casey, C. Oliva, B. Newstat, B. Imwalle, and C.E. Geyer. 2010. Lapatinib plus capecitabine in women with HER-2-positive advanced breast cancer: final survival analysis of a phase III randomized trial. *Oncologist*. 15:924-934.

Ceresa, B.P. 2006. Regulation of EGFR endocytic trafficking by rab proteins. *Histol Histopathol*. 21:987-993.

Chen, G., Y. Ito, and Y. Imanishi. 1997. Photo-immobilization of epidermal growth factor enhances its mitogenic effect by artificial juxtacrine signaling. *Biochim Biophys Acta*. 1358:200-208.

Chen, P., J.E. Murphy-Ullrich, and A. Wells. 1996. A role for gelsolin in actuating epidermal growth factor receptor-mediated cell motility. *J Cell Biol*. 134:689-698.

Damke, H., T. Baba, D.E. Warnock, and S.L. Schmid. 1994. Induction of Mutant Dynamin Specifically Blocks Endocytic Coated Vesicle Formation. *J Cell Biol*. 127:915-934.

Dawson, J.P., M.B. Berger, C.C. Lin, J. Schlessinger, M.A. Lemmon, and K.M. Ferguson. 2005. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. *Mol Cell Biol*. 25:7734-7742.

Dinneen, J.L., and B.P. Ceresa. 2004. Constitutive activation of rab5 results in a ligand independent redistribution of the EGFR and attenuates its ability to signal. *Traffic*. 5:606-615.

Feugaing, D.D., R. Tammi, F.G. Echtermeyer, H. Stenmark, H. Kresse, M. Smollich, E. Schonherr, L. Kiesel, and M. Gotte. 2007. Endocytosis of the dermatan sulfate proteoglycan decorin utilizes multiple pathways and is modulated by epidermal growth factor receptor signaling. *Biochimie*. 89:637-657.

Filmus, J., M.N. Pollak, R. Cailleau, and R.N. Buick. 1985. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochem Biophys Res Commun*. 128:898-905.

French, A.R., G.P. Sudlow, H.S. Wiley, and D.A. Lauffenburger. 1994. Postendocytic Trafficking of Epidermal Growth Factor-Receptor Complexes is Mediated through Saturable and Specific Endosomal Interactions. *J Biol Chem*. 269:15749-15755.

Gill, G.N., and C.S. Lazar. 1981. Increase phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature*. 293:305-307.

Goh, L.K., F. Huang, W. Kim, S. Gygi, and A. Sorkin. 2010. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *J Cell Biol*. 189:871-883.

Grandal, M.V., R. Zandi, M.W. Pedersen, B.M. Willumsen, B. van Deurs, and H.S. Poulsen. 2007. EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. *Carcinogenesis*. 28:1408-1417.

Gruenberg, J., and F.R. Maxfield. 1995. Membrane Transport in the endocytic pathway. *Curr Opin Cell Biol*. 7:552-563.

Guerin, M., M. Barrois, M.J. Terrier, M. Spielmann, and G. Riou. 1988. Overexpression of either c-myc or c-erbB-2/neu proto-oncogenes in human breast carcinomas: correlation with poor prognosis. *Oncogene Res*. 3:21-31.

Harris, R.C., E. Chung, and R.J. Coffey. 2003. EGF Receptor ligands. *Exp Cell Res*. 284:2-13.

Haugh, J.M., A.C. Huang, H.S. Wiley, A. Wells, and D.A. Lauffenburger. 1999a. Internalized epidermal growth factor receptors participate in the activation of p21(ras) in fibroblasts. *J Biol Chem*. 274:34350-343560.

Haugh, J.M., K. Schooler, A. Wells, H.S. Wiley, and D.A. Lauffenburger. 1999b. Effect of epidermal growth factor receptor internalization on regulation of the phospholipase C-gamma1 signaling pathway. *J Biol Chem*. 274:8958-8965.

Herbst, J.J., L.K. Opresko, B.J. Walsh, D.A. Lauffenburger, and H.S. Wiley. 1994. Regulation of Postendocytic Trafficking of the Epidermal Growth Factor Receptor through Endosomal Retention. *J Biol Chem*. 269:12865-12873.

Hill, T.A., C.P. Gordon, A.B. McGeachie, B. Venn-Brown, L.R. Odell, N. Chau, A. Quan, A. Mariana, J.A. Sakoff, M. Chircop, P.J. Robinson, and A. McCluskey. 2009. Inhibition of dynamin mediated endocytosis by the dynoles--synthesis and functional activity of a family of indoles. *J Med Chem*. 52:3762-3773.

Huang, F., A. Khvorova, W. Marshall, and A. Sorkin. 2004. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interfernce. *J Biol Chem*. 279:16657-16661.

Hyatt, D.C., and B.P. Ceresa. 2008. Cellular localization of the activated EGFR determines its effect on cell growth in MDA-MB-468 cells. *Exp Cell Res*. 314:3415-3425.

Hynes, N.E., and G. MacDonald. 2009. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol*. 21:177-184.

Johanessen, L.E., T. Ringerike, J. Molnes, and I.H. Madshus. 2000. Epidermal growth factor receptor efficiently activates mitogen-activated protein kinase in HeLa cells and HepG2 cells conditionally defective in clathrin-dependent endocytosis. *Exp Cell Res*. 260:136-145.

Jorissen, R.N., F. Walker, N. Pouliot, T.P.J. Garrett, C.W. Ward, and A.W. Burgess. 2003. Epidermal Growth Factor Receptor: Mechanisms of Activation and Signalling. *Exp Cell Res*. 284:31-53.

Kempiak, S.J., S.-C. Yip, J.M. Backer, and J.E. Segall. 2003. Local signaling by the EGF receptor. *J Cell Biol*. 162:781-787.

King, A.C. 1984. Monensin, like methylamine, prevents degradation of 125I-epidermal growth factor, causes intracellular accumulation of receptors and blocks the mitogenic response. *Biochem Biophys Res Commun*. 124:585-591.

Kottke, T.J., A.L. Blajeski, L.M. Martins, P.W. Mesner Jr., N.E. Davidson, W.C. Earnshaw, D.K. Armstrong, and S.H. Kaufmann. 1999. Comparison of Paclitaxel-, 5-Fluoro-2'-deoxyuridine-, and Epidermal Growth Factor (EGF)-induced Apoptosis. *J Biol Chem*. 274:15927-15936.

Larkin, J.M., M.S. Brown, J.L. Goldstein, and R.G. Anderson. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell*. 33:273-285.

Liao, H.J., and G. Carpenter. 2007. Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. *Mol Biol Cell*. 18:1064-1072.

Luetteke, N.C., T.H. Qiu, S.E. Fenton, K.L. Troyer, R.F. Riedel, A. Chang, and D.C. Lee. 1999. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for

EGF receptor ligands in mouse mammary gland development. *Development*. 126:2739-2750.

Masui, H., L. Castro, and J. Mendelsohn. 1993. Consumption of EGF by A431 cells: evidence for receptor recycling. *J Cell Biol*. 120:85-93.

Matsui, Y., S.A. Halter, J.T. Holt, B.L. Hogan, and R.J. Coffey. 1990. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. *Cell*. 61:1147-1155.

McClintock, J.L., and B.P. Ceresa. 2010. Transforming Growth Factor-α (TGF-α) Enhances Corneal Epithelial Cell Migration by Promoting EGFR Recycling. *Inv Opth Vis Sci*. doi:10.1167/iovs.09-4386.

Miettinen, P.J., J.E. Berger, J. Meneses, Y. Phung, R.A. Pedersen, Z. Werb, and R. Derynck. 1995. Epithelial immaturity and multi-organ failure in mice lacking epidermal growth factor receptor. *Nature*. 376:337-341.

Mosesson, Y., G.B. Mills, and Y. Yarden. 2008. Derailed endocytosis: an emerging feature of cancer. *Nat Rev Cancer*. 8:835-850.

Nicholson, R.I., J.M. Gee, and M.E. Harper. 2001. EGFR and cancer prognosis. *Eur J Cancer*. 37 Suppl 4:S9-15.

Offterdinger, M., and P.I. Bastiaens. 2008. Prolonged EGFR signaling by ERBB2-mediated sequestration at the plasma membrane. *Traffic*. 9:147-155.

Presly, J.F., S. Mayor, T.E. McGraw, K.W. Dunn, and F.R. Maxfield. 1997. Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J Biol Chem*. 272:13929-13936.

Ray, E., and A.K. Samanta. 1996. Dansyl Cadaverine regulates ligand induced endocytosis of interleukin-8 receptor in human polymorphonuclear neutrophils. *FEBS Lett.* 378:235-239.

Ringerike, T., E. Stang, L.E. Johanessen, D. Sandnes, F.O. Levy, and I.H. Madshus. 1998. High-affinity binding of epidermal growth factor (EGF) to EGF receptor is disrupted by overexpression of mutant dynamin (K44A). *J Biol Chem*. 273:16639-16642.

Roepstorff, K., M.V. Grandal, L. Henriksen, S.L. Knudsen, M. Lerdrup, L. Grovdal, B.M. Willumsen, and B. van Deurs. 2009. Differential Effects of EGFR Ligands on Endocytic Sorting of the Receptor. *Traffic*.

Rowinsky, E.K. 2004. The erbB Family: Targets for Therapeutic Develpment Against Cancer and Therapeutic strategies Using Monoclonal Antibodies and Tyrosine Kinase Inhibitors *Ann Rev of Med*. 55:433-457.

Rutten, M.J., P.J. Dempsey, C.A. Luttropp, M.A. Hawkey, B.C. Sheppard, R.A. Crass, C.W. Deveney, and R.J. Coffey, Jr. 1996. Identification of an EGF/TGF-alpha receptor in primary cultures of guinea pig gastric mucous epithelial cells. *Am J Physiol*. 270:G604-612.

Sandgren, E.P., N.C. Luetteke, T.H. Qiu, R.D. Palmiter, R.L. Brinster, and D.C. Lee. 1993. Transforming growth factor alpha dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. *Mol Cell Biol*. 13:320-330.

Sibilia, M., and E.F. Wagner. 1995. Strain-dependent epithelial defects in mice lacking the EGFR. *Science*. 269:234-238.

Stefonek, T.J., and K.S. Masters. 2007. Immobilized gradients of epidermal growth factor promote accelerated and directed keratinocyte migration. *Wound Repair Regen*. 15:847-855.

Stoscheck, C.M., and G. Carpenter. 1984. Down regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. *J Cell Biol*. 98:1048-1053.

Threadgill, D.W., A.A. Dlugosz, L.A. Hansen, T. Tennenbaum, U. Lichti, D. Yee, C. LaMantia, T. Mourton, K. Herrup, R.C. Harris, and et al. 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science*. 269:230-234.

Tikhomirov, O., and G. Carpenter. 2004. Ligand-induced, p38-dependent Apoptosis in Cells Expressing High Levels of Epidermal Growth Factor Receptor and ErbB-2. *J Biol Chem*. 279:12988-12996.

Tullo, A.B., B. Esmaeli, P.I. Murray, E. Bristow, B.J. Forsythe, and K. Faulkner. 2005. Ocular findings in patients with solid tumour treated with the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Phase I and II clinical trials. *Eye*. 19:729-738.

Uberall, I., Z. Kolar, R. Trojanec, J. Berkovcova, and M. Hajduch. 2008. The status and role of ErbB receptors in human cancer. *Exp Mol Pathol*. 84:79-89.

Vanlandingham, P.A., and B.P. Ceresa. 2009. Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. *J Biol Chem*. 284:12110-12124.

Verveer, P.J., F.S. Wouters, A.R. Reynolds, and P.I.H. Bastiaens. 2000. Quantitative Imaging of Lateral ErbB1 Receptor Signal Propagation in the Plasma Membrane. *Science*. 290:1567-1570.

Vieira, A.V., C. Lamaze, and S.L. Schmid. 1996. Control of EGF Receptor Signaling by Clathrin-Mediated Endocytosis. *Science*. 274:2086-2089.

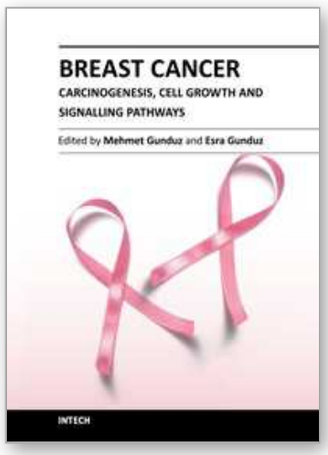
Wang, Y.N., H. Yamaguchi, J.M. Hsu, and M.C. Hung. 2010. Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. *Oncogene*. 29:3997-4006.

Wells, A., J.B. Welsh, C.S. Lazar, H.S. Wiley, G.N. Gill, and M.G. Rosenfeld. 1990. Ligand-Induced Transformation by a Noninternalizing Epidermal Growth Factor Receptor. *Science*. 247:962-964.

Wikstrand, C.J., L.P. Hale, S.K. Batra, M.L. Hill, P.A. Humphrey, S.N. Kurpad, R.E. McLendon, D. Moscatello, C.N. Pegram, C.J. Reist, and et al. 1995. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res*. 55:3140-3148.

Worthylake, R., L.K. Opresko, and H.S. Wiley. 1999. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. *J Biol Chem*. 274:8865-8874.

Zhang, G., S. Basti, and L.M. Jampol. 2007. Acquired trichomegaly and symptomatic external ocular changes in patients receiving epidermal growth factor inhibitors: case reports and a review of literature. *Cornea*. 26:858-860.



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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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