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Epidermal growth factor signaling in transformed cells

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Abstract

Members of the epidermal growth factor receptor (EGFR/ErbB) family play a critical role in normal cell growth and development. However, many ErbB family members, especially EGFR, are aberrantly expressed or deregulated in tumors and are thought to play crucial roles in cancer development and metastatic progression. In this chapter, we provide an overview of key mechanisms contributing to aberrant EGFR/ErbB signaling in transformed cells which results in many phenotypic changes associated with the earliest stages of tumor formation, including several hallmarks of epithelial-to-mesenchymal transition (EMT). These changes often occur through interaction with other major signaling pathways important to tumor progression resulting in a multitude of transcriptional changes that ultimately impact cell morphology, proliferation and adhesion, all of which are crucial for tumor progression. The resulting mesh of signaling networks will need to be taken into account as new regimens are designed for targeting EGFR for therapeutic intervention. As new insights into the molecular mechanisms of the cross-talk of EGFR signaling with other signaling pathways and their role in therapeutic resistance to anti-EGFR therapies are gained a continual reassessment of clinical therapeutic regimes and strategies will be required. Understanding the consequences and complexity of EGF signaling and how it relates to tumor progression is critical for the development of clinical compounds and establishing clinical protocols for the treatment of cancer.

Keywords

Transformation; Epithelial-Mesenchymal Transition (EMT); Epidermal Growth Factor Receptor (EGFR); Cancer

1. INTRODUCTION

The type of cell a tumor originates from is used to classify cancers. They include carcinomas derived from epithelial cells, sarcomas developing from cells of mesenchymal origin such as supportive and connective tissues, lymphoma and leukemia arising from hematopoietic cells, germ cell tumors from pluripotent cells and blastomas deriving from immature precursor cells or embryonic tissue. Common to all these cancers is a multi-step process that involves an accumulation of several genetic alterations and the acquisition of mutant alleles of proto-oncogenes, tumor suppressor genes, and other genes that control cell proliferation directly or

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indirectly (Hahn, Weinberg, 2002). Among these, the ErbB family of receptor tyrosine kinases, that includes epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4, regulates a complex signaling network that impacts a variety of cellular processes, including proliferation, survival, angiogenesis, and metastasis in many cancers. Mutations and/or aberrant activation of members of the ErbB family have been well described in carcinoma (Abhold *et al.,* 2012) and glioblastoma multiforme (Clark *et al.,* 2012) but have also been found in some blastomas such as medulloblastoma (Liu *et al.,* 2014b) and sarcomas like osteosarcoma (McCleese *et al.,* 2013) and rhabdomyosarcoma (Yamamoto *et al.,* 2013). In this review, we summarize some of the recent developments in understanding the role of EGFR/ErbB signaling in the transformation of normal cells to cancer cells, its contribution to cancer progression and the possibilities and challenges in targeting EGFR/ErbB signaling in cancer therapy.

2. HALLMARKS OF TRANSFORMATION

Carcinogenesis is a process by which normal, otherwise healthy cells break free of normal control mechanisms to acquire sustained proliferation, growth suppressor evasion, resistance to cell death, indefinite replication, invasion of neighboring tissues, and undergo angiogenesis to provide nutrients to support rapidly dividing cells. As these processes are important to normal cell growth and differentiation, one can therefore view cancer as deregulated differentiation, often occurring from “differentiation blocks” whereby faster growing less differentiated cell populations expand faster than neighboring differentiated cells (Greaves, 1982). Because transcription factors are at the heart of normal cellular development (Tenen *et al.,* 1997); (Shivdasani, Orkin, 1996), aberrant regulation or activation of physiologically important transcription factors often leads to tumor formation (Alcalay *et al.,* 2001). In the last decade, two additional hallmarks have been added: reprogramming of energy metabolism and evading immune destruction (Hanahan, Weinberg, 2011). In carcinoma, the most prevalent form of all human cancers (80 to 90 percent), malignant transformation is associated with the loss of differentiated epithelial characteristics and a coinciding increase of less mature mesenchymal traits, a process termed the epithelial-mesenchymal transition (EMT) (Figure 1). EMT occurs physiologically during embryonic development, but also plays a fundamental role later in life during pathological processes including cancer and fibrosis. In cancer, EMT contributes to tumor progression by conferring properties such as invasiveness, the ability to metastasize, resistance to therapy, and possibly the generation of stem-like cancer cells (Mallini *et al.,* 2014). Cells undergoing an EMT-like transition are believed to be more motile and invasive, thought to be a critical step in the progression toward metastasis (Garcia de Herreros, Moustakas, 2014). In addition to changes in adhesion, cancer cells also acquire the capability to sustain proliferative signaling in a number of alternative ways. Some tumor cells produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation. Alternatively, cancer cells may send signals to stimulate neighboring normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various growth factors. Receptor signaling can also be deregulated by elevating the levels of receptor proteins displayed at the cancer cell surface, rendering such cells hyper responsive to otherwise-limiting amounts of growth factor ligand;

the same outcome can result from structural alterations in the receptor molecules that facilitate ligand-independent firing (Hanahan, Weinberg, 2011). Growth factor independence may also derive from the constitutive activation of components of signaling pathways operating downstream of these receptors.

2.1. Epithelial-mesenchymal transition (EMT)

One of the earliest steps in tumor progression is when epithelial-like cells begin to take on the phenotypic traits of mesenchymal cells. EGFR and EGF signaling plays a critical role in the initiation of this process, termed the epithelial-mesenchymal transition (EMT). Epithelia are sheets of tightly associated specialized epithelial cells that line surfaces throughout the body. These cellular sheets perform vital functions as a barrier while also regulating nutrient and fluid exchange. To carry out these functions, epithelial cells possess highly specialized cell architecture and are polarized. The apical-basal polarity present in epithelial cells requires structural integrity of intercellular junctions and extracellular interactions between epithelial cells and substrates or neighboring cells (Martin-Belmonte, Perez-Moreno, 2012). These junctional complexes include tight junctions that physically separate the apical and basolateral plasma membranes to maintain the polarized protein/lipid composition of the respective membrane domains, adherens junctions essential for cell-cell adhesion, desmosomes involved in intercellular adhesion and gap junctions that facilitate intercellular communication. Together, these junctions restrict cell motility, preserve tissue integrity and permit individual cells to function as cohesive units (Martin-Belmonte, Perez-Moreno, 2012). As such, tumors must find ways, including activation of the EGF signaling pathway, to disrupt these junctions in order for tumors to progress and metastasize. Continued expression and functional activity of junctional complexes are required for polarized cells to remain tightly associated within the epithelium and to coordinate signaling pathways that regulate proliferation. Loss of junctional complexes is associated with depolarization, loss of differentiated characteristics, enhanced epithelial cell proliferation, and acquisition of an invasive potential. For these reasons, these junctional complexes are often aberrantly regulated during tumor formation and progression. Intracellular effector molecules orchestrate the transcriptional down regulation of cell adhesion molecules, disassembly of junctional complexes, and changes in cytoskeletal organization during EMT, that lead to the subsequent loss of intercellular junctions and cell polarity. These changes occur at multiple molecular levels, including gene regulation through promoter methylation/demethylation or histone acetylation/deacetylation, alternative splicing, protein translocation/sequestration, and transcriptional regulation of target genes. As epithelial cells lose intercellular adhesion, the cytoskeleton reorganizes and cells gain mesenchymal cell characteristics including increased motility and the expression of mesenchymal genes. Underscoring the important inter-relationship between EMT and adhesion, several key transcription factors that promote EMT directly regulate the expression of both cell adhesion and cell polarity complexes. For example, the EGF signaling intermediates Snail (now known as SNAI1), Slug (SNAI2), Sip1 (Zeb2), E47 (E2α), Twist1, FoxC2, FoxC1, GSC, β-catenin, and Zeb1 regulate E-cadherin gene repression and influence the gene-expression patterns that underlie EMT (Garcia de Herreros, Moustakas, 2014). Ultimately, the cellular changes resulting from EMT promote many hallmarks of cancer, including loss of contact inhibition, enhanced invasiveness, altered growth control, and increased resistance to apoptosis.

EMT is typically initiated by extracellular activation resulting from an intricate network of interactions amongst several signaling pathways and eventually stabilizes to lead to increased stability of the mesenchymal phenotype (Lamouille *et al.,* 2014). Many of these pathways have common endpoints, including E-cadherin down regulation and expression of EMT-associated genes. E-cadherin, a calcium-dependent adhesion molecule that mediates homophilic cell-cell adhesion, is a central regulator of the epithelial phenotype and its expression is lost in many tumors either through mutations in the *CDH1* gene, which encodes E-cadherin, or through transcriptional repression of *CDH1* during EMT. Down regulation of E-cadherin results in the loss of E-cadherin-dependent junctional complexes and of E-cadherin mediated sequestration of β-catenin. Unsequestered β-catenin activates transcriptional regulation through LEF/TCF4 (lymphoid-enhancer-binding factor/T-cell factor-4) and further drives the EMT process. Due to cross-talk between integrin and E-cadherin signaling, down regulation of E-cadherin is also involved in the switch from cadherin-mediated adhesion in epithelial cells to integrin-mediated adhesion predominant in mesenchymal cells (Reviewed in (Nagathihalli, Merchant, 2012)). Loss of expression or functional activity of many cell adhesion molecules and cell polarity proteins (e.g. PAR, crumbs (CRB) and scribble (SCRIB) complexes) during EMT are intricately related to advanced stages of tumor progression and invasiveness. Indeed, many of the proteins that control epithelial polarity are tumor suppressors or proto-oncoproteins and their contributions to the early stages of tumorigenesis has been described in an excellent review by Martin-Belmonte and Perez-Moreno (Martin-Belmonte, Perez-Moreno, 2012).

The initiation of most important cellular processes is under tight transcriptional control, mediated by of transcription factors that regulate the activation of a web of downstream targets and mediators. The cellular transition from an epithelial to mesenchymal phenotype is no exception. One of best described transcription factors involved in EMT is SNAI1, which has been characterized as a critical central regulator of EMT. SNAI1 binding to E-box consensus sequences in the E-cadherin promoter and repressing genes involved in cell polarity genes found in the Crumbs, Par, and Scribble complexes (Whiteman *et al.,* 2008). Binding of Snail to the E-cadherin promoter seems to be facilitated by local modifications of the CDN1 chromatin structure by SIN3A, histone deacetylases (HDAC)-1 and -2 and Polycomb 2 complex proteins (Herranz *et al.,* 2008) and posttranslational modifications of Snail such as phosphorylation (PAK, GSK3β)/dephosphorylation (SCP) and lysine oxidation (LOXL2) (Peinado *et al.,* 2008). However, the role of Snail in transformation extends beyond the regulation of cell adhesion and cell polarity proteins, as Snail activates immunosuppressive cytokines and T-cell responses during the immuno-suppression often observed in tumors (Kudo-Saito *et al.,* 2009; Pioli, Weis, 2014). While Snail is the best characterized transcription factor of EMT, many transcriptional regulators expressed in malignant tumors regulate one another and target the same genes (Hanahan, Weinberg, 2011). Overall, the net effect of this gene regulation is to orchestrate the steps involved in EMT: 1) loss of adherens junctions, 2) conversion of a well-differentiated phenotype to a mesenchymal/fibroblastic phenotype, 3) expression of matrix-degrading enzymes, 4) increased motility, 5) increased resistance to apoptosis, and ultimately 5) invasion and metastasis. Various signals trigger expression of these transcription factors including heterotypic interactions with neighboring cancer cells and interactions with adjacent tumor-

associated stromal cells or inflammatory cells. Indeed, tumor-associated macrophages secrete EGF to neighboring cancer cells, which in turn stimulate macrophages to facilitate intravasation and metastatic dissemination of the cancer cells (Zheng *et al.,* 2013). Together, these findings substantiate a role of EGF-mediated signaling not only in EMT and proliferative signaling itself, but also in the cross talk between tumor cells and the microenvironment.

2.2. Role of EGFR in EMT

Because EMT is often viewed as one of the earliest transforming events leading to tumorigenesis, it is critical to examine and understand potential molecular mechanisms by which this process is activated in cells. EGFR and ligands belonging to the EGF-like family of growth factors play important roles in tumorigenesis, especially during the initial stages of EMT and are over expressed in many cancers and the majority of carcinomas (Salomon *et al.,* 1995). EGFR function is frequently dysregulated in epithelial tumors; EGF has been shown to promote tumor cell migration and invasion (in part through dephosphorylation and inactivation of FAK), and EGFR signaling has been shown to play an important role both in cancer progression and in EMT-like transitions (Al Moustafa *et al.,* 2012) (Figure 1). EGFR activation correlates with neoplastic transformation of solid tumors, EGFR overexpression correlates with poor patient survival, and EGFR-driven autocrine mechanisms are involved in the initial stages of EMT during carcinoma development (Ardito *et al.,* 2012). In agreement with the hypothesis that EGF family members play an important role in the initial stages of EMT, transformation by Her2/neu resulted in increased CD44high/CD24low immortalized human mammary epithelial cells that possess many of the stem-like properties associated with the initial stages of EMT (Morel *et al.,* 2008). In oral squamous cell carcinoma cells, EGFR inhibition resulted in a transition from a fibroblastic morphology to a more epithelial phenotype as well as accumulation of desmosomal cadherins at cell–cell junctions (Lorch *et al.,* 2004). Thus, EGFR signaling mediates the initial stages of EMT and EGFR inhibition may restrain EMT in certain cellular contexts. In support of this hypothesis, ligand-independent, constitutively active forms of EGFR can increase motility and invasiveness of tumor cells and EGFR inhibitors block cancer cell migration *in vitro* (Liu *et al.,* 2013; Yue *et al.,* 2012)

3. THE ERBB/HER RECEPTOR FAMILY IN NORMAL AND TRANSFORMED CELLS

One of the hallmarks that distinguishes cancer cells from normal cells is growth factor independence, meaning that cancer cells do not need stimulation from external signals in the form of growth factors to multiply (Hanahan, Weinberg, 2000). One way this can occur is if cancer cells generate their own growth signal; glioblastomas can produce their own platelet-derived growth factor (PDGF), and sarcomas can produce their own tumor growth factor α (TGF-α) (Suzuki, Yamada, 1994; Venugopal *et al.,* 2012). Alternatively, growth factor receptors can be overexpressed within tumors, as in the case of epidermal growth factor receptor (EGF-R/ErbB) overexpression in stomach, brain and breast cancers, or HER2/neu receptor overexpression in stomach and breast cancers (Berghoff *et al.,* 2013; Menard *et al.,*

2001). Another possibility is that activating mutations arise within the growth factor receptors that lead to signal transduction in the absence of ligand (Frattini *et al.*, 2013).

3.1. Overview of EGFR receptors, ligands, and signaling

The ErbB family of receptor tyrosine kinases consists of four distinct members: EGFR (ErbB-1/HER1), ErbB-2 (neu/HER2), ErbB-3 (HER3), and Erb-4 (HER4). All members of the ErbB receptor family have an extracellular ligand-binding domain, a single hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase-containing domain (reviewed in (Tebbutt *et al.*, 2013)). The intracellular tyrosine kinase domain that mediates EGFR activation and downstream signaling is highly conserved among the ErbB receptor family members although the intracellular tyrosine kinase domain of ErbB-3 lacks kinase activity due to several critical amino acid substitutions (Guy *et al.*, 1994). ErbB extracellular binding domains are far more variable, because this is the region that confers ligand specificity and activation by different autocrine and paracrine secreted EGF-family growth factor ligands (Tebbutt et al., 2013).

ErbB receptors are recognized by different structurally related growth factors, but about ten EGFR ligands have been identified that contain a characteristic 55 amino acid region with three disulfide bonds and a loop-rich structure (Schneider *et al.*, 2008). The best known ErbB receptor family ligands are epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), neuregulins (NRG) 1 through 4, epiregulin (ERG), betacellulin (BTC), and heparin-binding epidermal growth factor (HB-EGF). Each ligand shows distinct affinity and binds to a specific ErbB receptor family member; some receptors share ligands and some ligands bind exclusive receptors (Carrasco-Garcia *et al.*, 2014). EGF, AR and TGFα bind EGFR; HRG, BTC and HB-EGF bind EGFR and erbB-4 (Riese *et al.*, 1996). NRG1 and NRG2 bind erbB-3 and erbB-4 and NRG3 and NRG4 bind only to erbB-4 (Stonecypher *et al.*, 2006). Unlike other EGF family receptors, the HER2 receptor extracellular domain has no identifiable ligand; HER2 is present in an active conformation and can undergo ligand-independent dimerization with other EGF receptors (Koutras, Evans, 2008).

Binding of ligands to the extracellular domain of ErbB receptor family members results in homo- or hetero- dimerization and subsequent activation of the intrinsic tyrosine kinase domain (Olayioye *et al.*, 2000). After ligand binding, the intracellular tyrosine kinase domain of the dimerized receptor is activated, leading to the phosphorylation of specific c-terminal tyrosine residues that serve as docking sites for proteins containing Src homology 2 (SH2) domains such as Grb2, Shc1, p85, PLCγ, and JAK1, leading to the activation of several intracellular signaling pathways (Chen *et al.*, 1987). These down-stream signaling cascades include the PI3K/Akt, JAK/STAT, NF-κB, PLCγ/protein kinase-C (PKC), and Ras/MAPK/ERK pathways and influence functions such as cell proliferation, survival, and motility (Sasaki *et al.*, 2013).

3.2. ErbB/HER family members in transformed cells

Members of the ErbB receptor family play prominent roles during carcinogenesis and most induce EMT when overexpressed both *in vitro* and *in vivo*. Simply overexpressing many of

these receptors into NIH/3T3 fibroblast cells confers a transforming phenotype (Al Moustafa et al., 2012). In agreement with the hypothesis that ErbB family members are oncogenic, EGFR blockade significantly inhibits *in vitro* and *in vivo* growth of several human carcinoma cell lines and anti-HER2 monoclonal antibodies block tumor progression in multiple cancer cell lines (Normanno *et al.,* 2003). Amplification or over-expression of *HER2* is strongly associated with increased disease recurrence and a poor prognosis in many cancers including ovarian, stomach, uterine cancer, and approximately 15–30 percent of breast cancers (Normanno *et al.,* 2006). EGFR is frequently over expressed in anal cancers and 54 percent of glioblastoma exhibit EGFR overexpression (Heimberger *et al.,* 2005; Walker *et al.,* 2009), further suggesting an *in vivo* role for ErbB receptor family members during carcinogenesis. Similarly, HER2 amplification occurs in 20 percent of breast cancers (Puglisi *et al.,* 2012) and ERBB4 confers metastatic capacity in Ewing sarcoma (Mendoza-Naranjo *et al.,* 2013). However, not all ErbB receptor family members are individually potent. Although HER3 is significantly elevated in several *in vitro* breast cancer cell lines, is overexpressed in colorectal, gastric, breast, and ovarian cancers, and HER3 overexpression is associated with worse patient survival, HER3 is not transforming on its own; HER3 appears to need cooperating mutations within other ErbB family members such as HER2 to confer oncogenic activity (Jaiswal *et al.,* 2013; Ocana *et al.,* 2013). The reason for this apparent dichotomy is being investigated and may mean either that the functions of HER proteins are interdependent or that certain ErbB heterodimer pairs are more potent than others. In support of the latter hypothesis, the most active and tumor promoting dimerization combination is thought to be the HER2/HER3 dimer (Pinkas-Kramarski *et al.,* 1998).

3.3. EGFR mutations

Although expressed in many cells, EGFR was first purified from the A431 human squamous carcinoma cell line, giving rise to the hypothesis that EGFR was an oncogene with tumorigenic capacity (Cohen *et al.,* 1982). In addition to frequent upregulation in various forms of cancer, mutations in EGFR that lead to aberrant or constitutive activation also play important roles in tumor progression. A constitutively active and highly tumorigenic EGFR form known as EGFRvIII or variant III that cannot bind ligands because it lacks the ligand-binding domain, is present in about 20 to 30 percent of glioblastomas and has also been found in medulloblastomas, breast, ovary and lung cancer, but not in healthy tissues (Ekstrand *et al.,* 1994; Nishikawa *et al.,* 1994). The progressive transformation of normal cells into highly malignant derivatives entails accumulation of a number of genetic changes. Mutations within the EGFR kinase domain resulting in constitutive activation may occur in as many as 40 percent of lung cancers (Herbst *et al.,* 2008). Roughly 20 percent of glioblastomas exhibit EGFR activating mutations due to a deletion of the ligand binding domain, resulting in constitutive EGFR receptor activation in the absence of ligand (Heimberger et al., 2005). Constitutively activating mutations within the EGFR kinase domain also decrease responsiveness to apoptotic agents and increased cell survival through the selective activation of the Akt and STAT signaling pathways in non-small cell lung cancers (Sordella *et al.,* 2004). Assumed to provide a selective advantage to tumor cells, these activating mutations within the EGFR kinase region were identified as potential therapeutic drug targets and eventually became the basis for the drug gefitinib, discussed in more detail later in this review. EGFR truncation mutants lacking the transmembrane or

tyrosine kinase domains are often observed in glioblastoma, non-small-cell lung carcinoma, breast cancer, and ovarian carcinomas and are believed to result in inappropriate receptor activation (Voldborg *et al.,* 1997). Deletion mutations within the extracellular domain of EGFR have also been found, although these typically do not result in increased activation and often occur at the same time as EGFR overexpression (Batra *et al.,* 1995).

3.4. EGFR polymorphism

Genotypic polymorphisms occur naturally and result in either single nucleotide changes within the genome or small truncations or insertions (Sachidanandam *et al.,* 2001). Single-nucleotide polymorphisms (SNPs) may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNPs that are not in protein-coding regions may still affect gene splicing, transcription factor binding, messenger RNA degradation, or the sequence of non-coding RNA. Genetic variations in the EGFR DNA sequence impact EGFR function, tumor initiation, tumor progression toward metastatic disease, and patient response to various therapeutic agents and/or treatment regimens. Because the first intron of many genes plays an important regulatory function in transcription, these regions are often polymorphic. The *EGFR* gene contains a highly polymorphic sequence in intron 1 with variable numbers of a dinucleotide simple repeat sequence, ranging from 9 to 22. Patients with CA-SSR1, a specific polymorphism resulting in a shorter EGFR gene product, demonstrated better responses and longer survival than those with longer repeats (Nie *et al.,* 2011; Nomura *et al.,* 2007). Two additional EGFR promoter region SNPs are associated with promoter activity and mRNA expression. One of the SNPs is located 191 base pairs upstream from the initiator ATG and may be correlated with increased protein expression (Han *et al.,* 2007). The other SNP, 216 base pairs upstream from the initiator, is in a region that encodes an important binding site for the transcription factor Sp1, which is necessary for activation of the *EGFR* gene (Puyo *et al.,* 2008). Interestingly, because this polymorphism influences EGFR activity, it has been suggested to be a potential predictor of clinical outcomes in non-small cell lung cancer patients treated with EGFR tyrosine kinase inhibitors (Jung *et al.,* 2012).

3.5. EGFR ligands

Under physiological conditions, growth factors typically act as signaling molecules between cells and regulate a variety of cellular processes. Non-transformed cells show an absolute requirement for growth factors in order to proliferate in culture; generally, more than one growth factor is required. Under usual culture conditions, growth factors are depleted faster than other media components and thus become rate limiting for proliferation. Decreased dependence for specific growth factors is a common occurrence in neoplastically transformed cells and may lead to a growth advantage, a cardinal feature of cancer cells (Goustin *et al.,* 1986). After initial mutations (first hit), premalignant epithelial cells may accumulate additional oncogenic mutations, but their expansion and progression to metastatic carcinomas depend on a multi-step process orchestrated primarily by growth factors. Growth factors, including those in the ErbB receptor family, support the consequent expansion of mutation-bearing clones, often leading to intraluminal lesions such as

carcinoma in situ, which are surrounded by the basal membrane (Witsch *et al.*, 2010). Unlike the paracrine mode of action of growth factors that dominates physiological processes like embryogenesis and wound healing, many cancer cells acquire the ability to synthesize growth factors to which they are responsive (Suzuki, Yamada, 1994; Venugopal et al., 2012). These autocrine loops lead to constitutive signaling via the tyrosine kinase domain and may provide the second hits that propel EMT and eruption of intracellular lesions. While growth factor receptor upregulation is far more common, some tumors also increase the expression of growth factors as another means to increase downstream signaling events that lead to increased proliferation and decreased apoptosis; amphiregulin is overexpressed in rapidly growing keratinocytic tumors and epiregulin overexpression results in aggressive non-small-cell lung cancer tumors (Billings *et al.*, 2003; Sunaga *et al.*, 2013). Studies using siRNA knockdown of EGF ligands indicate that in cancer cell lines HB-EGF and AR play pivotal roles in cancer cell proliferation (Yotsumoto *et al.*, 2009). Further supporting the hypothesis that EGF ligand overexpression plays an important role in tumor progression, overexpression of neuregulins, the natural ligands for ErbB-3 and ErbB-4, leads to increased breast cancer tumorigenicity (Atlas *et al.*, 2003).

4. EGFR SIGNALING IN NORMAL AND TRANSFORMED CELLS

Select growth factors and proto-oncogenes play pivotal roles in normal human development and are critical to normal embryonic organogenesis; deregulation of many of these genes can result in neoplastic transformation. In the case of EGFR, the posttranslational modifications, epigenetic influences, and microRNA often lead to aberrant EGFR compartmentalization, aberrant EGFR trafficking, and increased EGFR signaling due to transactivation (Figure 2). These molecular events are commonly associated with tumor formation in patient samples, most likely because the resulting constitutive activation of the EGF pathway leads to increased proliferation, increased invasiveness, increased motility, and decreased adhesion. As such, EGFR and the EGF signaling pathway appears critical to the initiation of EMT and tumor formation. At the receptor level, overexpression may enable cancer cells to become hyper-responsive to growth factors (e.g., EGFR in head and neck cancer, and ErbB-2/HER2 in breast cancer), whereas specific mutations or deletions can elicit ligand-independent signaling (e.g., brain tumor mutants of EGFR) (Hardy *et al.*, 2010). Mutations affecting downstream mediators may similarly confer growth autonomy. Signaling through the epidermal growth factor receptor (EGFR) is frequently deregulated in solid tumors, leading to abnormal activation of pro-proliferative and antiapoptotic pathways, notably the phosphatidylinositol 3-kinase/Akt, Ras/Raf/Mek/extracellular signal-regulated kinase, and the Jak/Stat pathways (Holbro, Hynes, 2004). EGFR signal transduction pathways contribute to the development of malignancies through various processes, such as effects on cell cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility and metastases (Al Moustafa et al., 2012).

4.1. Loss of cell adhesion and EGFR signaling

The majority of cancer deaths are not due to primary tumors, but occur after the primary tumor invades neighboring healthy tissue or metastasizes to distant organs within a patient's body. Under physiological conditions, migration is a highly coordinated process in normal

cells involving the precise regulation of cell adhesion and detachment from extracellular matrix (ECM) proteins (Lauffenburger, Horwitz, 1996). Functional regulation of the molecules involved in cell adhesion signaling should therefore be a key process in EGF-induced cell motility. Focal adhesion kinase (FAK) is a protein tyrosine kinase that localizes to focal adhesions, specific regions of cells that make close contacts with the ECM through transmembrane integrin molecules. Normal epithelial cells with functional adhesion receptors and FAK move as a coherent sheet, in which each cell keeps contact with its neighboring cells as well as the ECM; the ability to move individually appears to be an exclusive attribute of carcinoma cells (Danjo, Gipson, 1998). Cell motility and invasiveness are defining characteristics of tumors, which enable tumor cells to migrate into adjacent tissues or through limiting basement membranes and extracellular matrices. Invasive tumor cells are characterized by dysregulated cell motility in response to extracellular signals from growth factors and cytokines. Proliferating carcinoma cells traverse the subepithelial basement membrane by altering their attachment and secreting proteolytic enzymes that degrade the extracellular matrix (Barbolina *et al.,* 2007). Both alterations in tumor cell adhesiveness and tumor-associated protease activity facilitate migration to allow invasive tumor cells access to the surrounding stroma and vascular compartment, where they can metastasize to other sites. Invasion through the extracellular matrix is an active process that is accompanied by the destruction of some extracellular matrix structural components and modification of other components to reveal cryptic sites, such as those present in laminins and type IV collagen, that modify cell growth and migration (Davis *et al.,* 2000).

EGF promotes tumor cell motility and invasion (Al Moustafa et al., 2012). One mechanism by which growth factors, including EGFR ligands, enhance cell motility is by regulating the degree of adhesion a cell has toward its particular substrate (Manske, Bade, 1994). EGFR kinase activity, for example, stimulates migration by directly regulating focal adhesion disassembly and cell/substrate detachment (Xie *et al.,* 1998). EGF-induced inactivation of FAK results in cell detachment from the ECM, involving a disruption of cell-ECM contacts and cell-cell contacts (Lu *et al.,* 2001). EGFR regulation by other genes also impacts cell adhesion. Upon cell–cell contact the cytoplasmic NF2 gene product Merlin orchestrates adherens junction stabilization while simultaneously negatively regulating EGFR signaling by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized (Curto *et al.,* 2007). Through these actions, Merlin not only strengthens cadherin-mediated cell-cell adhesion, but also sequesters EGFR and effectively silences EGFR signaling. EGF also signals to SNAI1 through p21-activated kinase-1 (PAK1) leading to SNAI1 phosphorylation, accumulation in the nucleus and transcriptional repression of downstream target genes (Yang *et al.,* 2005).

4.2. Tumor microenvironment and EGFR signaling

It is becoming increasingly evident that tumor cells do not exist in isolation and that the interface between individual tumor cells and their immediate environment play a critical role in tumor development and metastasis. Cells neighboring the developing tumor secrete growth factors and inhibitory molecules that regulate tumor proliferation and apoptosis, while tumor cells simultaneously secrete factors to neighboring cells that regulate adhesion. As tumors develop, the integrity of the surrounding basement membrane plays a critical role

in invasion and metastasis. When intact, the basal membrane can serve to “pen in” a tumor, effectively limiting the potential for cellular damage by placing spatial constraints upon tumor cells. In this way, signals emanating from the tumor microenvironment play a critical role in tumor growth. In addition to these direct feedback mechanisms, nutrient availability is one of the first microenvironmental hurdles that tumor cells need to conquer before they can become large tumors. Data derived from examinations of human lung cancer brain metastases indicate that tumor cell division takes place within 75μm of the nearest blood vessel, whereas tumor cells residing beyond 150μm from a vessel undergo programmed cell death (Fidler *et al.,* 2002). The growth and spread of cancer is dependent on the formation of adequate vasculature to provide the nutrients needed to support tumor expansion and growth beyond 150μm (Folkman, 1992).

ErbB receptors and their ligands are involved in the cross-talk between cancer cells and different cell types of the tumor microenvironment. EGFR is activated in tumor-associated endothelial cells, but not in endothelial cells within uninvolved organ regions, suggesting that EGF receptor activation and expression of EGF receptors on endothelial cells is conditioned by the organ microenvironment (Kim *et al.,* 2003). It is also believed that cancer cells secrete EGF-like growth factors that can act directly on endothelial cells (Kuo *et al.,* 2012). The microenvironment can also send signals to tumor cells. Bone marrow stromal cells produce EGF-like peptides and angiogenic growth factors that can act on both endothelial cells and activate tumor cell EGFR to encourage synthesis of angiogenic growth factors (Fidler et al., 2002). EGFR activation in human carcinoma cell lines also increases matrix metalloproteinase-9 (MMP-9) (also known as gelatinase B or 92-kd type IV collagenase) activity, which is associated with increased *in vitro* cell invasion (Zuo *et al.,* 2011). The increased invasive activity after EGF-mediated induction of MMP-9 could be blocked by an anti-catalytic MMP-9 antibody or by synthetic low-molecular-weight or endogenous MMP inhibitors (known as the tissue inhibitors of MMPs or TIMPs). These findings indicate that EGFR activation can result in enhanced MMP-9 expression, which, in turn, facilitates removal of extracellular matrix barriers to tumor invasion.

4.3. Post-translational modifications and EGFR signaling

First identified when it was discovered that activating mutations in EGFR are frequently found in many cancers, the best-characterized posttranslational modification impacting EGFR signaling is phosphorylation, although additional modifiers of EGFR activity also influence EGFR signaling in normal and transformed cells. These findings led to the hypothesis that changes in equilibrium between EGFR active and inactive conformations were the root cause of the oncogenic potential of EGFR (Sutto, Gervasio, 2013). Indeed, the majority of known EGFR mutations linked to EMT influence the phosphorylation state of EGFR or its downstream effectors and are discussed further elsewhere in this review. The concept of EGFR phosphorylation impacting EMT is in line with previous studies that showed oncogenic Ha-Ras-transformed cells acquired characteristics of cells that have undergone EMT (Andreolas *et al.,* 2008). Although less studied, glycosylation of EGFR extracellular domains is an important mediator of ligand binding and signaling. EGFR mutations that block glycosylation at Asn^{420} and Asn^{579} alter ligand binding, decrease receptor dimerization within the membrane, and result in decreased EGFR activation

(Whitson *et al.*, 2005). Although the study of glycosylation patterns and their role in signaling is a relatively new area of exploration, it is clear that such study will be a necessary component in the characterization of EGFR and other tyrosine kinase receptors.

4.4. Epigenetic influences on EGFR signaling

Epigenetic deregulation of gene expression is involved in the initiation and progression of multiple cancers and an important initiator of EMT. Overexpression of the histone methyltransferase MMSET (multiple myeloma SET domain) in prostate cancer influences histone 3 lysine 36 dimethylation (H3K36me2) and lysine 27 trimethylation (H3K27me3). MMSET overexpression in immortalized prostatic epithelial cells leads to increased migration, increased invasion, changes in cell morphology, and changes in gene expression consistent with transition from an epithelial cell-like state to a mesenchymal cell-like state (Ezponda *et al.*, 2013). These effects are mediated by the ability of MMSET to activate the expression of *TWIST1*, a gene implicated in tumor-associated EMT and invasion and suggests that MMSET contributes to tumor progression via aberrant epigenetic regulation of genes that drive the metastatic phenotype (Yang *et al.*, 2004). Recent studies suggest that sustained activation of EMT leads to progressive epigenetic alterations and induce heritable effects that maintain the mesenchymal phenotype even after EMT-initiating signals are no longer present. Although EGFR is typically upregulated, clustered chromatin profiles using combinatorial patterns of posttranslational histone modifications and covalent changes to genomic DNA discovered a distinct chromatin signature among genes in well-established EMT pathways including EGFR, suggesting that chromatin remodeling of EGFR plays an important role in EMT (Cieslik *et al.*, 2013). Acetylation also affects EGFR expression and downstream signaling. HDAC6 regulates EGFR endocytic trafficking and degradation in renal epithelial cells; HDAC6 upregulation slows EGFR trafficking from early endosomes to late endosomes and HDAC6 inhibition results in decreased phosphorylation of ERK1/2, a downstream target of EGFR (Liu *et al.*, 2012).

4.5. MicroRNAs and EGFR

MicroRNAs (miRNAs) are a subset of small (approximately 22 nucleotides) noncoding RNA molecules found in plants, animals, and some viruses that play key roles in the regulation of transcriptional and post-transcriptional gene expression (Bartel, 2004). miRNA is transcribed by RNA polymerase II as large primary transcripts (pre-miRNA) that are approximately 70 nucleotides in length named precursor miRNA (pre-miRNA). Mature miRNAs are cleaved from this 70- to 100-nucleotide hairpin pre-miRNA by RNase III Dicer to form a miRNA duplex. One strand of the short-lived duplex is degraded, whereas the other strand serves as the mature miRNA. The mature miRNA is then incorporated into a ribonuclear particle to form the RNA-induced silencing complex, RISC, which mediates gene silencing. Similar to RNA interference, miRNAs function via base-pairing with complementary sequences within mRNA molecules. As a result, these mRNA strands are often actively disassembled by the cell because they can no longer be translated into proteins by ribosomes, resulting in gene silencing (Hutvagner, Zamore, 2002). miRNA-mediated regulation has been shown to be involved in a wide range of biological processes such as cell cycle control, apoptosis and several developmental and physiological processes including stem cell differentiation, hematopoiesis, hypoxia, cardiac and skeletal muscle development,

neurogenesis, insulin secretion, cholesterol metabolism, aging, immune responses and viral replication. In addition, highly tissue-specific expression and distinct temporal expression patterns during embryogenesis suggest that miRNAs play a key role in the differentiation and maintenance of tissue identity.

In addition to their important roles in healthy individuals, miRNAs have also been implicated in a broad range of cancers, heart disease and neurological diseases (Steinfeld *et al.,* 2013). Consequently, miRNAs are intensely studied as candidates for diagnostic and prognostic biomarkers and predictors of drug response. Non-coding miRNAs are increasingly recognized as important players in EMT and EGFR regulation. Because a single miRNA can target several messenger RNAs, dysregulation of miRNAs can effectively affect multiple signaling pathways leading to tumor formation and metastasis. In particular, the miR-200 micro RNA family has been identified as new epithelial markers and repressors of EMT by directly targeting two genes that repress E-cadherin expression (Park *et al.,* 2008). Another important EMT-related miRNA is miR-7, which regulates EGFR and consequently the PI3K/Akt pathway in lung cancer to influence differentiation, proliferation, and cell survival (Webster *et al.,* 2009). Also involved in proliferation, self-renewal, differentiation, and tumor growth, miR-128 expression is repressed in glioblastoma, targets EGFR, and may be a candidate glioma tumor suppressor (Papagiannakopoulos *et al.,* 2012). A genome-wide miRNA (miRNome) screen coupled with high-throughput protein level monitoring identified three miRNAs (miR-124, miR-193a-3p, and miR-147) as novel tumor suppressors that co-target EGFR-driven cell-cycle network proteins and inhibit cell-cycle progression and proliferation in breast cancer (Uhlmann *et al.,* 2012). Supporting a role as a tumor suppressor, ectopic expression of miR-124 inhibits tumor migration and invasion and miR-124 expression is reduced in glioblastoma (Fowler *et al.,* 2011). Similarly, miR-193 is epigenetically silenced in acute myeloid leukemia (AML) and targets the c-Kit oncogene leading to apoptosis (Gao *et al.,* 2011). NF-κB and STAT1α bind to the miR-147 promoter in as part of the inflammatory response, resulting in the inhibition of cytokine expression and prevention of excessive inflammatory responses (Liu *et al.,* 2009). Other miRNAs, such as *miR-195* and *miR-122*, have been proposed as clinical markers of *EGFR* mutation status and may provide prognostic value in predicting survival in non-smoking female patients with lung adenocarcinoma (Zhang *et al.,* 2013). In addition to being regulated by miRNA, EGFR also modulates miRNA maturation in response to hypoxia through phosphorylation of AGO2 (Shen *et al.,* 2013).

4.6. Compartmentalization and trafficking of EGFR

The duration of EGFR signaling is regulated by the internalization and degradation of the receptor; the activated receptor/ligand complex is endocytosed and either degraded within the lysosomes, or recycled to the plasma membrane (Goh, Sorkin, 2013). EGFR endocytosis and degradation induces down-regulation of the growth factor induced signal and requires EGFR kinase activity and N-terminal transmembrane dimerization motif (Heukers *et al.,* 2013). Ubiquitination of the receptor and endocytic adaptor proteins are critically important in mediating the endocytic pathways that regulate EGFR internalization and downstream signal transduction, especially by the Cbl family of E3 ubiquitin ligases. The Cbl family consists of three mammalian homologs (c-Cbl, Cbl-b and Cbl-3) and contain conserved

regions that enable Cbl family members to recognize and interact with phosphotyrosine-containing proteins (Liu *et al.,* 2014a). Following EGFR activation, Cbl is recruited through its constitutive binding partner Grb2 which directly binds to the EGFR tyrosine kinase binding domain via its SH2 domain (Waterman *et al.,* 2002). Recent structural studies suggested that once bound, Cbl becomes phosphorylated, which enables binding of the ubiquitin-loaded E2 complex and leads to stimulation of Cbl E3 ligase activity, resulting in the subsequent multi-monoubiquitination and polyubiquitination of the EGFR (Kobashigawa *et al.,* 2011). Under normophysiologic conditions, EGFR can translocate to the nucleus upon ligand binding, where it can modulate gene transcription by interacting with STAT3 (Lo *et al.,* 2005). Nevertheless EGFR also colocalizes with focal adhesions in glioblastoma samples from patients before it receives activating signals, thus acting as a constitutively active receptor (Dasari *et al.,* 2012). The BS-153 glioblastoma cell line overexpresses normal EGFR as well as a mutant, EGFRvIII, which is constitutively activated and poorly internalized (Huang *et al.,* 1997), resulting in enhanced tumorigenicity.

4.7. EGFR transactivation

Transactivation occurs when the expression rate of a gene is increased by the induced expression of an intermediate transactivator protein, and is another mechanism that can induce EGFR tyrosine phosphorylation and subsequent stimulation of intracellular signaling pathways. EGFR signal transactivation by G-protein coupled receptors (GPCRs) was originally described by Daub and colleagues (Daub *et al.,* 1996), who found that treatment of Rat fibroblasts treated with LPA (lysophosphatidic acid), ET-1 (endothelin-1) or thrombin resulted in rapid, transient EGFR phosphorylation and subsequent activation of downstream signaling events such as MAPK phosphorylation or c-*fos* gene expression. These signaling events depend on EGFR function, as the specific EGFR kinase inhibitor AG1478 and a dominant-negative EGFR mutant abrogated this GPCR-induced signaling. Various studies further demonstrated that GPCR-induced EGFR signal transactivation occurs in a variety of cell types, including vascular smooth muscle cells, human keratinocytes, primary mouse astrocytes and PC12 cells (George *et al.,* 2013). In cells treated with a GPCR receptor agonist, GPCRs stimulate metalloproteinases, which induce cleavage of EGF-like ligand precursors, leading to phosphorylation of ErbB receptors (Prenzel *et al.,* 1999). Cytokines such as growth hormone (GH) and prolactin (Prl) can indirectly activate ErbB receptors through Janus tyrosine kinase 2 (Jak2), which phosphorylates specific tyrosine residues in the cytoplasmic domains of EGFR or ErbB-2 (Yamauchi *et al.,* 1997), (Yamauchi *et al.,* 2000). Similarly, Src phosphorylates various residues on EGFR, leading to enhanced receptor signaling (Biscardi *et al.,* 1999). For these reasons, the ErbB receptors also function as signal integrators through their interaction with different signaling proteins and membrane receptors.

5. CROSSTALK BETWEEN EGFR SIGNALING AND OTHER MAJOR SIGNALING PATHWAYS IN TRANSFORMED CELLS

EMT can be initiated by multiple extracellular signals and there is a significant crosstalk among the downstream intracellular signaling pathways and transcription factors that together choreograph this process. During the last decade, it has become obvious that

progression and severity of malignant diseases is often not caused by a single genetic aberration or deregulation of a single signaling pathway, but actually requires the cooperation of oncogenic signaling pathways in cancer cells and the ErbB receptors can function as signal integrators. As such, it is only natural that crosstalk exists between EGFR signaling and other major signaling pathways (Figure 3).

5.1. MET

EGFR and c-MET (Mesenchymal epithelial transition factor, aka hepatacyte growth factor receptor) trigger the same signal transduction pathways and elicit similar molecular responses suggesting that both of these signaling pathways converge on the same downstream mediators. In agreement with the hypothesis that signaling crosstalk exists between the MET and EGFR signaling pathways, MET is known to interact with EGFR and acts as a compensatory pathway for EGFR signaling (Jun *et al.,* 2014). The only known ligand for the MET receptor is the hepatocyte growth factor (HGF), which activates MET upon binding and triggers the signaling of MAPK and AKT, common downstream targets of the EGFR family. Crosstalk between EGFR and c-MET induces proliferation, invasion and migration in glioblastoma cells and therefore contributes to tumorigenesis (Dasari et al., 2012). MET co-immunoprecipitates with EGFR regardless of the existence of their ligands in tumor cells, but not in normal human hepatocytes, and this association facilitates the phosphorylation of MET in the absence of hepatocyte growth factor (Jo *et al.,* 2000). The cross-talk between MET and EGFR has significant implications for resistance to chemotherapy and altered growth control during tumorigenesis. Studies in lung, breast, and colon cancer cells have shown that activation of MET can lessen the inhibitory effects of drugs designed specifically to target members of the EGFR family (Liska *et al.,* 2011), while HER kinase activation confers resistance to MET inhibition in some gastric cancer cells (Corso *et al.,* 2010).

5.2. TGF-β

While members of the transforming growth factor-beta (TGF-β) receptor family can either display context-dependent tumor suppressive or tumor-promoting activity, TGF-β family members are the best-characterized inducers of EMT. TGF-β activates multiple distinct signaling mechanisms that are either Smad-dependent or independent. A genetic modifier screen in nontumorigenic mammary epithelial cells identified TGF-β1 and TGF-β3 as molecules that cooperate with HER2 in inducing cell motility and invasion (Seton-Rogers *et al.,* 2004). Furthermore, TGF-β can activate signaling pathways downstream of ErbB receptor tyrosine kinases such as Ras/mitogen-activated protein kinase and phosphoinositide-3 kinase (PI3K)/Akt (Biver *et al.,* 2014). Overexpression of active TGF-β1 or active mutants of the type I TGF-β receptor (Alk5) in the mammary gland of bitransgenic mice also expressing mouse mammary tumor virus-Neu (ErbB2) accelerates metastases from Neu-induced mammary cancers (Slattery *et al.,* 2013). Inhibition of HER2 with the HER2-neutralizing antibody trastuzumab blocked the pro-migratory effect of TGF-β on HER2-overexpressing mammary epithelial cells (Ueda *et al.,* 2004), suggesting that oncogene function is required for the transforming effect of TGF-β. TGF-β induces HER2 translocation to the lamellipodia through a PI3K-dependent mechanism that involves

activation of Rac1 and Rak1 and reorganization of the actin cytoskeleton, ultimately prolonging Rac1 activation and decreasing apoptosis (Wang *et al.*, 2006).

5.3. IGFR

Crosstalk between the IGF-induced EMT suppresses the erlotinib-sensitizing effect of EGFR exon 19 deletion mutations in non-small cell lung carcinoma (NSCLC) cells (Cufi *et al.*, 2013). *IGF1R* gene silencing in two human squamous cancer cell lines (SKUT-1 and MDA-MB-468) led to significant enhancement of EGFR phosphorylation, although this phenomenon did not abrogate the inhibitory effects of IGF1R knockdown on tumor cell survival. Interaction was abolished by knockdown of either receptor, and EGFR knockdown also suppressed IGF1R protein levels. EGFR depletion also induced enhancement of IGF1R ubiquitylation and degradation (Riedemann *et al.*, 2007). Reciprocal co-precipitation between the IGF1R and EGFR could be detected in two squamous cancer cell lines and clinical samples of breast cancer (Riedemann et al., 2007). Clinical therapies targeting EGFR (described in more detail below) exhibit significant anti-cancer activity, but resistance to these drugs has developed through compensatory activation of IGF signaling (Chakravarti *et al.*, 2002).

5.4. SHH

The first suggestion that the Sonic hedgehog (SHH) pathway and EGFR signaling pathways could crosstalk came when it was found that activation of either pathway resulted in a malignant transformation of human keratinocytes through induction of the MEK/ERK/JUN pathway (Schnidar *et al.*, 2009). The interaction of HH/GLI with EGF-induced signaling has since been described in esophageal, pancreatic, and skin cancer (Aberger, Ruiz, 2014). Additional studies indicated that several genes possess binding sites for GLI and EGF-regulated transcription factors, such as c-JUN/AP-1 (Gotschel *et al.*, 2013) and provided further evidence supporting the hypothesis that both pathways may merge at the level of transcriptional regulation. Cooperative effects between the SHH and EGFR pathways were also seen at the level of protein activation. SHH activation results in murine embryonic stem cell proliferation, but these effects are mediated by cooperation between the EGFR, SHH, and PKC signaling pathways (Heo *et al.*, 2007). Mechanistically, it was found that SHH pathway activation in neural stem cells and in HeLa cells resulted in EGFR internalization and transient activation of the MAPK/ERK signaling cascade, without detectable ubiquitination (Reinchisi *et al.*, 2013). EGF also impacts SHH signaling, as GLI1 transcription factors need to be stabilized through direct phosphorylation by ERK, a known downstream target of the EGFR pathway (Whisenant *et al.*, 2010).

5.5. Wnt

ErbB transactivation has been shown to involve Wnt, which binds to frizzled (Fz) receptors and stimulates EGFR tyrosine kinase activity through metalloproteinase-mediated cleavage of EGF-like ligands (Civenni *et al.*, 2003). Wnt signaling can lead to EMT through inhibition of glycogen synthase kinase-3β (GSK-3β) mediated degradation of β-catenin which then translocates into the nucleus to activate transcription factors involved inducing EMT associated genes (Yang *et al.*, 2011). EGFR signaling can promote the Wnt/β-catenin signaling through the stabilization and subsequent nuclear accumulation of β-catenin, which

depends on several EGFR-regulated mediators including ERK and MAPK (Krejci *et al.*, 2012). The EGFR signaling pathway also leads to downregulation of caveolin-1, which leads to loss of E-cadherin, transcriptional activation of β-catenin and enhanced invasiveness (Lu *et al.*, 2003).

5.6. Notch

Dependent on the cell type, Notch signaling can be either oncogenic or tumor suppressive due to the complexity of signaling involving multiple receptors, ligands and downstream mediators. Aberrant Notch signaling is found in several solid cancers including breast, colon, and colorectal cancer as well as medulloblastoma and melanoma and in certain leukemias and crosstalk between EGFR and Notch pathways occurs in gliomas, lung, skin cancers, and breast cancers (Guo *et al.*, 2012). Inhibition of either EGFR or Notch signaling alone was insufficient to suppress basal-like breast tumor cell survival and proliferation. However, simultaneous inhibition of EGFR and Notch signaling uncovered crosstalk between these two oncogenic pathways (Dong *et al.*, 2010). Forced overexpression of Notch1 by transfection increased EGFR expression in human breast cancer cells and overexpression of Notch1 reversed EGFR inhibitor–induced cell toxicity, suggesting that Notch and EGFR signaling may be positively crosslinked in human breast cancer (Dai *et al.*, 2009). Down-regulation of Notch1 by RNA interference had little or no suppressive effects on the proliferation of either ErbB2-positive or ErbB2-negative cell lines. In contrast, down-regulation of Notch3 significantly suppressed proliferation and promoted apoptosis of ErbB2-negative tumor cells. Targeted suppression of the Notch3 signaling pathway may be a promising strategy for the treatment of ErbB2-negative breast cancer (Yamaguchi *et al.*, 2008). Notch3 rather than Notch1-mediated signaling plays an important role in the proliferation of ErbB2-negative breast tumor cells. HER2-overexpressing cells display activated Notch1 signaling and Notch1 signal inhibition by small interfering RNA or γ-secretase inhibitor resulted in down-regulation of HER2 expression and decrease of sphere formation of carcinoma cell lines (Magnifico *et al.*, 2009). Also, Notch signaling regulates HER2 activity through Notch-binding sequences contained in the HER2 promoter (Chen *et al.*, 1997).

6. THERAPY

A finite number of major signaling pathways appear to control carcinogenesis, suggesting that a cure for cancer should be achievable by simply suppressing these signaling pathways. However, clinical success has not matched scientific expectations, most likely because many oncogenic signaling pathways can either promote or suppress carcinogenesis depending on tissue type, cancer stage, genetic mutations and crosstalk within the signaling pathways. The molecular mechanisms that promote cell proliferation and mediate cell survival differ among tumor cells, but are responsible for the varying degrees of sensitivity to EGFR inhibition. As tumor cells use varying mechanisms for proliferation and survival, even among tumors of the same primary origin, identifying biomarkers is critical for selecting patients who are likely to receive the most clinical benefit from EGFR inhibitors. Mutations in EGFR, KRAS, and anaplastic lymphoma kinase (ALK) are mutually exclusive in non-small cell lung cancer (NSCLC) patients. Since the presence of one mutation in lieu of another can drastically

influence therapeutic responses testing for these mutations and tailoring therapy accordingly is widely accepted as standard practice (Savas *et al.,* 2013).

6.1. EGFR as target for cancer therapy

ErbB receptors are expressed at high levels in various types of cancer, and their gene/protein expression levels are correlated with the growth, state, and aggressiveness of cancer (Wieduwilt, Moasser, 2008). Specifically, EGFR overexpression has been reported in non-small cell lung, head and neck, pancreas, and breast, and has been shown to correlate with poor survival (Grandis, Sok, 2004). For these reasons, EGFR became an attractive target for cancer therapy (Normanno et al., 2003). The discovery that somatic mutations in the EGFR gene are found in a subset of lung adenocarcinomas and are associated with sensitivity to the EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib generated excitement among clinicians and researchers studying non–small cell lung cancer (Riely *et al.,* 2006). Expression of other ErbB family members is also correlated with various forms of cancer. For example, HER2/neu is amplified and/or overexpressed in 7 percent to 35 percent of invasive gastric cancers, and high levels of HER2 are associated with worse clinical outcome (De Vita *et al.,* 2010). However, care should be taken when using ErbB family members as therapeutic targets. Her3 is expressed in normal human tissues, with a high density on mature and differentiated cells of the gastrointestinal tract and in the neurons of the central nervous system (Rajkumar, Gullick, 1994). Without proper specificity, therapeutics targeting Her2 could theoretically also impact Her3, leading to gastrointestinal and neuronal side effects.

6.2. Anti-EGFR therapy approaches

There are 2 main therapeutic strategies which have been implemented to inhibit EGFR: (a) monoclonal antibodies directed at the EGFR extracellular domain such as cetuximab (Imclone/Bristol Myers) and panitumamab (Abgenix/Amgen) and (b) small molecule selective EGFR and Her family antagonists such as gefitinib (Astra Zeneca), lapatinib (GlaxoSmithKline) and erlotinib (OSI Pharmaceuticals/Genetech/Roche). Anti-EGFR antibodies have shown clinical utility, including cetuximab and panitumamab which are approved for the treatment of EGFR-expressing, metastatic colorectal carcinoma (Baselga, Arteaga, 2005). Small molecule inhibitors are also a promising strategy, as treatment with gefitinib significantly reduced the expression and activation of uPA and MMP-9 in prostate cancer cells, and this phenomenon was associated with a significant decrease in the ability of these cells to form bone metastases (Angelucci *et al.,* 2006). Lapatinib, a dual inhibitor of EGFR and Her2, delays progression of trastuzumab-refractory breast cancer by interrupting the downstream signaling pathways such as MAPK and AKT (Wainberg *et al.,* 2010). Additional small molecule dual EGFR-Her2 inhibitors which bind irreversibly are in earlier stages of clinical development, because although the current EGFR inhibitors provide significant clinical benefit when compared to the current standard of care, not all patients derive a benefit in terms of overall survival (Shepherd *et al.,* 2005). The mAb cetuximab is a human-mouse anti-ErbB-1 chimeric IgG1 antibody, which has shown efficacy in colorectal cancer and in head and neck cancer (Bonner *et al.,* 2006; Cunningham *et al.,* 2004). Cetuximab binds to the ligand-binding domain of ErbB-1 and prevents dimerization and subsequent activation by autophophorylation (Li *et al.,* 2005). Panitumumab is a fully human

IgG2 antibody specific to ErbB-1, which is effective and well tolerated in colorectal cancer (Carteni *et al.,* 2007). Nimotuzumab is yet another humanized mAb that inhibits EGF binding and shows effectiveness in nasopharyngeal cancer and in glioma. One striking feature of Nimotuzumab is the absence of severe adverse effects, such as skin rash, which commonly associate with similar mAbs (Perez-Soler *et al.,* 2005). Low toxicity of nimotuzumab might be due to intermediate affinity and incomplete abrogation of the active conformation (Talavera *et al.,* 2009). Trastuzumab is an ErbB-2/HER2-specific mAb, which was approved in 1998 for the treatment of metastasizing breast cancer, only if tumors overexpress ErbB-2/HER2 and secrete no soluble version of this protein (Slamon *et al.,* 2001). By suppressing ErbB-2/HER2 signaling, trastuzumab interferes with cell cycle control, angiogenesis, and the PI3K pathway. Another mechanism of action of trastuzumab involves the induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (Musolino *et al.,* 2008). Yet another potential mechanism entails antibody-induced degradation of ErbB-2/HER2, a process enhanced on combining two antibodies directed at distinct sites of the oncoprotein (Ben-Kasus *et al.,* 2009). Compared with mAbs, TKIs are low molecular weight mimics of ATP. Along with mono-specific inhibitors like the EGFR inhibitor erlotinib (approved for treatment of lung and pancreatic cancer), pan-ErbB, or dual-specificity TKIs, like lapatinib, show encouraging clinical efficacies. Moreover, lapatinib holds promise for treatment of trastuzumab-resistant patients (Xia *et al.,* 2007). Another class of experimental therapeutics comprises inhibitors of heat shock proteins (HSPs), chaperones involved in the folding and conformational maturation of signaling proteins, including ErbB2. Disruption of HSP90 results in ubiquitylation and proteasomal degradation of ErbB2, leading to abrogation of the PI3K/AKT and cyclin D pathways (Citri *et al.,* 2004). EMT influences the response of certain cancers to EGFR-targeted therapeutics. For instance, the sensitivity of cancer cell lines to erlotinib or gefitinib depends on the EMT status rather than EGFR levels (Thomson *et al.,* 2005). The restoration of E-cadherin alleviated resistance to these kinase inhibitors (Witta *et al.,* 2006). Treatment of human squamous cell carcinoma of head and neck cells with the anti-EGFR blocking antibody C225 (cetuximab) significantly reduced their ability to invade surrounding tissues, including bone, and this inhibition was associated with downregulation of MMP-9 expression (Huang *et al.,* 2002). Conversely, head and neck squamous cell carcinoma, pancreatic, colorectal and bladder carcinoma that express EMT markers are more resistant to EGFR antagonists (Buck *et al.,* 2007).

6.3. Resistance to EGFR therapy

Despite the success in some cancers, targeted ErbB therapy has remained a challenge, mainly due to intrinsic or acquired resistance to TKIs and therapeutic antibodies. Single agents targeting EGFR have shown only modest activity in clinical trials for head and neck squamous cell carcinomas due to intrinsic and acquired resistance, resulting in a need to develop more effective strategies to improve EGFR-targeted therapy (Chen *et al.,* 2010). Similarly, gefitinib and erlotinib effectively target EGFR in individuals with non–small cell lung cancer, but these therapeutic agents are ultimately limited by the emergence of mutations and other molecular mechanisms conferring drug resistance (Politi *et al.,* 2010). Early clinical studies with anti-HER2 therapy such as lapatinib have shown promising results; however, many of the patients who initially responded eventually developed

resistance. One possible mechanism of resistance is activation of an alternate receptor tyrosine kinase that restores the signaling pathways. Treatment of HER2-amplified tumor cells with small molecule EGFR inhibitors leads to a compensatory increase in HER3 expression, HER3 membrane localization, and decreased HER3 dephosphorylation, resulting in significantly enhanced HER3 signaling (Sergina *et al.,* 2007). MET activation abrogates the sensitivity of non–small cell lung cancer cells to an analogue of lapatinib (Agarwal *et al.,* 2009), exemplifying the concept that that mutations in EGFR signaling cascade downstream of the drug target can also lead to drug resistance. The selection of patients who will most benefit from treatment with specific molecularly targeted therapies is an increasingly important goal.

7. CONCLUDING REMARKS

Understanding the consequences and complexity of EGF signaling and how it relates to tumor progression is critical for the development of clinical compounds and establishing clinical protocols for the treatment of cancer. EGFR and other members of the ErbB/Her receptor family are often aberrantly regulated in tumors, resulting in many of the phenotypic changes that are hallmarks of EMT and the initial stages of tumorigenesis. Altered EGFR signaling results in transcriptional changes that increase proliferation in cellular changes in adhesion that result in decreased cellular adhesion to substrates, increased invasiveness and increased motility. These cellular changes are especially critical for tumor progression, because they mark a transition toward metastasis. EGFR signaling interacts with a multitude of other important signaling pathways, resulting in complex crosstalk that needs to be taken into account as therapeutic regimens are designed for patients. Indeed, single chemotherapeutic regimens targeting EGFR may not be as effective as dual-agent regimens meant to restrict EGFR and a compensatory signaling mechanism, such as MEK. As more insights into how the molecular mechanisms of EGFR signaling impacts cellular transformation and tumor progression, therapeutic regimes must be reassessed.

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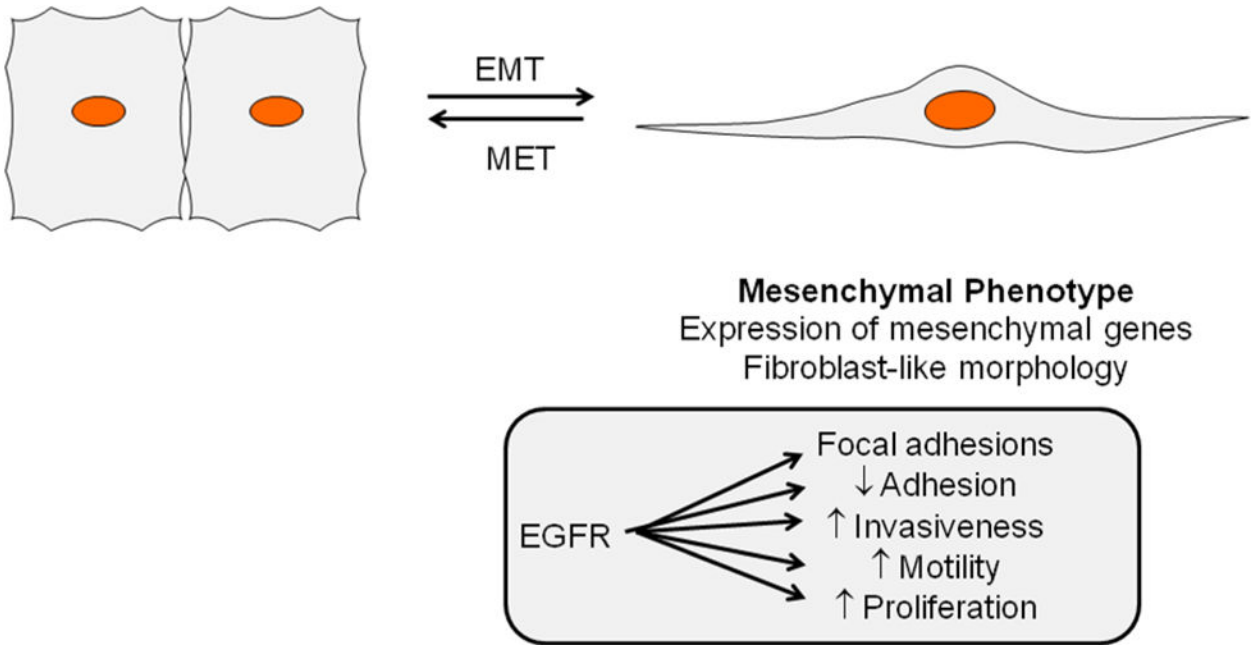

Figure 1. EGFR and the hallmarks of EMT

Schematic highlighting the phenotypic changes cells undergo during EMT. Several of these phenotypic changes are directly regulated by EGFR.

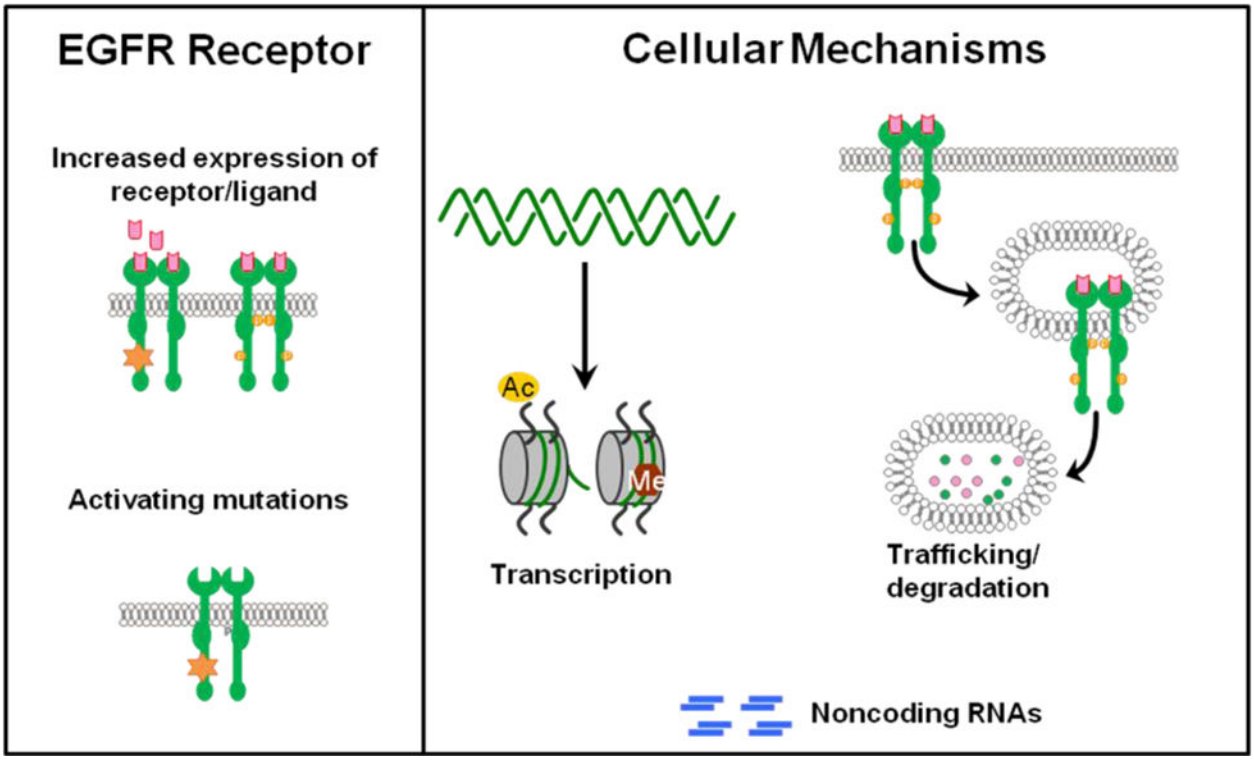

Figure 2. Changes in transformed cells that can affect EGFR signaling

Representative molecular mechanisms resulting in increased EGFR signaling in transformed cells. At the receptor level, upregulation of EGFR or its ligands and activating mutations of EGFR are shown. Cellular mechanisms impacting EGFR signaling in transformed cells include epigenetic histone modifications (including acetylation and methylation) to regulate transcription, and decreased degradation either through altered endocytosis or miRNA expression regulating EGFR levels and signaling.

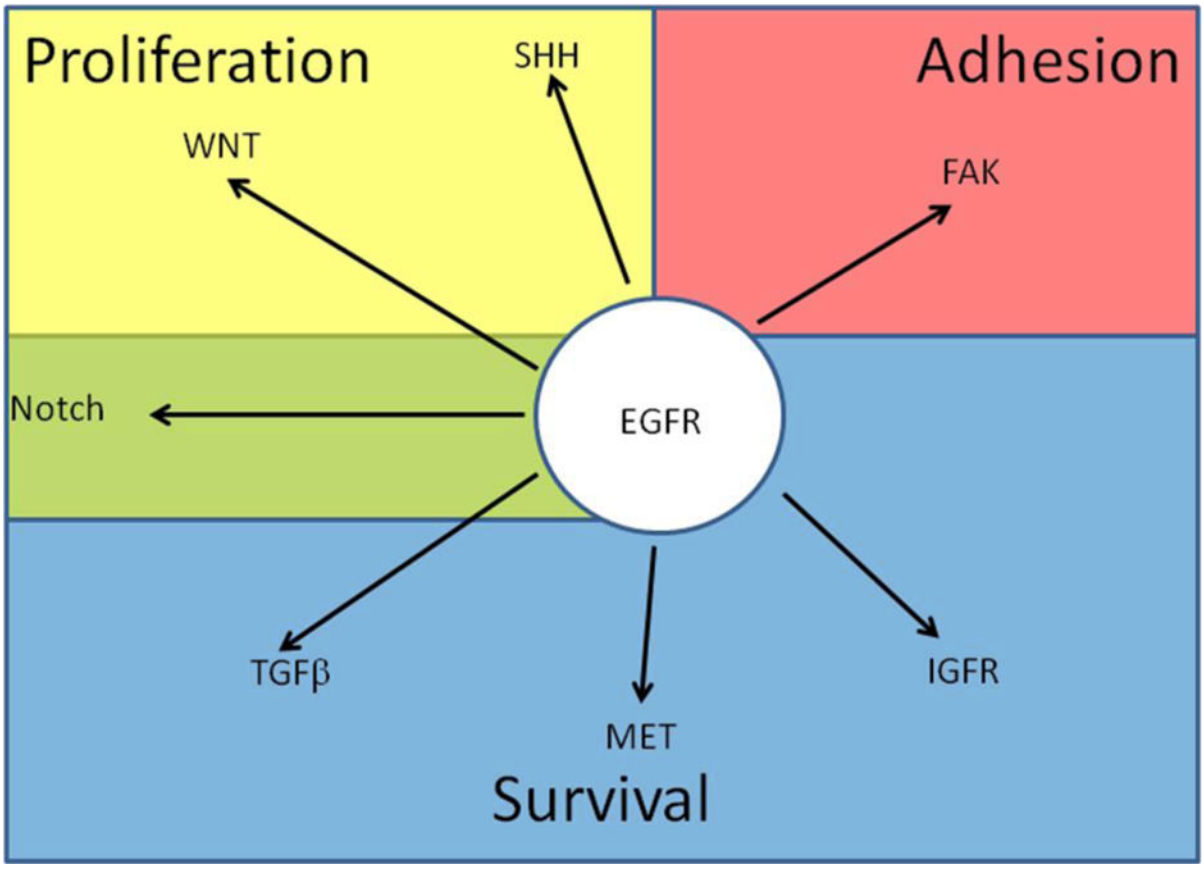

Figure 3. Crosstalk between EGFR signaling and other signaling pathways affected in cancer

Highlighted are several key signaling pathways known to interact with EGFR signaling and their broad impact on cellular processes in transformed cells.