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Epidermal Growth Factor Receptor (EGFR) Phosphorylation, Signaling and Trafficking in Prostate Cancer

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1. Introduction

The molecular mechanisms of prostate cancer are still poorly understood, despite the threat that prostate cancer poses to the health of men worldwide. As prostate tumors are initially dependent on androgens for growth and survival, androgen deprivation therapy is the first-line treatment for prostate cancer patients. However, a hormonal-refractory (androgen independent) state often develops afterwards, and principal treatment options are palliative because of the tumor progression, which is characterized by uncontrolled growth and metastasis associated with androgen independence. To date, no effective therapy can abrogate prostate cancer progression to advanced, invasive forms. Recent evidence suggests that acquisition of androgen-independence may be due to upregulation of growth factor receptor signaling pathways, principally the epidermal growth factor receptor (EGFR)/ErbB/human epidermal receptor (HER) family (Craft et al., 1999), making it an attractive target for therapeutic intervention. EGFR/ErbB/HER signaling in cancer has been extensively studied for decades, and there have been a number of excellent reviews on the roles of ErbB receptors in the initiation and progression of a wide variety of cancers, including prostate cancer (Laskin & Sandler, 2004; Ratan et al., 2003; Yarden & Sliwkowski, 2001). Thus, this review chapter will focus more narrowly on EGFR phosphorylation, signaling, and trafficking, and their specific roles in prostate cancer development and progression (tumor growth and metastasis) given the growing literature in this area. Better understanding of the precise roles of divergent EGFR signaling pathways and their phenotypic consequences in prostate cancer (and normal prostate) will enable the development of more effective and selective therapies for this urologic disease.

2. Overview of the EGF/EGFR signaling system

2.1 The EGFR/ErbB/HER family and ligands

EGFR/ErbB1/HER1 is the prototype of the EGFR or ErbB family, which also includes other three receptor tyrosine kinases, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Figure 1). All four members have in common an extracellular ligand-binding domain, a single hydrophobic transmembrane domain, and a cytoplasmic region that contains a highly conserved tyrosine kinase domain and C-terminal tail (Wells, 1999). However, ErbB3 lacks intrinsic tyrosine kinase activity due to substitutions of critical amino acids within the kinase.
domain (Guy et al., 1994). The extracellular domains are less conserved among the four, suggesting their ligand binding specificity (Yarden, 2001; Yarden & Sliwkowski, 2001).

ErbB receptors are activated by a number of ligands that belong to the EGF family of peptide growth factors (Citri & Yarden, 2006; Yarden, 2001). The EGF-related growth factors are characterized by the presence of an EGF-like domain consisting of three disulfide-bonded intramolecular groups conferring binding specificity, and additional structural motifs such as immunoglobulin-like domains, heparin-binding sites and glycosylation sites. They are produced as transmembrane precursors that are biologically active and able to interact with receptors expressed on adjacent cells, and the ectodomains are processed by proteolysis, resulting in the shedding of soluble growth factors (Massagué & Pandiella, 1993). Based on their affinity for one or more ErbBs, the EGF-related growth factors are generally classified into three groups (Yarden & Sliwkowski, 2001) (Figure 1). The first group includes EGF, transforming growth factor-α (TGF-α) and amphiregulin (AR), which bind specifically to EGFR. The second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), which exhibit dual specificity for both EGFR and ErbB4 (Yarden, 2001). The third group includes nuregulins (NRG, also called Neu differentiation factors (NDF) or heregulins (HRG)) that can be divided into two subgroups based on their binding specificity to both ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4) (Harari et al., 1999; Zhang et al., 1997). Despite intensive efforts, no direct ligand for ErbB2 has yet been discovered. Increasing evidence suggests that ErbB2 primarily functions as a coreceptor for other ErbB family members (Graus-Porta et al., 1997; Tzahar et al., 1996).
2.2 Major EGF/EGFR signaling pathways

The repertoire of ErbB ligands and the combinatorial properties of ligand-induced receptor dimers give rise to the signaling diversity of the ErbB family. Ligand binding drives receptor homo- or hetero-dimerization, leading to activation of the intrinsic tyrosine kinase and subsequent auto- or trans-phosphorylation of specific tyrosine residues in the cytoplasmic tail (Citri & Yarden, 2006; Olayioye et al., 2000; Yarden & Śliwkowski, 2001), which provide the docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Shoelson, 1997; Sudol, 1998). These proteins generally include the adaptor proteins such as Src homology domain-containing adaptor protein C (Shc), Crk, growth factor receptor-bound protein 2 (Grb2), Grb7, Grb2-associated binding protein 1 (Gab1), phospholipase C γ (PLCγ), Cbl, Esp15; the kinases such as Src, Chk and phosphatidylinositol-3-kinase (PI3K; via the p85 regulatory subunit); and the protein tyrosine phosphatases such as PTP1B, SHP1 and SHP2 (Olayioye et al., 2000; Sebastian et al., 2006), suggesting diversity and complexity of ErbB signaling networks. Among them, the signaling elicited by EGF-induced EGFR homodimers is perhaps the best studied and has served as the prototype for other cases.

Adaptor proteins, kinases, and phosphatases recruited by the activated EGFR transmit signals from the receptor through different downstream signaling pathways to the nucleus to regulate various biological functions such as cell proliferation, differentiation, anti-apoptosis (survival), adhesion, migration, and angiogenesis (Baselga & Hammond, 2002; Laskin & Sandler, 2004; Morandell et al., 2008; Yarden & Śliwkowski, 2001). So far with the numbers still growing, over one hundred EGFR interacting proteins have been described in the literature, of which many were discovered by proteomics approaches (Morandell et al., 2008). Approximately twenty phosphotyrosine residues located within the EGFR cytoplasmic tail have been identified as specific docking sites for above-mentioned EGFR interacting partners to engage various signaling cascades (Figure 2). The major EGF/EGFR signaling pathways include Ras/Raf/MAPK kinase (MEK)/extracellular-related kinase (ERK) and PI3K/Akt (Hirsch et al., 2003; Singh & Harris, 2005), although other pathways such as PLCγ/protein kinase C (PKC), signal transducer and activator of transcription (STAT) (Andl et al., 2004; Kloth et al., 2002), c-Jun terminal kinase (JNK) and p38 MAPKs (Johnson et al., 2005), and Ca²⁺-calmodulin-dependent protein kinase (CaMK) (Sengupta et al., 2009) have been reported. It is also known that upon EGF binding, EGFR undergoes a process of internalization, ubiquitination (via Cbl), destruction (namely EGFR endocytosis and trafficking), resulting in temporary EGFR downregulation (Citri & Yarden, 2006; Sebastian et al., 2006; Wiley, 2003). This will be discussed in Section 3.

2.3 EGFR signaling in prostate cancer development

Both clinical and experimental data have established the importance of ErbB3s, especially EGFR and ErbB2, in carcinogenesis and progression of various types of solid tumors including prostate cancer (Harari, 2004; Laskin & Sandler, 2004; Sebastian et al., 2006; Yarden & Śliwkowski, 2001). Increased expression and signaling of EGFR and/or ErbB2 are associated with a more aggressive clinical behavior of tumors, and correlate with a poor prognosis (Alroy & Yarden, 1997; Hatake et al., 2007; Lichtner, 2003; Nicholson et al., 2001). There are estimated 40-80% of prostate tumors with expressed EGFR (Kim et al., 1999; Sebastian et al., 2006). Studies mainly from breast cancer, lung cancer, and glioma have suggested many potential mechanisms related to aberrant EGFR signaling (quantitatively and/or qualitatively). These include elevated expression of ligands and/or receptors, enhanced autocrine signaling loop, constitutive activation of EGFR mutants, impaired endocytosis and trafficking of the ligand-
receptor complex, hetero-dimerization with other ErbBs (Ciardiello & Tortora, 2003; Grandal & Madshus, 2008; Huang et al., 2009; Olayioye et al., 2000; Roepstorff et al., 2008; Sebastian et al., 2006; Sharma et al., 2007), as well as crosstalk with other receptor signaling systems such as type 1 insulin-like growth factor receptor (IGF-1R), G-protein-coupled receptors (GPCRs), and cytokine receptors (Adams et al., 2004; Gee et al., 2005; Prenzel et al., 2000).

Fig. 2. EGF/EGFR-mediated signaling pathways and cellular effects. Major tyrosine phosphorylation sites in the EGFR cytoplasmic tail, possible adaptors and signaling proteins, and signaling cascades are indicated. See the text for more details.

Progression from normal prostate epithelium to an androgen-responsive tumor, and finally to hormone-refractory carcinoma is a multistep process, usually accompanied by the
upregulation of growth factor receptors and/or their ligands, and downregulation of tumor suppressor gene products (Djakiew, 2000; Ware, 1999). EGFR ligands, such as EGF, HB-EGF, and TGF-α, are expressed in the prostate and prostatic carcinomas (Elson et al., 1984; Freeman et al., 1998). In particular, the expression of TGF-α (signaling merely through EGFR) has been found to be greater in some higher grade and metastatic prostate cancers than in primary low grade tumors (Scher et al., 1995). It is now more widely believed that EGF is the predominant EGFR ligand in early, localized prostate cancer, and that TGF-α becomes more abundant than EGF at advanced, metastatic stages (Liu et al., 1993; Scher et al., 1995; Seth et al., 1999). This is the so-called EGFR ligand switch (DeHaan et al., 2009). Overexpression of EGFR and/or ErbB2 would have been expected in prostate carcinomas, as seen in breast cancer (Hatake et al., 2007; Lichtner, 2003; Nicholson et al., 2001). However, current data regarding ErbB receptor overexpression in prostate cancer appear to conflict with each other, possibly due to technical reasons and lack of standardized measurement and evaluation methods (Marks et al., 2008; Neto et al., 2010; Salomon et al., 1995; Schlomm et al., 2007; Sherwood & Lee, 1995). Nevertheless, several lines of evidence strongly support the important role of EGFR signaling in prostate cancer development. For example, autocrine activation of EGFR signaling by EGF and TGF-α most likely drives the autonomous growth of human prostate cancer (Hofer et al., 1991; Scher et al., 1995). Expression of mutant EGFRs also contributes to prostate carcinogenesis and malignant progression (Cai et al., 2008; Douglas et al., 2006; He & Young, 2009; Olapade-Olaopa et al., 2000). Taken together, studies over the years have suggested that both EGFR and ErbB2 signaling play important roles in prostate cancer development and, more specifically, in the progression from an androgen-dependent to a hormone-refractory state.

3. EGF/EGFR endocytosis and trafficking

As just described, aberrant EGFR signaling is frequently associated with carcinogenesis and cancer progression. This can be the result of several unbalanced mechanisms controlling the quantitative and qualitative output of EGFR, such as elevated expression of receptors and ligands, activating receptor mutations, and impaired endocytic receptor downregulation. Proper endocytic uptake and endosomal sorting of signaling receptors have been considered as a crucial step for precisely controlling cellular processes such as growth, differentiation, and survival. Our current understanding of ligand-induced receptor endocytic downregulation is largely from the knowledge of EGFR trafficking routes following EGF binding, which has historically been and remains to be the most popular experimental system for studies in this field. In contrast, very little is known about endocytosis of ErbB2-4, as well as about EGFR endocytosis following binding of ligands other than EGF. It is generally believed that EGFR is present at the plasma membrane as a monomer prior to activation. Ligand (EGF) binding triggers EGFR dimerization and activation of its intrinsic kinase, leading to signaling and relocation to invaginating clathrin-coated pits (CCPs) on the plasma membrane. The CCPs give rise to clathrin-coated endocytic vesicles. The vesicles are then released from the membrane and fuse with early endosomes. Thus, EGFR is delivered to this compartment by these sequential processes. From here the receptor is sorted for further transport, either back to the cell surface by recycling, or to the multivesicular bodies (MVBs), a pathway for eventual delivery of EGFR to late endosomes and lysosomes for degradation, which results in temporary EGFR downregulation (Figure 3). Under most physiological
conditions, clathrin-dependent pathways are considered to be the main routes of EGFR internalization and downregulation. However, clathrin-independent pathways have also been reported and suggested as alternative mechanisms for EGFR endocytosis (Orth et al., 2006; Sigismund et al., 2005; Yamazaki et al., 2002), which will not be discussed here.

Fig. 3. EGFR endocytosis, trafficking, and turnover. EGF engagement results in EGFR activation and signaling from the cell surface. Upon EGF binding, EGFRs are internalized into clathrin-coated pits (CCP). Activated EGFR recruits the E3 ubiquitin (Ub) ligase Cbl, which ubiquitinates EGFR. EGFRs are delivered to early endosomes. From here, the receptors are sorted for either recycling back to the plasma membrane or transferring via multivesicular bodies (MVB) to late endosomes/lysosomes for degradation. The activated EGFR can continuously signal from endosomes or during its postendocytic trafficking. See the text for more details.

3.1 Ubiquitination and internalization of EGFR

Although the major steps of EGF/EGFR endocytosis and trafficking pathways are well established (Grandal & Madshus, 2008; Wiley, 2003), the molecular machinery controlling these processes remains poorly understood. It is believed that ubiquitination plays a key role in “tagging” or sorting EGFR for endocytosis and degradation (Hicke, 1999). Cbl is a ring-finger domain E3 ubiquitin ligase that is mainly responsible for EGFR ubiquitination (Levkowitz et al., 1998; Waterman et al., 1999). Upon EGF binding to EGFR, Cbl proteins are tyrosine phosphorylated by Src kinases (Feshchenko et al., 1998) and recruited rapidly to the
activated EGFR to mediate the receptor ubiquitination (Sebastian et al., 2006). Cbl can bind to EGFR either directly at phosphorylated tyrosine residue 1045 (Y1045) or indirectly via adaptor protein Grb2, which binds to phosphorylated EGFR at Y1068 and Y1086 (Levkowitz et al., 1999; Waterman et al., 2002). However, these two Cbl-EGFR interaction mechanisms have different effects on EGFR endocytosis. Direct binding via Y1045 may not be necessary for EGFR endocytosis, as the Y1045F mutation of EGFR results in impaired ubiquitination but does not affect receptor internalization (Grøvdal et al., 2004; Jiang et al., 2003). In contrast, Grb2-mediated binding is essential and sufficient for EGFR internalization. This is supported by the fact that Grb2 knockdown inhibits EGFR endocytosis and a chimeric protein consisting of the Y1068/Y1086-binding domain of Grb2 fused to Cbl can rescue the EGFR internalization in Grb2-depleted cells (Huang & Sorkin, 2005).

In addition to acting as an E3 ubiquitin ligase, Cbl may have other functions in EGFR endocytic signaling. Phosphorylated Cbl can bind to the 85 kDa Cbl interacting protein (CIN85) that is constitutively associated with endophilin (Soubeyran et al., 2002), a known regulator of clathrin-mediated endocytosis (CME) (Reutens & Begley, 2002). The recruitment of CIN85 and endophilin to EGFR by Cbl plays an important role in EGF-induced EGFR internalization and downregulation (Soubeyran et al., 2002). Furthermore, Eps15 and epsin, the two adaptor proteins with ubiquitin binding capacity and known to localize to CCPs, are required for EGFR internalization and possibly form a complex with ubiquitinated EGFR (Polo et al., 2002; Roepstorff et al., 2008; Salcini et al., 1999). Interestingly, it has been reported that Esp15 localizes at the rim of CCPs (Stang et al., 2004; Tebar et al., 1996), while epsin localizes along the entire CCP curvature (Stang et al., 2004). These findings suggest that EGFR (ubiquitinated by Cbl) is captured by Eps15 and subsequently handed off to epsin deeper in the coated pits, which could be a more efficient way of EGFR progression into CCPs (Grandal & Madshus, 2008).

As shown above, many lines of evidence indicate that functional Cbl is a prerequisite for EGFR internalization and that Cbl ubiquitinates EGFR. However, the role of EGFR ubiquitination as an internalization signal remains controversial. One study reported that an ubiquitination-deficient mutant of EGFR with full kinase activity can still undergo normal internalization (Huang et al., 2007). Several studies also showed that siRNA depletion of epsin and/or Eps15 did not specifically affect the clathrin-mediated EGFR internalization (Chen & Zhuang, 2008; F. Huang et al., 2004; Sigismund et al., 2005; Vanden Broeck & De Wolf, 2006). A very recent study has demonstrated that CME of activated EGFR is regulated by four mechanisms, which function in a redundant and cooperative fashion (Goh et al., 2010). All these imply that the EGFR endocytosis is a rather complicated process whose molecular mechanisms deserve further investigation.

3.2 Ubiquitination and endosomal sorting of EGFR
Upon EGF binding, activated EGFR undergoes CME at a much enhanced rate compared to the constitutive (ligand-independent) rate (Wiley, 2003). Immediately after internalization by CME, EGFR is delivered to early endosomes for sorting to either recycled back to the plasma membrane or transferred via MVBs to late endosomes/lysosomes for degradation (Figure 3). If not recycled back to the cell surface (as in the absence of EGF stimulation), EGFRs are sorted for lysosomal degradation. The latter is initiated by forming a complex with Esp15, signal transduction adaptor molecule (STAM), and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Bache et al., 2003). Hrs directs the receptors to
tumor susceptibility gene-101 (TSG101). The endosomal sorting complex required for transport (ESCRT) complexes (ESCRT-I to III) are sequentially recruited. These processes eventually lead to the translocation of EGFRs into the intraluminal vesicles (ILVs) of MVBs and MVS fusion with lysosomes for receptor degradation and signal termination (Bache et al., 2006; Katzmann et al., 2002; Q. Lu et al., 2003; Williams & Urbé, 2007).

In contrast to its controversial role in EGFR internalization (see above), it is clear that Cbl-mediated EGFR ubiquitination plays a pivotal role at the early endosome to the late endosome/lysosome sorting step of EGFR downregulation (Duan et al., 2003). EGFR mutants with reduced ubiquitination display impaired downregulation or degradation (Grøvdal et al., 2004; Huang et al., 2007; F. Huang et al., 2006; Jiang & Sorkin, 2003; Levkowitz et al., 1999), and the Y1045F mutant can not translocate to ILVs (Grøvdal et al., 2004). Thus, it can be concluded from these studies that Cbl-associated ubiquitination is the signal for EGFR downregulation.

3.3 EGFR signaling from the endosome

EGF binding leads to and accelerates internalization and lysosomal degradation of EGFR. The most obvious function of receptor endocytosis is to remove activated EGF/EGFR complexes from the cell surface to achieve consumption of ligand and activated receptors and to prevent excessive signaling. Thus, the canonical view holds that endocytosis is a mechanism to attenuate receptor signaling via receptor downregulation. On the other hand, it has been known for many years that activated EGFR following EGF stimulation remains at the cell surface only briefly (5-10 min), and the majority of activated receptors are located in endosomes for a much longer time (1 h) (Lai et al., 1989; Sebastian et al., 2006; Wiley, 2003). Accumulating evidence indicates that the activated EGFR can continuously signal from endosomes or during its postendocytic trafficking (Baass et al., 1995; Carpenter, 2000; Pennock & Wang, 2003; Wang et al., 2002a).

Studies of EGFR signaling in the context of endocytosis have uncovered that endosome-associated EGFR is linked to many, if not all, of its downstream signaling cascades, suggesting the complex and multifaceted effects of EGFR endocytosis on its signaling. Early work done in rat liver parenchyma (in vivo) has demonstrated that, shortly after EGF administration (1 min), internalized activated EGFR recruits a protein complex of Shc, Grb2, and the son of sevenless (Sos) to endosomes, leading to endosomally localized activation of the Ras/Raf/MEK/ERK pathway (Di Guglielmo et al., 1994). In mice, the MEK1 binding partner (MP1), adaptor protein p14, and MEK1 form a complex in endosomes. Such endosomal p14-MP1-MEK1 signaling plays an important role in cell proliferation during tissue homeostasis (Teis et al., 2006; Teis et al., 2002). Further, appropriate trafficking of activated EGFRs through endosomes ensures spatial and temporal fidelity of MAPK signaling (Taub et al., 2007). An elegant work in which EGFR is specifically activated when it is endocytosed into endosomes, has established that internalized EGFR can exert signals from endosomes to control cell survival (Wang et al., 2002a; Wang et al., 2002b), possibly by stimulating the PI3K/Akt pathway (Haugh & Meyer, 2002; Sorkin & von Zastrow, 2009). Finally, it has been reported that EGFR endocytosis is essential for STAT3 nuclear translocation and STAT3-dependent gene regulation, suggesting that endocytosis is the transport machinery for STAT3 translocation through the cytoplasm to the nucleus (Bild et al., 2002). Collectively, ligand-activated EGFR has been demonstrated to continue to signal along the endocytic pathway, which contributes to the spatio-temporal regulation of signaling, i.e. determining the specificity of signals and controlling the strength and duration of signaling.
4. ERK-dependent EGFR phosphorylation and its impact on EGFR trafficking

4.1 Ras/Raf/MEK/ERK signaling pathway

The Ras/Raf/MEK/ERK cascade is one of the major and best studied EGFR downstream pathways, which links extracellular signals to the machinery that can regulate diverse and fundamentally important cellular processes such as cell proliferation, differentiation, migration, apoptosis, angiogenesis, and chromatin remodeling (Dunn et al., 2005; Yoon & Seger, 2006). Upon ligand binding, receptor dimerization, and EGFR intrinsic kinase activation and auto-phosphorylation, activation of the ERK pathway is triggered by Grb2 binding directly to EGFR at Y1068 and Y1086 and indirectly through Shc binding at Y1148 and Y1173 (Batzer et al., 1994; Lowenstein et al., 1992) (Figure 2). Grb2 recruits Sos guanine nucleotide exchange factor to the receptor complex and Sos mediates the route of activation of Ras proteins (H-Ras, K-Ras, and N-Ras) at the plasma membrane (Downward, 1996; Quilliam et al., 1995). Ras activation induces the activation of Raf family kinases including A-Raf, B-Raf, and C-Raf (Raf-1) (Marais et al., 1997; Marais & Marshall, 1996). The active Raf then activates MEK1 and MEK2 by phosphorylating serines 218 and 222 in the activation loop, which further phosphorylate and activate ERK1 and ERK2 (Dhillon et al., 2007; McKay & Morrison, 2007). These active ERKs phosphorylate numerous cytoplasmic and nuclear targets including kinases, phosphatases, transcription factors, and cytoskeletal proteins (Yoon & Seger, 2006). We have recently uncovered ERK activation-dependent phosphorylation of EGFR in several cell systems including human prostate cancer cells, which can have profound feedback to EGFR signaling and trafficking and EGFR-driven cell migration. These previously understudied aspects are further discussed below.

4.2 ERK activity-dependent phosphorylation of EGFR at threonine-669

As illustrated in Figure 2, EGF binding to EGFR causes activation of the receptor tyrosine kinase and phosphorylation at multiple tyrosine residues in the cytoplasmic tail. Besides the tyrosine phosphorylation events, EGFR can be phosphorylated at several serine and threonine residues (Bao et al., 2000; Countaway et al., 1990; Theroux et al., 1992a), which may influence the EGFR kinase activity (Countaway et al., 1992; Theroux et al., 1992b). We have recently uncovered a previously unappreciated type of EGFR phosphorylation induced by EGF stimulation in several cell types. By employing a state-specific monoclonal antibody (mAb), PTP101, which specifically recognizes phosphorylation of the consensus site(s) (serine or threonine residues) in the substrates for proline-directed protein kinases such as ERKs (Pearson & Kemp, 1991), we initially observed that upon EGF stimulation, both EGFR and ErbB2 undergo PTP101-reactive phosphorylation in addition to tyrosine phosphorylation in murine 3T3-F442A preadipocytes (Huang et al., 2003). Such PTP101-reactive phosphorylation seems to correlate well with EGF-induced ERK activation, as the phosphorylation can be specifically inhibited by pretreatment of the cells with two separate MEK1 inhibitors, PD98059 and UO126 (Huang et al., 2003). Furthermore, we found that peptide hormones, such as growth hormone (GH) and prolactin, can activate ERKs and cause PTP101-reactive phosphorylation of both EGFR and ErbB2 in 3T3-F442A (Huang et al., 2003) and human T47D breast cancer cells (Y. Huang et al., 2006), respectively. Previous studies suggested that serine/threonine phosphorylation of EGFR and ErbB2/Neu induced by the phorbol ester (PMA) and platelet-derived growth factor (PDGF) are attributable to the activation of PKC (Bao et al., 2000; Davis & Czech, 1985; Davis & Czech, 1987; Epstein et al., 1990; Hunter et al., 1984; Lund et al., 1990). Our data showed that neither GH-induced
ERK activation nor EGFR and ErbB2 PTP101 reactivity are affected by the PKC inhibitor (GF109203X), though the MEK1 inhibitors (PD98059 and UO126) are indeed inhibitory (Huang et al., 2003). Similar results have been obtained for prolactin-induced ERK activation and PTP101-reactivity of EGFR in T47D cells (Y. Huang et al., 2006). Collectively, our data suggests that the mAb PTP101 detects ERK-dependent, rather than PKC-dependent, serine/threonine phosphorylation of EGFR and ErbB2, and that EGF/GH/prolactin-induced and PMA-induced phosphorylation may have distinct mechanisms (Huang et al., 2003; Y. Huang et al., 2006). Interestingly, we have recently demonstrated that such an EGF-induced serine/threonine phosphorylation of EGFR also occurs in human prostate cancer cells, which requires activation of ERK pathway but not Akt pathway (Gan et al., 2010) (Figure 4).

Fig. 4. EGF-induced PTP101-reactive threonine phosphorylation of EGFR is ERK pathway dependent. Serum-starved DU145 cells were pretreated with vehicle control, MEK/ERK pathway inhibitors (PD98059 or UO126) or PI3K/Akt pathway inhibitor (LY294002) for 1 h prior to stimulation with EGF for 15 min. Protein extracts were either immunoprecipitated (IP) with anti-EGFR antibody, followed by immunoblotting (IB) with PTP101 (A) or anti-EGFR (B), or directly immunoblotted with anti-phospho-ERK (C) or anti-total ERK (D). For more details see (Gan et al., 2010). Reprinted with permission from Oncogene.

Two major threonine phosphorylation sites are known in the EGFR juxtamembrane cytoplasmic domain, Thr-654 and Thr-669 (Davis & Czech, 1985; Heisermann & Gill, 1988; Hunter et al., 1984; Takishima et al., 1988). PKC may directly mediate Thr-654 phosphorylation (Davis & Czech, 1985; Hunter et al., 1984), whereas Thr-669 is thought to be phosphorylated by ERKs (Northwood et al., 1991; Takishima et al., 1991). The human EGFR cytoplasmic tail contains only one ERK consensus phosphorylation site [PX(S/T)P], i.e. PL669TP (Li et al., 2008). In reconstituted Chinese hamster ovary (CHO) cells, we showed that only wild-type EGFR, but not EGFR mutant (EGFR-T669A in which Thr-669 is mutated to alanine), underwent PTP101-reactive phosphorylation upon EGF stimulation, although EGF can cause tyrosine phosphorylation of both forms of receptors (Li et al., 2008). In a comparison experiment, in distinction to EGFR-T669A, a different EGFR mutant (EGFR-T654A in which Thr-654 is mutated to alanine) can undergo PTP101-reactive phosphorylation after EGF treatment, which was abolished by the MEK/ERK pathway inhibitor PD98059 (Li et al., 2008). These findings indicate that Thr-669, but not Thr-654, is required for EGF-induced, ERK activity-dependent PTP101-reactive (threonine) phosphorylation of EGFR.
4.3 Impact of Thr-669 phosphorylation on EGFR tyrosine phosphorylation (activation) and trafficking

EGF binding triggers EGFR kinase activation and phosphorylation, and also initiates the process of EGFR endocytosis and degradation, leading to temporary downregulation of EGFR (Wiley, 2003; Wiley et al., 2003). Previous views held that signaling emanated only from activated cell-surface EGFRs and that internalization terminated signaling (Wells et al., 1990). However, it is now more widely believed that signaling can also emanate from the EGFR in the process of postendocytic trafficking and thus, altered postendocytic trafficking of the activated EGFR may quantitatively and/or qualitatively influence its net signaling (Burke et al., 2001; Ceresa & Schmid, 2000; Di Fiore & De Camilli, 2001; Sebastian et al., 2006; Wiley, 2003).

We previously reported that GH pretreatment lessens EGF-induced EGFR downregulation in murine 3T3-F442A preadipocytes (Huang et al., 2003). Further, GH-mediated attenuation of EGF-induced EGFR downregulation is ERK pathway-dependent, correlating with GH-induced threonine phosphorylation of EGFR and signaling synergy of GH and EGF (Y. Huang et al., 2004; Huang et al., 2003). Similarly, in human T47D breast carcinoma cells, prolactin-induced, ERK activation-dependent, PTP101-reactive phosphorylation of EGFR retards subsequent EGF-induced receptor downregulation and potentiates acute EGF/EGFR signaling (Y. Huang et al., 2006). Furthermore, in T47D cells, EGF itself causes PTP101-reactive threonine phosphorylation of EGFR, and inhibition of the MEK/ERK pathway enhances EGF-induced EGFR downregulation (Y. Huang et al., 2006). Similar results were obtained in a human fibrosarcoma cell line that harbors an activating Ras mutation and subsequent basal activation of ERK and ERK-dependent PTP101-reactive EGFR phosphorylation (Li et al., 2008). Recently, we have demonstrated that in two human prostate cancer cell lines, DU145 and PC-3, pharmacological blockade of MEK/ERK pathway, but not PI3K/Akt pathway, results in accelerated EGF-induced EGFR downregulation (Figure 5), which negatively correlates with ligand-induced ERK-dependent threonine phosphorylation of EGFR (Figure 4) (Gan et al., 2010). Taken together, these results strongly suggest that ERK-mediated threonine phosphorylation of EGFR, whether accomplished by GH or prolactin (via crosstalk), or as a result of EGF-induced ERK activation, may serve as a “brake” on ligand-induced EGF downregulation. Indeed, elimination of EGFR phosphorylation at threonine-669 by a point mutation (threonine to alanine) resulted in accelerated EGF-induced EGFR loss in CHO reconstitution cell system (Li et al., 2008).

Fig. 5. Inhibition of ERK pathway but not Akt pathway accelerates EGF-induced EGFR downregulation. (A) Serum-starved DU145 cells were pretreated with vehicle (DMSO), PD98059 or LY294002 for 1 h prior to stimulation with EGF for 0-30 min. Protein extracts were subjected to immunoblotting (IB) with anti-EGFR or anti-β-actin. (B) Statistical analysis of pooled data from five independent experiments indicated that PD98059 significantly enhances EGF-induced EGFR downregulation at 15 and 30 min (**, P < 0.01). For more details see (Gan et al., 2010). Reprinted with permission from Oncogene.
Early studies of EGFR phosphorylation at serine and threonine sites, including serine-1046, serine-1047, and threonine-654, revealed that mutations at these sites can modulate EGFR signaling and downregulation (Bao et al., 2000; Countaway et al., 1990; Countaway et al., 1992; Theroux et al., 1992a). When examining the impact of ERK-mediated EGFR phosphorylation at threonine-669 on EGFR signaling, we found that in the CHO cell reconstitution system, the mutant EGFR-T669A exhibits enhanced tyrosine phosphorylation (reflecting EGFR kinase activation) compared to wild-type EGFR upon EGF stimulation (Li et al., 2008). Interestingly, coexpression of wild-type EGFR and EGFR-T669A, presumably resulting in a hybridimer of wild-type and mutant EGFR, does not dampen the propensity of EGFR-T669A to enhance EGF sensitivity (reflected in enhanced EGFR kinase activation) (Li et al., 2008). This led us to conclude that, in the hybridimer, the mutant EGFR-T669A exerts dominance regarding the EGF-induced EGFR activation (Li et al., 2008). More recently, in human prostate cancer cells (DU145 and PC-3) where the endogenous EGFR level is high, we have shown that pharmacological inhibition of the MEK/ERK pathway, but not the PI3K/Akt pathway, significantly augments the EGF-induced EGFR phosphorylation at multiple tyrosine residues including Y845, Y1045, and Y1068 (Gan et al., 2010).

The EGF-induced downregulation of EGFR is a complex, tightly regulated process, and impaired endocytic downregulation is often associated with malignancy (Grondal & Madshus, 2008; Polo et al., 2004; Roepstorff et al., 2008). The molecular machinery controlling ligand-induced EGFR endocytic trafficking remains poorly understood. It is believed that ubiquitination plays a key role in “tagging” EGFR for endocytosis. Subsequent to EGF binding to EGFR, the activated receptor is rapidly ubiquitinated by Cbl, an ubiquitin ligase that binds to phosphorylated EGFR, promoting post-inteernalization EGFR sorting to lysosomes for degradation (see Sections 3.1 and 3.2 for details). In human prostate cancer cells, we uncovered that blockade of the MEK/ERK pathway, but not the PI3K/Akt pathway, significantly enhanced EGF-induced ubiquitination of EGFR, correlating with increased Cbl tyrosine phosphorylation level and degree of physical association between tyrosine phosphorylated Cbl and activated EGFR (Gan et al., 2010). This phenomenon in prostate cancer cells resembles the effects of mutant EGFR-T669A in the CHO reconstitution system, in which EGFR-T669A underwent more robust ubiquitination than wild-type EGFR did upon EGF stimulation, due to the loss of phosphorylation at Thr-669 in EGFR-T669A cells (Li et al., 2008).

Emerging evidence suggests that Cbl can bind to EGFR directly at phosphorylated Y1045 or indirectly through Grb2, which binds to phosphorylated Y1068 and Y1086 in the EGFR cytoplasmic tail (Levkowitz et al., 1999; Waterman et al., 2002). As described above, our data in prostate cancer cells indicated that inhibition of ERK activity enhances the EGF-induced tyrosine phosphorylation of EGFR at multiple sites, at least including Y1045 and Y1068 (Gan et al., 2010). This raises several interesting questions, such as through which site(s) or tyrosine residue(s) within the EGFR cytoplasmic domain is the effect of the ERK activation-dependent Thr-669 phosphorylation exerted; whether Cbl is the sole factor in EGFR ubiquitination or are there any other contributors, such as CIN85, Grb2, Eps15, epsin, Hrs, and ESCRT complexes (see Sections 3.1 and 3.2 for details); and finally whether two completely different types of EGFR phosphorylation (tyrosine versus threonine phosphorylation) exist and how they are balanced under physiological and pathological conditions. More detailed studies are required to decipher these mechanisms. Taken together, our recent experimental data from multiple cell systems strongly support the notion that ERK-mediated Thr-669 phosphorylation of EGFR may serve as a “brake” on EGF-induced EGFR activation, signaling, and trafficking (ubiquitination and downregulation) (Figure 6).
Fig. 6. Schematic model of how ERK activity-dependent threonine phosphorylation of EGFR modulates EGF-induced EGFR ubiquitination and downregulation. Based on our published data (Gan et al., 2010; Huang et al., 2003; Y. Huang et al., 2006; Li et al., 2008), ERK activation results in PTP101-reactive phosphorylation of EGFR at Thr-669. Such threonine phosphorylation serves as a “brake” on EGF-induced EGFR tyrosine phosphorylation (kinase activation), ubiquitination, and downregulation (A). Mutation of Thr-669 to alanine (B), or blockade of the ERK pathway by PD98059 (C) abolishes the threonine phosphorylation of EGFR, which releases the “brake”, resulting in enhanced EGF-induced EGFR tyrosine phosphorylation/activation, ubiquitination, and downregulation.

5. Akt signaling, EGF/EGFR-driven epithelial-mesenchymal transition (EMT) and tumor metastasis

5.1 PI3K/Akt signaling pathway
The PI3K/Akt pathway plays an important role in human cancers including prostate carcinoma (Chin & Toker, 2009; de Souza et al., 2009; Morgan et al., 2009; Qiao et al., 2008). Akt was initially identified as an oncogene within the murine leukemia virus AKT8 (Staal, 1987; Staal & Hartley, 1998). It is a serine/threonine kinase and also called protein kinase B (PKB) because its catalytic domain is related to PKA and PKC family members (Jones et al., 1991). In humans, there are three highly homologous isoforms of Akt (Akt1, Akt2, and Akt3) (Nicholson & Anderson, 2002). However, it remains controversial whether all three are equally important in human malignancies (Chin & Toker, 2009; Le Page et al., 2006; Maroulakou et al., 2008). PI3K and the tumor suppressor, phosphatase and tensin homolog...
deleted on chromosome 10 (PTEN), are two well-known upstream components of Akt. Receptor tyrosine kinases such as EGFR and IGF-1R can activate PI3K at the cell membrane, initiating the PI3K/Akt signaling cascade. Once activated, PI3K phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2), leading to accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3) (Morgan et al., 2009). PIP3 recruits Akt and phosphoinositide dependent protein kinase 1 (PDK1) to the cell membrane, where Akt is phosphorylated at Thr-308 by PDK1 and at Ser-473 via an unknown mechanism (de Souza et al., 2009). Activated Akt translocates to the nucleus, resulting in downstream effects, such as cell survival (anti-apoptosis), cell motility, angiogenesis, proliferation, and metabolism (Chin & Toker, 2009; de Souza et al., 2009; Morgan et al., 2009). PTEN is the primary negative regulator of Akt (Li et al., 1997). Loss of PTEN or PTEN mutation is the most common cause of hyperactivation of the PI3K/Akt pathway in many human cancers (Sansal & Sellers, 2004). Most recently, we have demonstrated that the Akt pathway plays a central role in EGFR-driven prostate cancer cell migration by activating epithelial-mesenchymal transition (EMT) (Gan et al., 2010), which is discussed in detail below.

5.2 EMT and tumor metastasis

EMT is a pivotal physiological process involved in embryogenesis, wound healing, and tissue remodeling (Thiery, 2003), and is regulated by complex signaling networks (Thiery & Sleeman, 2006). It is now recognized that EMT may be an important mechanism for carcinoma progression given EMT-like phenotypes of epithelial cancers (Klymkowsky & Savagner, 2009; Thiery, 2002). Acquisition of migratory properties is a prerequisite for cancer progression and for invasive migration of tumor cells into surrounding tissue. Within carcinoma (cancer of epithelial origin) cells, acquisition of invasiveness requires a dramatic morphological alteration similar to EMT, wherein carcinoma cells lose their epithelial characteristics of cell polarity and cell-cell adhesion and switch to a motile mesenchymal phenotype (Thiery, 2002; Thiery, 2003; Thiery & Sleeman, 2006). Disruption of cell-cell adherens junctions mediated by E-cadherin (one of the epithelial markers) is considered a crucial step in EMT and the downregulation of E-cadherin is common in metastatic carcinomas (Cavallaro & Christofori, 2004). Reduced E-cadherin expression has been found in high-grade prostate cancers and is associated with poor prognosis (Umbas et al., 1994; Umbas et al., 1992), reflective of its critical role in tumor progression. It is widely believed that downregulation of E-cadherin occurs via transcriptional repression mediated by the protein, Snail (Cano et al., 2000; Moreno-Bueno et al., 2008; Peinado et al., 2007). Accumulating evidence indicates that the EGFR family and PI3K/Akt signaling pathway can regulate Snail expression (Hipp et al., 2009; Lee et al., 2008; Qiao et al., 2008), suggesting that inhibition of the EGFR signaling pathways may prevent the loss of E-cadherin function and thereby acquisition of invasive motility (metastasis).

5.3 Role of Akt signaling in EGF/EGFR-driven EMT and prostate cancer cell migration

To understand which pathway(s) may have significant impact on EGFR-driven migration, we have recently probed this issue in human prostate cancer cells. The two cell lines, DU145 and PC-3, are both androgen insensitive (van Bokhoven et al., 2003), and are excellent models for studying EGFR signaling in hormonal-refractory prostate cancer. We showed that the two cell lines predominantly expressed EGFR but not ErbB-2 when compared to an androgen-responsive prostate cancer cell line, LnCap (van Bokhoven et al., 2003), in which
both EGFR and ErbB-2 were expressed (Gan et al., 2010). EGF activated EGFR and its downstream ERK and Akt pathways, and markedly promoted cell migration in both DU145 and PC-3. Using pharmacological inhibitors, LY294002 and PD98059, to specifically block PI3K/Akt and MEK/ERK pathways, respectively, we further demonstrated that LY29004, but not PD98059, significantly inhibited EGF/EGFR-driven cell motility. In parallel, we observed that DU145 cells expressing constitutively activated (myristoylated) Akt (Myr-Akt) migrated much faster than control cells (Gan et al., 2010). Taken together, our data suggests that Akt activation is critical for EGFR-mediated prostate cancer cell migration.

As described above, tumors of epithelial origin, as they transform to malignancy, appear to exploit the innate plasticity of epithelial cells, with EMT conferring increased invasiveness and metastatic potential. Previous studies have implicated the involvement of ErbBs in EMT and E-cadherin downregulation in breast, lung, and cervical cancer cells (Lee et al., 2008; Lu et al., 2009; Z. Lu et al., 2003). Our recent work has clearly demonstrated that prostate cancer cells undergo EMT-like morphological changes after EGF treatment, accompanied by the loss of E-cadherin at cell-cell junctions (Gan et al., 2010). Interestingly, these EGF-induced phenomena were markedly prevented when the cells were exposed to the PI3K/Akt pathway inhibitor LY294002 (Figure 7). Consistent with downregulation of E-cadherin (an epithelial marker), we further showed an upregulation of vimentin (a mesenchymal marker) induced by EGF treatment. Similarly, LY294002 pretreatment abolished the EGF-induced quantitative (mass) changes of both E-cadherin and vimentin (Figure 8) (Gan et al., 2010). All these findings suggest that Akt activation is required for EGFR-driven EMT.

Fig. 7. Effect of inhibition of Akt pathway on EGF-induced EMT and loss of E-cadherin at cell-cell junction. Serum-starved DU145 cells were treated with vehicle (-) or EGF for 24 h in the presence or absence of LY294002. Inhibition of the Akt pathway by LY294002 prevents EGF-induced EMT (A) and loss of E-cadherin expression at cell-cell adherens junctions (B). For details see (Gan et al., 2010). Reprinted with permission from Oncogene.
Snail is one of the several transcriptional factors that can suppress E-cadherin gene expression (Batlle et al., 2000; Cano et al., 2000) via binding to E-box sequences in the proximal E-cadherin promoter (Hemavathy et al., 2000). Snail is regulated by glycogen synthase kinase 3β (GSK3β, a downstream effector of Akt) by direct binding and phosphorylation, and inhibition of GSK3β results in upregulation of Snail and downregulation of E-cadherin (Zhou et al., 2004). This implies that Snail and GSK3β together function as a molecular switch for many signaling pathways leading to EMT, and may provide a new connection of Akt to EMT. Along this line, we uncovered that in prostate cancer cells, EGF induced robust GSK3β phosphorylation (inactivation) and LY294002 markedly inhibited this phosphorylation, which correlated with the Akt activity. Consistent with Akt-mediated inactivation of GSK3β, Snail was upregulated upon EGF stimulation. Intriguingly, LY294002 pretreatment abolished such an EGF-induced upregulation of Snail, presumably by inactivating Akt and restoring GSK3β activity (Figure 8). As an alternative approach, we also demonstrated that knockdown of endogenous Snail in DU145 cells significantly prevented the EGF-induced loss of E-cadherin expression and concurrently suppressed EGF-driven EMT, which correlated with a decrease in EGF-directed cell migration (Figure 9) (Gan et al., 2010). These results implicate Snail as a central effector of EMT and cell motility mediated by EGF/EGFR-activated Akt within prostate cancer cells. Collectively, our findings that EGF-mediated Akt signaling affects both phenotypic and molecular attributes, typical of EMT, provide new insights into the molecular mechanisms of EGF-driven prostate cancer progression and metastasis.
6. Negative feedback loop between EGFR-directed ERK and Akt signaling

As described above, Ras/Raf/MEK/ERK and PI3K/Akt signaling pathways play central roles in many aspects related to tumorigenesis and cancer progression. Thus, inhibition of these signaling cascades could hold powerful therapeutic potentials. Given that many receptors utilize the common downstream pathways such as MEK/ERK and PI3K/Akt, targeting these kinases is expected to have greater therapeutic efficacy and broader applicability. For example, blockade of signaling through MEK offers the potential advantage of inhibiting both proliferation-promoting and anti-apoptotic signals originating from either activated receptors or mutation of RAS/Raf in breast cancer (Adeyinka et al., 2002). However, clinical studies of MEK inhibitors have only shown limited antitumor effects (Adjei et al., 2008; Rinehart et al., 2004). The underlying mechanisms remain poorly understood.

The molecular features of breast cancer cells that determine sensitivity to pharmacological inhibition of the Ras/Raf/MEK/ERK signaling pathway have been recently examined. Using a large set of human breast cancer cell lines as a model system, it was found that activation of PI3K/Akt pathway in response to MEK inhibition through a negative MEK-EGFR-PI3K feedback loop counteracts the efficacy of MEK inhibition on cell cycle and apoptosis induction (Mirzoeva et al., 2009). In concert with this finding, we uncovered that in prostate cancer cells, in contrast to inhibition of PI3K/Akt pathway, inhibition of MEK/ERK pathway rather enhanced EGF-directed cell motility, accompanied by enhanced EGF-induced Akt activation (Figure 10) (Gan et al., 2010). This phenomenon highly supports the notion that Akt is the key node in EGFR-mediated migratory pathways (see Section 5.3). It also raises a key question as to how ERK inactivation exerts its feedback effect to EGF-induced Akt activation. Based on our data, we believe that one mechanism could be through the feedback of ERK on EGFR phosphorylation (Figure 6). One can envision that inhibition
of ERK activity eliminates EGFR threonine-669 phosphorylation, resulting in enhanced EGFR tyrosine phosphorylation (kinase activation), and subsequently augmented activation of the downstream PI3K/Akt pathway. The discovery of the negative feedback loop of MEK/ERK-EGFR-PI3K/Akt on several cellular aspects implies that targeting single MEK/ERK pathway in some cancers (e.g., breast and prostate carcinomas) may have undesirable outcomes, which deserves further investigation.

Fig. 10. Effects of ERK and Akt pathways on EGF-driven prostate cancer cell migration. (A) Inhibition of the ERK pathway by PD98059 augments EGF-induced Akt activation in both DU145 and PC3 cells, revealed by immunoblotting (IB) with anti-phospho-Akt antibody (top panel). (B) Transwell assay shows that blockade of the Akt pathway by LY294002 significantly inhibits EGF-driven cell migration. In contrast, blockade of the ERK pathway by PD98059 rather enhances EGF-induced migration. **, \( P < 0.01 \). For details see (Gan et al., 2010). Reprinted with permission from Oncogene.

7. Concluding remarks

Recent advances in the ErbB field have broadened our understanding of the important roles of EGFR/ErbB signaling in human cancer. However, the complexity of the ErbB signaling network, which involves numerous ligands, multiple dimerization partners, and a variety of downstream signaling components, makes it a real challenge to establish which pathways are activated or critical in the context of tumorigenesis and progression of specific cancer types. In this chapter, several aspects of EGFR/ErbB signaling and their potential roles in prostate cancer initiation and progression are discussed. In particular, we focus on the mechanisms of how Ras/Raf/MEK/ERK and PI3K/Akt pathways impact EGFR phosphorylation, trafficking, and cell motility. New insights into prostate cancer biology gained from our own work and the studies of other investigators highlight the importance of ERK activity-dependent threonine-669 phosphorylation of EGFR and its profound feedback on EGFR tyrosine phosphorylation/kinase activation, ubiquitination, and trafficking. Recent data from our group demonstrates that the Akt pathway plays a pivotal role in EGFR-driven prostate cancer cell migration by activating EMT. In particular, our results in prostate cancer (Gan et al., 2010) and data from a recent study in breast cancer (Mirzoeva et al., 2009) suggest that therapeutic targeting of ERK signaling may have undesirable outcomes. For example, inhibition of the MEK/ERK pathway conversely
activates the PI3K/Akt pathway through a negative MEK/ERK-EGFR-PI3K/Akt feedback loop. We believe that ERK-mediated threonine-669 phosphorylation is critically involved in such a negative feedback and thereby contributes to invasive migration (metastasis). Thus, inhibition of the MEK/ERK-EGFR-PI3K/Akt feedback loop is likely to result in therapeutic synergism. Future detailed studies along these lines and a deeper understanding of various mechanisms of cell signaling from EGFR and other ErbBs will undoubtedly generate new avenues for drug and biomarker development to combat cancers including prostate cancer.

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9. References


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The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the-art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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