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Landscape of EGFR Signaling Network in Human Cancers: Biology and Therapeutic Response in Relation to Receptor Subcellular Locations

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Abstract

The epidermal growth factor receptor (EGFR) pathway is one of the most dysregulated molecular pathways in human cancers. Despite its well-established importance in tumor growth, progression and drug-resistant phenotype over the past several decades, targeted therapy designed to circumvent EGFR has yielded only modest clinical success in cancer patients, except those with non-small cell lung cancer (NSCLC) carrying EGFR activation mutations. However, almost all of these NSCLC patients eventually developed resistance to small molecule EGFR kinase inhibitors. These disappointing outcomes are, in part, due to the high complexity and the interactive nature of the EGFR signaling network. More recent compelling evidence further indicates that EGFR functionality can be dependent on its subcellular location. In this regard, EGFR undergoes translocation into different organelles where it elicits distinctly different functions than its best known activity as a plasma membrane-bound receptor tyrosine kinase. EGFR can be shuttled into the cell nucleus and mitochondrion upon ligand binding, radiation, EGFR-targeted therapy and other stimuli. Nuclear EGFR behaves as transcriptional regulator, tyrosine kinase, and mediator of other physiological processes. The role of mitochondrial EGFR remains poorly understood but it appears to regulate apoptosis. While studies using patient tumors have shown nuclear EGFR to be an indicator for poor clinical outcomes in cancer patients, the impact of mitochondrial EGFR on tumor behavior and patient prognosis remains to be defined. Most recently, several lines of evidence suggest that mislocated EGFR may regulate tumor response to therapy and that plasma membrane-bound EGFR elicits survival signals independent of its kinase activity. In light of these recent progresses and discoveries, we will outline in this minireview an emerging line of research that uncovers and functionally characterizes several novel modes of EGFR signaling that take center stage in the cell nucleus, mitochondrion and other subcellular compartments. We will also discuss the clinical implications of these findings in the rationale design for therapeutic strategy that overcomes tumor drug resistance.

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1. Introduction

Epidermal growth factor receptor (EGFR) was isolated approximately two decades after the discovery of its ligand EGF in 1962 [1; 2; 3]. The importance of EGFR in protein phosphorylation [1; 4; 5] and in tumorigenesis [6] was later-established. Since then, the EGF-EGFR signaling axis has taken the center stage of not only cancer research, but also developmental biology for over three decades. EGFR is best known for its classical function as a receptor tyrosine kinase localized on the plasma membrane and activated upon ligand binding (Figure 1). Activated EGFR recruits a number of downstream signaling molecules, leading to the activation of several major pathways that are important for tumor growth, progression, and survival [7; 8; 9]. The main pathways downstream of EGFR activation include those mediated by PLC-γ–PKC, Ras-Raf-MEK, PI-3K-Akt-mTOR and JAK2-STAT3. Similar to EGFR, the EGFRvIII variant is primarily localized on the cell-surface where it activates several signaling modules. However, unlike EGFR, EGFRvIII is constitutively active independent of ligand stimulation, in part, due to its loss of a portion of the ligand-binding domain [10; 11; 12; 13].

While EGFR overexpression is found in many types of human cancers, EGFRvIII is predominantly detected in malignant gliomas [10; 11; 12; 13]. Both EGFR and EGFRvIII play critical roles in tumorigenesis and in supporting the malignant phenotypes in human cancers. Consequently, both receptors are targets of anti-cancer therapy. Several EGFR-targeting small molecule kinase inhibitors and therapeutic antibodies have been approved by the FDA to treat patients with breast cancer, colorectal cancer, non-small cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck, and pancreatic cancer. Despite extensive efforts invested in the preclinical and clinical development of EGFR-targeted therapy, the current treatments have demonstrated only modest effects on most cancer types [9; 14; 15; 16], with the exception of NSCLC that expresses gain-of-function EGFR mutants [17; 18; 19]. However, almost all of these NSCLC patients eventually developed resistance to small molecule EGFR kinase inhibitors [20; 21]. This acquired resistance has been shown to be linked to a secondary EGFR T790M mutation in about a half of patients [22; 23; 24]. The resistance can be attributed to other potential mechanisms, such as, uncontrolled activation of MET [25; 26] and subsequent MET-mediate HER3 activity [27; 28], and activated insulin-like growth factor-1 receptor [29; 30]. Since lung cancer-associated EGFR mutations are either absent or very rare in other tumor types, there is an imminent need to indentify the mechanisms underlying tumor resistance to anti-EGFR agents in order to derive sensitization strategies that can be used to overcome the resistance [31].

In addressing the need of gaining a deeper understanding of the EGFR pathway and EGFR-associated malignant biology in human cancer, compelling evidence indicates that plasma membrane-bound EGFR can mediate cellular processes independent of its kinase activity [32; 33; 34; 35; 36; 37; 38]. This atypical mode of EGFR signaling could contribute to the failure of the majority of EGFR-targeted agents that are designed to inhibit its kinase activity. Also compelling are the facts that both EGFR and EGFRvIII undergo nuclear and mitochondrial transport and that within these organelles, the receptors exert novel functions that are distinctly different from their classical role as a receptor tyrosine kinase [31; 39]. To date, EGFR nuclear accumulation has been linked to several malignant phenotypes of human cancers, including proliferation, inflammatory response, DNA repair and therapeutic resistance and poor clinical outcomes in cancer patients [40; 41; 42; 43]. Over the past few years, it has become clear that both EGFR and EGFRvIII undergo ligand- and treatment-induced mitochondrial localization. However, regulation and consequences of the mitochondrial mode of EGFR signaling are still poorly understood despite being actively investigated. These exciting discoveries and recent advances in the landscape of the EGFR signaling provide the foundation for this minireview in which we will summarize our current

knowledge of the classical and atypical modes of the EGFR pathway and provide a timely outline of their impact on malignant tumor biology as well as therapeutic response of human cancers to currently available agents.

2. Cell-surface and Cytoplasmic Modes of EGFR Signaling

2.1. Kinase-dependent functions

The best known ligands of EGFR are EGF, transforming growth factor-α, and heparin-binding EGF-like growth factor. As summarized in Figure 1, upon ligand binding, activated EGFR recruits, phosphorylates and activates a number of important signaling molecules such as PLC-γ, Ras, PI-3K and JAK2 [9; 44; 45; 46; 47]. Activated EGFR also phosphorylates signal transducer and activator of transcription-3 (STAT3) at Y705 and activates its dimerization, nuclear transport and subsequent gene regulation [46; 48; 49; 50; 51]. For example, EGFR-activated STAT3 has been shown to activate the expression of an E-cadherin repressor, TWIST, and thereby-, promote epithelial-mesenchymal transition [51]. These EGFR downstream signaling cascades can also be activated via EGFR-independent mechanisms whereby regulating tumorigenesis, tumor proliferation and progression, and therapeutic resistance [6; 52; 53; 54; 55].

2.2. Kinase-independent functions

Independent of kinase activity or ligand activation, EGFR can mediate cellular processes mostly through its ability to physically interact with other proteins (Figure 1). One of the first observations suggesting this interesting phenomenon derived from the notion that loss of EGFR kinase activity did not lead to the phenotypes similar to ablation of EGFR expression. In this context, EGFR knockout animals were found to survive for up to eight days after birth and suffer from impaired epithelial development in several organs including skin, lung and gastrointestinal tract [56], whereas the animals with kinase-dead EGFR were viable despite having skin and eye abnormalities [57]. In line with these findings, Coker et al [32] showed that the kinase-dead EGFR D813A mutant retained the ability to stimulate DNA synthesis. Co-expression of the kinase-dead EGFR K721M mutant with HER2 rescued the inability of the mutant EGFR to activate Akt and MAPK, suggesting that heterodimerization with other members of the ErbB family of receptors may help support the kinase-independent function of EGFR [33].

In agreement with these reports, Ewald et al [34] showed that the kinase-dead EGFR K721R mutant retained the ability to survive serum starvation-induced death, while losing its ability to respond to EGF or to stimulate cell growth. Interestingly, the same study found another kinase-dead EGFR mutant D813A to lose both growth-stimulating and prosurvival properties, suggesting that the prosurvival activity of EGFR is independent of the kinase activity but likely dependent of its unique structural properties to associate with other cellular proteins. This notion is in line with a more recent report by Weihua et al [35] showing that loss of expression of EGFR, but not its kinase activity, resulted in autophagic cell death. The authors found that reduced intracellular glucose levels, leading to autophagy in EGFR-deficient cells, was due to the degradation of sodium/glucose cotransporter 1, SGLT1, a plasma membrane-bound protein that enables glucose uptake. Interestingly, cell-surface EGFR was found to physically interact with and stabilize SGLT1 independent of its kinase activity, thereby maintaining high glucose levels in the cells. Conversely, EGFR expression knockdown, but not kinase inhibition, led to SGLT1 degradation, reduction in intracellular glucose and subsequent autophagic cell death [35]. In support of these observations, co-expression of EGFR and SGLT1 was found to be frequent in cell lines and specimens of oral squamous cell carcinoma [58].

Through physical associations but not kinase activity, EGFR can modulate protein subcellular trafficking. Our laboratory recently reported that both EGFR and EGFRvIII associate with p53-upregulated modulator of apoptosis (PUMA), a proapoptotic member of the Bcl-2 family of proteins primarily located on the mitochondria [36]. PUMA is a potent apoptosis inducer that binds to and inhibits all five anti-apoptotic proteins [59; 60], while most BH3-only proteins selectively engage anti-apoptotic proteins. PUMA also directly binds to the apoptotic executor BAX [61; 62] to induce mitochondrial outer membrane permeabilization. PUMA strongly induces apoptosis in colorectal cancer [63; 64], malignant gliomas [65], and in adult stem cells [66]. Importantly, our study [36] further demonstrated that the EGFR-PUMA and EGFRvIII-PUMA interactions are independent of EGF stimulation or kinase activity and that these interactions are constitutive and only modestly reduced following apoptotic stress. The same study also found that EGFR/EGFRvIII did not significantly interact with other proa poptotic members of Bcl-2 family of proteins, Bax and Bmf. As a consequence of the EGFR-PUMA and EGFRvIII-PUMA interactions, PUMA is sequestered in the cytoplasm and unable to translocate onto the mitochondria to initiate apoptosis. This interesting observation is in agreement with the evidence showing that PUMA is highly co-expressed with EGFR/EGFRvIII in cell lines and primary specimens of malignant gliomas and that this particular tumor type is highly resistant to apoptosis-inducing treatments [36]. We further reported in this study that a Bcl-2/Bcl-xL inhibitor that mimics PUMA's proapoptotic activity sensitized EGFR- and EGFRvIII-expressing glioblastoma cells to an EGFR kinase inhibitor Iressa, suggesting that targeting both kinase-dependent and -independent functions of EGFR/EGFRvIII could be an effective strategy to overcome tumor resistance to the agents that solely inhibit the kinase function of the receptors.

Interestingly, two recent studies indicate that EGFR localized within the lipid raft microdomain of the plasma membrane could activate Akt without EGFR kinase activity [37; 38]. Breast cancer cell lines with higher levels of EGFR in the lipid rafts appeared to be more resistant to Iressa [38]. Treating resistant cells with lovastatin to deplete cholesterol, an essential component of the lipid rafts, sensitized the cells to EGFR kinase inhibition. The mechanisms for these interesting observations remain unclear. However, it has been shown that PI-3K and c-Src co-localized and associated with EGFR in the lipid rafts [37]. These findings suggest that the lipid raft microdomain may serve as a platform for EGFR and other signaling molecules to interact with each other to transmit survival signals, independent of EGFR kinase function, and that pharmacological inhibitors for cholesterol biosynthesis may be useful in targeting some of the kinase-independent activities of cell-surface localized EGFR.

3. Nuclear Mode of EGFR Signaling

3.1. Detection of nuclear EGFR and EGFRvIII

Nuclear existence of EGFR was first observed in hepatocytes that underwent regeneration more than two decades ago [67]. EGFR ligands, EGF and pro-TGF-α, were also found to translocate into the nucleus of proliferating hepatocytes [68; 69]. Nuclear expression of EGFR was further detected in other types of normal cells and tissues, such as placenta, thyroid, immortalized epithelial cells of ovary and kidney origins, and keratinocytes [70; 71; 72; 73]. More recently, nuclear EGFR has been shown to be detected in many different types of cancer cells and specimens, including those of breast [71; 74], epidermoid [75; 76], bladder [75], ovary [77], oral cavity [74; 78], lungs [79], and pancreas [80], and also in malignant gliomas [81; 82]. Nuclear EGFR can be localized within the nucleoplasm [70; 71; 83; 84] and on the inner nuclear membrane [73; 85]. Evidence to date indicates nuclear EGFR to be the full-length receptor that originates from the cell-surface [70; 71; 74; 82]. Analysis for nuclear presence of EGFRvIII has not been extensively conducted; the

presently available information from our laboratory and those of others showed that EGFRvIII can be detected in prostate cancer [86] and in malignant gliomas [81; 82].

3.2. Nuclear EGFR and EGFRvIII as transcriptional regulators

The role of EGFR in regulating gene regulation independent of its kinase activity was first suggested by a 1994 study by Eldredge et al [87]. The authors showed that a kinase-dead EGFR mutant transcriptionally activated c-fos gene expression. This interestingly finding is in line with another 1994 report by Xie et al [88] demonstrating that rat neu/p185 contained a transactivation domain in the C-terminus and that the full-length receptor underwent nuclear transport. A milestone study in 2001 by Mien-Chi Hung's group [70] defined nuclear EGFR as a transcriptional co-factor that contains a transactivation domain in its C-terminus, like rat neu/p185. This study also showed that nuclear EGFR associated with a consensus A/T-rich sequence within the human cyclin D1 promoter and that following binding, cyclin D1 gene expression was upregulated. As summarized in Figure 2, the transcriptional targets of nuclear EGFR that have been identified to date include cyclin D1 [70], inducible nitric oxide synthase (iNOS) [71], B-Myb [76], cyclooxygenase-2 (COX-2) [82], aurora A [89], c-Myc [80], and breast cancer resistance protein, BCRP [90]. Through increasing the expression of these target genes, nuclear EGFR has been linked to several malignant phenotypes of human cancers, including proliferation, inflammation and tumor drug resistance [40; 41; 42].

Given the fact that EGFR lacks a DNA-binding domain, extensive efforts have been focused on finding its transcriptional co-regulators with DNA-binding capability. These efforts have opened up a new avenue of research. In 2005, Lo et al [71] reported that nuclear EGFR is able to associate with STAT3 oncogenic transcription factor to enhance expression of iNOS, a protein involved in inflammation, tumor progression and metastasis. The same group further reported that nuclear EGFR interacted with E2F1 to activate human B-Myb gene expression, leading to uncontrolled proliferation [76]. Nuclear EGFR has also been shown to also interact with STAT5 to enhance human aurora A gene expression, leading to chromosome instability [89].

Our laboratory recently reported, for the first time, a systemic unbiased approach to identify nuclear EGFR target genes [82]. This was accomplished using a set of three isogenic glioblastoma cell lines expressing vector control, EGFR, and nuclear entry-defective EGFR (lacking the functional nuclear localization signal within the juxtamembrane region) followed by DNA microarray for over 47,000 gene transcripts. The results indicated 19 potential target genes of nuclear EGFR of which COX-2 was subsequently validated to be a novel transcriptional target of nuclear EGFR. Our results further demonstrated that STAT3 greatly synergized with nuclear EGFR to enhance COX-2 gene expression. Importantly, we found nuclear EGFRvIII to also activate COX-2 gene expression. The impact of STAT3 on nuclear EGFRvIII-mediated COX-2 expression was found to be only modest, which is in contrast to the significant positive impact of nuclear EGFR-STAT3 complex on COX-2 gene activation [82]. Ongoing efforts are being invested on validating other potential nuclear EGFR target genes that have been identified by the gene expression profiling.

Another mechanism for nuclear EGFR-associated transcriptional regulation was suggested by Huo et al [91] that RNA helicase A serves as a DNA-binding partner for nuclear EGFR. Knockdown of RNA helicase A expression in cancer cells abolished nuclear EGFR binding to its target gene promoters and reduced EGFR-induced gene expression. Interestingly, a most recent study by Jaganathan et al [80] showed that EGFR, Src and STAT3 form a heteromeric complex in the nucleus. This nuclear complex is bound to the c-Myc gene, which may contribute to c-Myc gene overexpression in pancreatic cancer cells. Also interesting and indicative of a possible mechanism underlying the ability of nuclear EGFR to regulate gene transcription is the ability of nuclear EGFR to interact with MUC1, which may

promote both the accumulation of chromatin-bound EGFR and the significant co-localization of EGFR with phosphorylated RNA polymerase II [92].

In line with the observation on rat neu/p185 [88], its human homolog HER2 can also be detected in the cell nucleus and activates COX-2 gene expression by binding to HER2-associated sequences [93]. Nuclear HER2 has been shown to associate with STAT3 to upregulate cyclin D1 gene expression [94]. This study also showed that progesterone receptor induces HER2 nuclear translocation. Interestingly, a recent report by Li et al [95] demonstrated that nuclear HER2 enhanced translation by activating transcription of ribosomal RNA genes. Taken together, these findings indicate that nuclear EGFR and EGFRvIII function as transcriptional regulators that cooperate with their transcriptional co-factors to mediate the expression of a number of important cancer-related genes and thereby, regulate many physiological and pathological processes.

3.3. EGFR as a nuclear tyrosine kinase

Evidence to date indicates that nuclear EGFR retains its tyrosine kinase activity. Wang et al [96] reported that nuclear EGFR phosphorylates proliferating cell nuclear antigen (PCNA) to promote cell proliferation and DNA repair (Figure 2). Chromatin-bound PCNA protein is phosphorylated on the Tyr-211 residue by nuclear EGFR and becomes stabilized. This important finding raised the possibility that additional nuclear proteins may be phosphorylated by both nuclear EGFR and HER2, and that the functions, stability, and/or intracellular trafficking of these target proteins may be altered as a consequence of tyrosine phosphorylation. Future efforts are needed to explore these exciting possibilities.

3.4. Nuclear EGFR as a modulator of DNA repair

Nuclear EGFR also plays an essential role in DNA repair following radiation therapy (Figure 2). The first two studies that reported radiotherapy-induced EGFR nuclear transport in cancer cells and the consequences of this process were conducted by Dittmann and colleagues [97; 98]. They showed that upon radiation therapy induced EGFR nuclear entry, EGFR localized in the nucleus interacts with DNA-dependent protein kinase (DNA-PK), leading to repair of radiation-induced DNA double-strand breaks in bronchial carcinoma cells. A non-steroid anti-inflammatory drug celecoxib has been shown to facilitate tumor cell radiosensitization by inhibiting radiation-induced nuclear EGFR transport and DNA repair [99]. This action of celecoxib appears to be independent of its COX-2 inhibitory effect since radiosensitization was correlated with neither COX-2 expression nor prostaglandin E2 levels. Another study by Hsu et al [100] further showed that nuclear EGFR is required for tumor resistance to DNA damage induced by the DNA alkylating agent, cisplatin. Collectively, these studies suggest a negative impact of nuclear EGFR on tumor sensitivity to DNA-damaging radiation therapy and anti-cancer alkylating agents. A more recent study by Liccardi [101] provided a potential mechanism for nuclear EGFR-mediated tumor resistance to cisplatin. This study showed that cisplatin induces binding of nuclear EGFR and EGFRvIII to DNA-PK, leading to DNA repair. Similar to EGFR, HER2 nuclear transport can be induced by radiation [102]. Interestingly, Herceptin appears to inhibit radiation-induced HER2 nuclear accumulation, suggesting a potential benefit of combining Herceptin with radiation in treating breast cancer patients with HER2-positive tumors.

Conversely, nuclear EGFR may protect normal cells from unwanted DNA damage caused by ultraviolet and γ irradiations. Ultraviolet irradiation has been shown to induce EGFR nuclear translocation in human keratinocytes [103]. The mechanisms for the observed protective effects of nuclear EGFR in normal skin cells are still unclear. However, it has been shown that following irradiation and treatment of the radioprotector Bowman-Birk protease inhibitor, nuclear EGFR is able to associate with p53 and MDC1 protein, both of

which are essential for formation of DNA repair foci [104]. Another radioprotector O-phospho-l-tyrosine has been shown to activate PKC-epsilon and to trigger nuclear EGFR import and phosphorylation of DNA-PK, leading to repair of DNA double-strand breaks [105].

3.5. Nuclear EGFR and EGFR-targeted therapy

Presently, five EGFR-targeted agents have been approved by the FDA for treating cancer patients. Among them, three are small molecule tyrosine kinase inhibitors and two are therapeutic antibodies. (1) Gefitinib (ZD1839, Iressa), a small molecular weight EGFR kinase inhibitor, is being used for locally advanced and metastatic NSCLC. (2) Erlotinib (OSI-774, Tarceva), a sma ll molecule EGFR kinase inhibitor has been approved both to treat metastatic NSCLC as single agent and to be used in combination with gemcitabine for pancreatic cancer that cannot be removed by surgery or has metastasized. (3) Lapatinib (GW572016, Tykerb/Tyverb) is an EGFR/HER2-dual targeting small molecule inhibitor [106] that is used in combination with capecitabine in women with HER2-positive breast cancer whose disease (or condition) has not responded to other chemotherapy. Lapatinib is also approved for combined therapy with letrozole in postmenopausal women with HER2-positive and hormone receptor-positive breast cancer who need hormonal therapy. (4) Cetuximab (C225, Erbitux), a humanized monoclonal antibody that recognizes the extracellular domain of both EGFR [106] and EGFRvIII [107], has been approved for squamous cell carcinoma of the head and neck that has metastasized or recurred after chemotherapy, and as a first-line treatment with radiation therapy for advanced squamous cell carcinoma of the head and neck. Cetuximab is also used for colorectal cancer that has metastasized after chemotherapy has failed and, in combination with irinotecan, for metastatic colorectal cancer patients who have not responded to irinotecan alone. (5) Panitumumab (ABX-EGF, Vectibix), a humanized monoclonal antibody, has been approved to treat metastatic colorectal cancer that has failed other therapies and has metastasized [108].

Constitutive nuclear presence of EGFR can be constitutive, in part, attributed to ligand-activated nuclear transport and the ability of some cancer cells to secret EGF and to activate an EGF-EGFR autocrine loop [70; 71; 76; 84]. Constitutive presence of EGFR in the tumor nuclei may contribute to intrinsic therapeutic resistance. In this context, nuclear existence of EGFR may be beneficial to the tumors encountering EGFR-targeted therapeutic antibodies and small molecule inhibitors [16; 43]. Furthermore, a study by Li et al [79] demonstrated that NSCLS cells that had acquired resistance to cetuximab expressed increased levels of nuclear EGFR and that forced expression of a nuclear localization sequence-tagged EGFR rendered cetuximab-sensitive cells resistant to cetuximab, both in vitro and in mouse xenografts.

The effects of EGFR-targeted therapy and other anti-cancer treatments on the extent of EGFR nuclear translocation are still unclear, with mixed results being reported. Ionizing radiation induces EGFR nuclear transport [97; 104; 109], which can be inhibited by cetuximab [98]. Both lapatinib [110] and the Src family kinase inhibitor, dasatinib [79] block EGFR nuclear entry (Figure 2). Celecoxib has been shown to inhibit radiation-induced nuclear EGFR transport [99]. In contrast, Liao and Carpenter [111] showed that cetuximab is able to activate EGFR nuclear transport by promoting receptor endocytosis and activating receptor intracellular trafficking to the endoplasmic reticulum.

Overall, these observations have provided rationales for selecting novel combination treatments that can overcome nuclear EGFR-mediated therapeutic resistance. For example, celecoxib [99] and dasatinib [79] could be useful in blocking EGFR nuclear entry. Akt inhibition could be used to suppress EGFR nuclear transport because Akt-mediated EGFR

phosphorylation at Ser-229 has been shown to be required for EGFR nuclear entry [90]. In summary, despite significant advances in our understanding of the impact of nuclear EGFR on intrinsic and acquired tumor drug resistance as well as the reciprocal effects of anti-cancer agents on EGFR nuclear transport, our current knowledge regarding both aspects remains very limited. In particular, no information is available, to date, on the relationship between nuclear EGFRvIII and tumor drug resistance.

3.6. Nuclear EGFR and EGFRvIII as indicators for poor clinical outcome

There are only a few studies that have been conducted to elucidate the prognostic value of nuclear EGFR and EGFRvIII, with the first study published by Mien-Chi Hung's group in 2005 using 130 primary breast carcinomas [74]. In this study, immunohistochem ical staining was performed to detect the levels of nuclear and non-nuclear EGFR, as well as expression of cyclin D1 and Ki-67 in the tumors. The study found for the first time that 37.7% of the cohort was immunostained positively for nuclear EGFR with 6.9% having high levels of expression. Importantly, a significant inverse correlation was observed between high nuclear EGFR expression and overall survival of breast cancer patients [74]. In contrast, expression of non-nuclear EGFR did not significantly correlate with the overall survival rate of breast cancer patients. There were also positive associations of nuclear EGFR with cyclin D1 and Ki-67. In agreement with previous findings reported by Lo et al [74], Hadzisejdic et al [112] found in a cohort of 113 breast carcinomas that nuclear EGFR was detected in 40% of cases with 12% expressing high degrees of nuclear EGFR. Nuclear EGFR was found to be positively correlated with tumor size, lymph node metastasis, Nottingham prognostic index, estrogen receptor expression, and shortened overall survival.

In oral squamous carcinomas, Lo et al [74] analyzed 37 cases and reported that 24.3% of the samples contained moderate/high levels of nuclear EGFR and that those with high nuclear EGFR had the tendency to survive poorly. In line with these observations, Psyrri et al [78] analyzed 95 oropharyngeal carcinomas and observed an inverse correlation of nuclear EGFR with disease-free survival. In ovarian cancer, Xia et al. [77] investigated 221 cases of patient tumors and observed that 28.3% of the cohort had high levels of nuclear EGFR and that there was an inverse correlation of high nuclear EGFR with overall survival. Using 74 matched hormone-sensitive and hormone-refractory prostate tumors, Edwards et al [86] reported that in patients with hormone-refractory tumors, high levels of nuclear EGFRvIII were associated with poor overall survival. These promising results not only support future uses of nuclear EGFR and nuclear EGFRvIII as prognostic indicators for poor clinical outcome, but also validate the unique ability of nuclear EGFR/EGFRvIII to support the aggressiveness of tumor cells.

3.7. Trafficking of cell-surface EGFR to the nucleus

The mechanisms underlying nuclear transport of EGFR begin with endocytosis, which occurs following ligand-induced activation, as the ligand-bound receptors are internalized through clathrin-coated pits that pinch off from the plasma membrane in a dynamin-dependent manner [113]. After the endocytic vesicle fuses with the early endosome, the internalized EGFR can be (a) recycled back to the plasma membrane, (b) sorted to late endosomes and, eventually, to lysosomes for degradation, or (c) further transported into the nucleus. Playing a crucial role in the first two possible outcomes are the various members of the Rab family GTPases [114]. GTP-bound Rab5, for example, assembles on the membrane of early endosomes and recruits Rab tethering proteins to capture the initial clathrin-coated vesicles that pinch off from the cell surface. Additionally, Rab4 and Rab11 have been implicated to play a role in mediating the budding of recycling vesicles that return EGFR back to the plasma membrane [115]. Rab7 has also been shown to mediate the flow, and subsequent degradation, of EGFR out of the late endosome [116].

Early endosomal EGFR destined for the nucleus can undergo transport via several proposed models, each of which is dependent on the interaction between a nuclear localization signal (NLS) within EGFR and importin proteins [83; 84; 117]. Importin-β, either alone or as a heterodimer with importin-α, can bind to NLS of NLS-containing proteins as well as to components of nuclear pore complexes (NPCs), thereby directing these proteins for entry into the nucleus [118]. In the case of EGFR and HER2, a putative NLS has been both identified within the juxtamembrane region [71; 93; 119] and shown to interact with importin-β [84; 117]. For HER2, one proposed model suggests that importin-β associates with the NLS of endosome-bound HER2 and directs it to the nucleus by interacting with the nuclear pore protein Nup358 [117], a constituent of NPCs.

Another proposed model involving EGFR retrograde transport suggests that after early endosomal sorting, ErbB family of proteins destined for the nucleus are trafficked via the Golgi to the ER in COPI-coated vesicles [120]. ER-bound EGFR then interacts with Sec61 translocon [83; 121], passing through the channel in a similar manner as misfolded proteins undergoing ER-associated protein degradation (ERAD) and entering into the cytosol where it can be picked up by importin-β and transported into the nucleus. This retrotranslocation from the ER to the cytosol of full-length EGFR with its hydrophobic transmembrane domain requires the presence of cytosolic chaperone HSP70 [83], which may possibly play a role in solubilizing the receptor and preventing aggregation. Alternatively, ER-bound EGFR may also enter the nucleus via lateral diffusion from the ER membrane through the nuclear pore complex and into the inner nuclear membrane mediated by NLS-importin interaction, as suggested by evidence showing EGFR localized at the inner nuclear membrane [85] and nuclear matrix [122]. Although nuclear export signals have yet to be identified in ErbB family of receptors, the exportin CRM1 has been found to interact with EGFR and HER2 [84; 117], and inhibition of CRM1 using leptomycin B has led to increased accumulation of nuclear EGFR, HER2, and HER3 [84; 117; 123].

Other proteins reported to be involved in EGFR nuclear trafficking include Epstein-Barr virus (EBV) encoded latent membrane protein 1 [124], which was shown to regulate nuclear EGFR translocation in a dose-dependent manner, and PIKfyve kinase [75], which has been demonstrated to play a role in nuclear transport of EGFR via its interaction with cytoplasmic EGFR upon HB-EGF induced activation. Interestingly, a recent study found that Akt phosphorylation of EGFR is required for both EGFR nuclear translocation and acquisition of Iressa resistance via upregulation of BCRP by nuclear EGFR in breast cancer cells [90], indicating that advances in our understanding of nuclear EGFR trafficking can lead to further insight into the various approaches to EGFR-targeted therapy.

4. Mitochondrial Mode of EGFR Signaling

Mitochondrial detection of EGFR was first reported by Sarah Parsons' group in 2004 [125]. This important study demonstrated that EGFR translocated to the mitochondria after EGF stimulation (Figure 3). While localized on the mitochondria, EGFR interacts with cytochrome c oxidase subunit II (CoxII), a mitochondrion-encoded protein and a critical component of the oxidative phosphorylation pathway. CoxII binds to EGFR but not mutant EGFR (Y845F) that can not be phosphorylated by c-Src. EGFR Y845F mutant undergoes EGF-induced mitochondrial transport similar to EGFR, suggesting that c-Src-mediated EGFR phosphorylation is not required for EGFR mitochondrial import. This group further reported that c-Src translocated to the mitochondria with similar kinetics as EGFR after EGF stimulation, and that c-Src kinase activity/overexpression enhanced EGFR localization to the mitochondria [126]. This study also showed that CoxII can be phosphorylated by EGFR and c-Src; however, the consequences of this phosphorylation are still unknown. The authors also reported that clathrin-mediated endocytosis was shown to be essential in regulating

EGFR translocation to the mitochondria, suggesting the origin of mitochondrial EGFR to be the plasma membrane-bound EGFR [126]. In contrast to this finding, Yao et al [127] reported that EGFR mitochondrial transport is independent of endocytosis.

Demory et al [126] reported the identification of a potential mitochondrial localization signal contained in the juxtamembrane region of EGFR (residues 645–666). The authors also showed that EGFR decreased ATP biosynthesis in the cells under serum-starved conditions; expression of an EGFR deletion mutant (Δ645–666) restored ATP levels to about 80% [126]. Similarly, Yao et al [127] reported that EGFR deletion mutant (Δ646–660) was unable to enter the mitochondria. Since the nuclear localization signal of EGFR is also localized in the same juxtamembrane region (residues 645–657), [71; 82; 119] overlapping with the potential mitochondrial localization signal (Δ645–666), it remains unclear if the changes in ATP levels were attributed to mitochondrial or nuclear EGFR, or potentially to both. Also unclear is whether the region of interest (residues 645–666) is responsible for both nuclear and mitochondrial transports of EGFR. Furthermore, Yue et al [128] reported that EGFR mitochondrial translocation can be increased by a mTOR inhibitor, rapamycin. Autophagy inhibition by 3-methyladenine (an inhibitor of autophagy and a PI-3K inhibitor) and Beclin 1 expression knockdown leads to a reduction of rapamycin-induced mitochondrial import of EGFR. Etoposide also appears to decrease EGFR mitochondrial transport [128].

Most recently, our laboratory reported for the first time that EGFRvIII can be detected in tumor cell mitochondrion [129]. We showed that both EGFR and EGFRvIII are constitutively present in the mitochondria. Importantly, the degrees of EGFR and EGFRvIII mitochondrial transport were greatly enhanced following treatments with the apoptosis inducers, staurosporine and anisomycin, and with an EGFR kinase inhibitor, Iressa (Figure 3). Using mutant EGFR/EGFRvIII receptors engineered to undergo enriched intracellular trafficking into the mitochondria (but not into the cell nucleus), we showed that glioblastoma cells expressing the mitochondrially enriched EGFRvIII were more resistant to staurosporineand anisomycin-induced growth suppression and apoptosis. The tumor cells with mitochondrial EGFRvIII accumulation were highly resistant to Iressa-mediated growth inhibition. These findings indicate that apoptosis inducers and EGFR-targeted inhibitors enhance mitochondrial translocation of both EGFR and EGFRvIII, and that mitochondrial accumulation of these receptors contributes to tumor drug resistance. The findings also provide evidence for a potential link between the mitochondrial EGFR/EGFRvIII pathway and apoptosis.

Interestingly, Dreier et al [130] recently reported that cetuximab induced mitochondrial transport of EGFRvIII, but not EGFR, while we showed that Iressa increased mitochondrial transport of both receptors [129]. This could be attributed to the ability of cetuximab to increase EGFRvIII internalization. These findings together suggest that different EGFR-targeted treatments, e.g. tyrosine kinase inhibitors versus therapeutic antibodies, may differentially impact EGFR and EGFRvIII in their capability to undergo mitochondrial transport. Future investigations are clearly needed in this area to understand the mechanisms in order to provide better clinical therapeutic guidance.

HER4 intracellular domain (4ICD) has been shown by Frank Jones' group to be a BH3-only protein that undergoes ligand-induced mitochondrial transport [131]. The authors showed that 4ICD interacts with the anti-apoptotic protein Bcl-2 in a similar fashion to the BH3-only proteins. However, unlike other BH3-only proteins that depend on BAX/BAK to initiate apoptosis, 4ICD is essential for BAK-transmitted apoptosis. The same group further reported that mitochondrial 4ICD contributes to tamoxifen sensitivity in breast cancer cells [132].

In summary, several recent reports have uncovered a novel mode of EGFR signaling that takes place in the mitochondria, the central organelles that produce energy and initiate apoptosis. The mitochondrial transport of EGFR and/or EGFRvIII can be constitutive and further enhanced by EGF, rapamycin, apoptosis inducers, c-Src and EGFR inhibition (Figure 3). Conversely, the receptor mitochondrial import can be suppressed by 3-methyladenine and by etoposide. The origin of mitochondrial EGFR and EGFRvIII remains to be defined given the mixed results. While localized to the mitochondria, EGFR interacts with and phosphorylates CoxII though its impact on CoxII and CoxII-mediated ATP biosynthesis are still not known. Accumulation of EGFR and EGFRvIII in the tumor mitochondria could contribute to tumor resistance to apoptosis although the underlying mechanisms have yet to be defined. The anti-apoptotic effects of mitochondrial EGFR/EGFRvIII are in contrast to the pro-apoptotic effects of 4ICD. Overall, the nature and consequences of the mitochondrial mode of EGFR signaling are still not well understood. Consequently, there is an urgent need to further investigate this exciting new avenue of cancer research.

5. Perspectives and Future Directions

The EGFR signaling pathway is one of the most dysregulated and most extensively investigated molecular pathways in human cancers. Despite significant advances made in our understanding of the EGFR signaling network, studies in the past ten years have uncovered several atypical modes of the pathway that are intriguing and of both biological importance and therapeutic implications. These studies have helped the scientific community to reshape the subcellular landscape of the EGFR signaling network, in particular, by expanding from its classical location on the plasma membrane to the nucleus and mitochondrion, as well as, to microdomains within the plasma membrane.

Despite much skepticism, this unique unconventional field of EGFR research has attracted substantial positive attentions and tremendous interests. Consequent to this, the landscape of the EGFR pathway is now viewed as a web of signaling networks that has three major subcellular hubs. In addition to the obvious differences in the subcellular location, the evidence to date indicates that EGFR at the different subcellular locations elicits distinctly different and also overlapping signals. In this regard, mitogenic signals can be emitted from the plasma membrane-bound EGFR in both kinase-dependent and -independent fashions in which the kinase-independent activity can originate from the EGFR within the lipid rafts. Pro-survival signals can be transmitted from the EGFR/EGFRvIII on the cell-surface as well as from those on the mitochondria. The kinase-independent activity of cell-surface EGFR can modulate glucose uptake and autophagy, while EGFR mitochondrial import may be modulated by autophagy inhibition. Overall, these newly uncovered atypical modes of EGFR signaling have been established to control an array of cellular processes that post critical importance in tumor cell growth, progression, death and survival.

Several major knowledge gaps still exist in our understanding towards the nuclear and mitochondrial modes of the EGFR and EGFRvIII signaling pathways. (1) It remains unknown the relationship between the atypical EGFR/EGFRvIII pathways and tumorigenesis in any type of cancers. (2) The biological and pathological consequences of nuclear EGFRvIII in human cancers are still largely unknown. (3) The exact modes of actions of mitochondrial EGFR and EGFRvIII have remained undefined. (4) The impacts of nuclear and mitochondrial EGFR/EGFRvIII on tumor response to therapy and on intrinsic and acquired resistance to treatments are still poorly understood. Gaining a deeper insight into these processes could help us derive rationales to overcome the drug resistant phenotype frequently observed in tumors with dysregulated EGFR and EGFRvIII signaling. (5) Are there common factors beside ligands that activate EGFR nuclear and mitochondrial

transport? Targeting these factors could potentially overcome atypical EGFR signaling mediated resistance to various therapies. (6) Are the atypical EGFR and EGFRvIII pathways involved in embryonal development or cancer stem cell biology? (7) It remains uninvestigated whether the classical and atypical EGFR/EGFRvIII pathways cooperate to regulate the malignant phenotypes of human cancers. Likely, tumor cells work to mobilize and/or switch off different modes of EGFR signaling in order to maximize its growth and survival. (8) How do we derive therapeutic strategy that will simultaneously and fully inhibit a diverse array of EGFR/EGFRvIII activities that are elicited from different subcellular locations in tumor cells?

Indeed, addressing the above mentioned knowledge gaps and unanswered questions will not only better our biological understanding of the EGFR signaling network in human cancers, but also help us to generate novel insights into the nature of other plasma membrane-bound receptors that also undergo nuclear transport. These include receptor tyrosine kinases, such as, rat p185neu, HER2, HER3, HER4, FGFR, TrkA/B, FGFR, VEGFR-2, c-Met and orphan receptor 1, as well as cytokine receptors for IL-1, IL-5, interferon-γ, type I TGF-β and prolactin [69; 88; 123; 133; 134; 135; 136; 137; 138; 139; 140; 141; 142; 143; 144]. Undoubtedly, the newly gained knowledge will facilitate future development of more effective treatments for tumors with hyperactive EGFR and EGFRvIII pathways.

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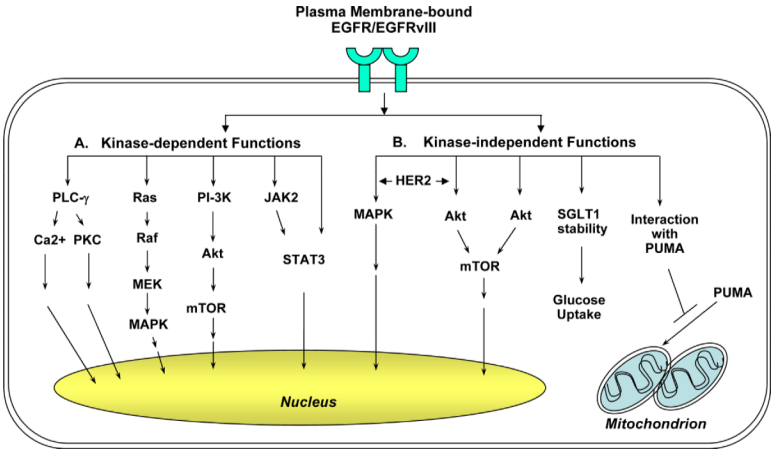

Figure 1. The plasma membrane-bound EGFR/EGFRvIII signaling is consisted of the kinase-dependent and -independent modes of actions

A: Kinase-dependent functions. Upon ligand binding, EGFR becomes activated and phosphorylated at multiple tyrosine residues including those within its kinase domain. Phosphorylated EGFR then recruits and phosphorylates downstream signaling molecules. The major pathways downstream of EGFR include those mediated by PLC-γ–PKC, Ras-Raf-MEK, PI3-K-Akt-mTOR and JAK2-STAT3. In addition, EGFR can directly interact with and phosphorylate STAT3 transcription factor. EGFRvIII is constitutively active independent of ligand stimulation.

B: Kinase-independent functions. Co-expression of the kinase-dead EGFR K721M mutant with HER2 rescued the inability of the mutant EGFR to activate Akt and MAPK. Kinase-dead EGFR D813A mutant may activate Akt via undefined mechanisms. Independent of its kinase activity, EGFR also interacts with and stabilizes plasma membrane-bound SGLT1, leading to glucose uptake and increased intracellular glucose levels. Our laboratory recently reported that EGFR and EGFRvIII associated with and sequestered the proapoptotic protein PUMA in the cytoplasm independent on EGF stimulation or its kinase activity. The EGFR-PUMA and EGFRvIII-PUMA interactions contribute to reduced apoptosis and survival.

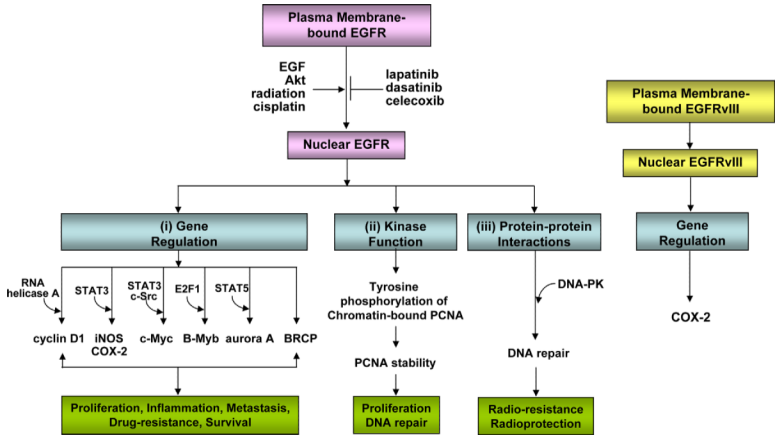

Figure 2. The nuclear mode of EGFR/EGFRvIII signaling network

EGFR nuclear transport can be induced by EGF, Akt phosphorylation, radiation and cisplatin, and conversely, inhibited by lapatinib, dasatinib and celecoxib. Nuclear EGFR has three major functions: (i) gene regulation, (ii) kinase function, and (iii) protein-protein interactions. Via these actions, nuclear EGFR is implicated in a number of physiological and pathological processes, such as proliferation, inflammation, metastasis, DNA repair, and resistance to DNA-damaging radiation and alkylating anti-cancer agents. Nuclear EGFRvIII activates COX-2 gene expression.

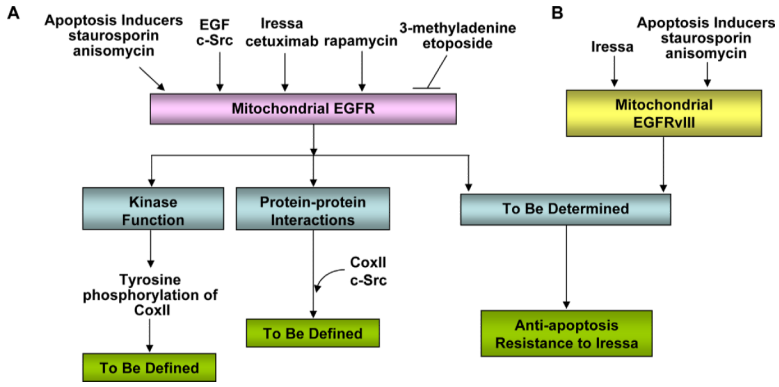

Figure 3. The mitochondrial mode of EGFR/EGFRvIII signaling pathway

A: EGFR mitochondrial import can be constitutive and the extent can be enhanced by apoptosis inducers (staurosporine and anisomycin), EGF, c-Src, Iressa, cetuximab and rapamycin. Conversely, EGFR mitochondrial transport can be blocked by 3-methyadenine (inhibitor of autophagy and PI-3K) and etoposide. Mitochondrial EGFR retains its tyrosine kinase activity and phosphorylates CoxII; however, the consequence of the phosphorylation is not yet defined. The mitochondrial EGFR-CoxII complex can include c-Src; however, the effects of this interaction are still unknown. Furthermore, mitochondrial accumulation of EGFR led to compromised apoptotic response and resistance to Iressa treatments, while the underlying mechanisms are still undetermined.

B: EGFRvIII mitochondrial import is constitutive and can be further enhanced by apoptosis inducers (staurosporine and anisomycin) and by Iressa. Mitochondrial accumulation of EGFRvIII rendered tumor cells highly resistant to apoptotic death and to Iressa treatments, while the mechanisms are still not identified.