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CURRENT CHALLENGES IN **PERSONALIZED CANCER MEDICINE**

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PREFACE

December 23rd 2011 marked the 40th year since President Richard Nixon signed the National Cancer Act declaring the "war on cancer". Despite significant progress being made, cancer still remains the second leading cause of death in Western Societies, with 1 in 2 of all Americans expected to develop cancer at some point in their lifetimes. With growing rates of obesity, diabetes and poor nutrition afflicting society, the future incidence of cancer seems likely to increase and new strategies for the treatment and management of patients with advanced malignancies are urgently required.

For many years the only therapies available for the treatment of advanced cancer have been cytotoxic drugs with modest selectivity for killing rapidly growing cancer cells over normal host cells. The general failure of these agents in most cancers, coupled with their narrow therapeutic windows and significant levels of toxicity, has led to the search for more selective anti-cancer drugs. The long held dream of cancer therapy, first espoused by Paul Ehrlich, has been the "magic bullet"; the ability to selectively kill malignant cells and to leave the healthy tissue unharmed (1). Thanks to the discoveries of the oncogene "revolution" and high throughput genomic sequencing we now understand a great deal about the underlying molecular basis of cancer and are coming closer to the reality that Ehrlich first postulated. It is now widely accepted that cancer is a disease of the genes and that tumors arise as a result of acquired genetic mutations. This realization has led to a shift from an "organ-centric" view of cancer to a more pathway-based, "oncogene-centric" view. Of therapeutic importance, it is now known that many types of cancer are uniquely dependent upon or "addicted" to signals from one oncogene for their survival and that dramatic anti-tumor responses can be achieved provided the correct oncogenic mutations are targeted (2). To date, small molecule inhibitors of Bcr-Abl, oncogenic *BRAF*, EGFR and Hedgehog signaling have been FDA-approved for chronic myeloid leukemia, *BRAF* mutant melanomas, sub-sets of non-small cell lung cancers (NSCLC) and locally advanced basal cell carcinoma, respectively (3-6). Although these new therapies have shown incredible promise in the clinic, responses have been have been mostly short-lived and resistance and disease relapse has been common (7). Strategies to further personalize cancer medicine, so that durable responses can be attained is likely to be a major

research theme for both academic and industrial scientists for many years to come.

For this volume of Advances in Pharmacology we have brought together some of the foremost basic science and clinical researchers to discuss some of the new frontiers in the development of targeted cancer therapy. The new age of personalized cancer therapy comes with a unique set of challenges for which the era of chemotherapy has provided little precedent. Data are already emerging showing that the inhibition of one signaling pathway or receptor tyrosine kinase (RTK), such the inhibition of BRAF in melanoma and EGFR in NSCLC, triggers compensatory signaling in parallel signaling pathways and RTKs, requiring rationally designed drug combinations. In other cases, the compensatory "escape" signaling may occur within the same pathway, as the result of altered feedback inhibition, so that one pathway will have to be targeted at multiple points (so-called vertical pathway inhibition). As we become better at targeting bulky disseminated disease, the chances of selecting for tumor cell clones that seed to therapeutically privileged sites such as the brain and the bone marrow are likely to increase, requiring novel strategies to co-target both the tumor and its sanctuary environment. Despite a drive towards more potent and specific therapies, a need still remains for more broadly targeted therapeutic agents, particularly for overcoming drug resistance. There is already good evidence from HER2 positive breast cancer and melanoma suggesting that resistance to EGFR and BRAF inhibitors could be overcome through combination with less specific agents, such as histone deacteylase inhibitors (HDAC) and heat shock protein (HSP)-90 inhibitors. At this stage, it is still not clear whether acquired drug resistance to targeted therapies arises following a process of adaptation and evolution or whether resistant clones (or even cancer stem cells) are already present prior to the initiation of therapy. The identification of the sub-population of cells within a tumor responsible for mediating resistance will prove critical in defining how therapeutic escape will be managed.

Although still in its formative stages, the development of targeted cancer therapies has already shown incredible promise in a limited number of cancer types. As basic cancer research and drug development continues, we expect this number to grow and more patients to benefit from these exciting advances. Through better patient selection and novel strategies to manage resistance, a future can be envisaged in which cancer can be reduced to the level of a chronic, manageable disease.

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Keiran S.M. Smalley

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CHAPTER ONE

Vertical Pathway Targeting in Cancer Therapy

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Abstract

Malignant cells arise from particular mutations in genes controlling cell proliferation, invasion, and survival. Older antineoplastic drugs were designed to target vital cellular processes, such as DNA maintenance and repair and cell division. As a result, these drugs can affect all proliferating cells and are associated with unavoidable toxicities. Recent discoveries in cancer research have identified "driver" mutations in some types of cancer, and efforts have been undertaken to develop drugs targeting these oncogenes. In most cases, due to escape mechanisms and adaptive responses, single oncogene targeting is insufficient to induce prolonged responses in solid tumors. Drug combinations are therefore used to enhance the growth inhibitory and cytotoxic effects of the targeted therapies. Depending on the position of additional targets within the signaling network, drug combinations may target either different signaling pathways (parallel targeting) or the same pathway at several fragile nodes (vertical targeting). In this review, we discuss strategies of multitarget inhibition with a focus on vertical signaling pathway targeting.

1. INTRODUCTION

Cancer is the second most frequent cause of mortality worldwide and the leading cause of death in developed countries (Jemal et al., 2011). While early stage solid tumors are curable by surgical resection and/or adjuvant therapy, disseminated cancers typically have a poor prognosis and require alternative therapeutic approaches, including the targeting of cellular mechanisms supporting uncontrolled cellular proliferation and metastasis.

There are over 100 types of cancer which affect virtually every organ or tissue in the human body. Until recently, most chemotherapies used in clinical practice targeted vital cellular processes such as DNA replication/repair (e.g., nucleotide analogs and intercalating agents) and cell division (including drugs inhibiting polymerization of cytoskeletal proteins) in a nonspecific fashion. This indiscriminate approach is toxic to any

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proliferating cell in the body. Most malignant cells derived from solid tumors have a number of mutations, which are necessary for tumorigenesis (Hahn & Weinberg, 2002). It has been proposed that given the genetic instability of cancers, every tumor might be unique and the repertoire of genetic changes that culminate in human tumors is infinitely variable. Mutations can be divided into three categories: "driver mutations" (hyperactivation of strong oncogenes and loss of tumor suppressors), "weak oncogenes," and "passengers" (accidentally co-selected mutations). The latter two categories are hard to distinguish since up to 5–10% of the entire exome is mutated in some tumor specimens (Salk et al., 2010). Hahn et al. have proposed that most of the driver mutations have been already discovered, and additional weaker oncogenes and passenger mutations in tumors might not be important drug targets (Hahn & Weinberg, 2002). Despite the complexity and heterogeneity of the genomic mutations in tumor specimens, some mutations render cancer cells dependent upon, or "addicted to", the activity of certain signaling cascades. For instance, the same oncogene may be responsible for proliferation and survival of different types of cancer. Protein kinases represent a significant fraction of these oncogenes, and fortunately, these enzymes are conducive to the development of small molecule inhibitors that target their kinase domains. In the case of receptor tyrosine kinases (RTKs), monoclonal antibodies can either block ligand-binding extracellular domains or neutralize their ligands. Examples of these phenomena are mutated ALK in subpopulations of patients with lung cancer, neuroblastoma, and anaplastic large cell lymphoma, and FGFR2 mutations in some endometrial and gastric cancers (Carpenter et al., 2012; Gatius et al., 2011; Kwak et al., 2010; Matsumoto et al., 2012; Merkel et al., 2011). Identification of aberrant molecular signaling mechanisms driving particular types of cancer has led to development of "smart" drugs that target a single oncogenic kinase or neutralize its ligand in the cancer cell. So far, the best clinical responses are seen when tumor cell survival is highly dependent on the targeted oncogene (Janne et al., 2009). This phenomenon is frequently referred to as "oncogene addiction" (Weinstein, 2002). Good examples are EGFR in subsets of non–small cell lung cancer (NSCLC), and BCR-Abl in chronic myelogenous leukemia (CML) (Sharma & Settleman, 2007). However, the assortment of oncogenic mutations is wide and triaging of genetically defined patient cohorts is of paramount importance in personalized medicine. In addition, single molecule targeting in most tumors is insufficient to induce prolonged tumor regression due to escape mechanisms and bypass signaling. One of the successful approaches to overcome

escape mechanisms via activation of downstream mediators is vertical pathway targeting. The following sections review and discuss approaches utilized to target signaling pathways in anticancer therapy, starting with single molecule targeting and finishing with multilevel pathway targeting.

2. MITOGENIC SIGNALING IN CANCER

Mitogenic growth factors and hormones promote cell proliferation by binding their cognate receptors and eliciting downstream signaling (Takeuchi & Ito, 2011). Many receptors are transmembranal RTKs. Typically, ligand binding results in conformational changes of the RTK and its dimerization (or in some cases oligomerization) which juxtaposes cytoplasmic kinase domains. This facilitates stabilization of the kinase in active conformation through phosphorylation of key tyrosine residues and results in full activation of the RTK (Hubbard & Miller, 2007). This creates phosphotyrosine sites on the cytoplasmic tail of the receptor and recruits phosphotyrosine-binding (SH2 or PTB domain-containing) adapter proteins. These adapters amplify the signal and recruit additional downstream effectors which activate major proliferative and survival signaling modules (e.g., Ras-MAPK and PI3K-mTOR cascades, Fig. 1.1). Upon deregulation, RTKs can support uncontrolled proliferation by constitutive mitogenic signaling. Receptors can become hyperactivated due to overexpression of the ligand or the receptor itself, or gain-of-function mutations driving ligand-independent constitutive activation of the receptor. Examples of strategies targeting ligands and receptors are described in the following sections.

3. MONOCLONAL ANTIBODIES AND SMALL MOLECULE INHIBITORS TARGETING ONCOGENIC SIGNALING IN CANCER

3.1. Monoclonal Antibodies Raised Against RTK Ligands

Several neutralizing monoclonal antibodies targeting RTK ligands have been developed for anticancer therapy. For instance, VEGF signaling not only promotes tumor vascularization, but also induces survival signaling in an autocrine manner (Amini et al., 2012; Breen, 2007). The addition of monoclonal antibodies that neutralize VEGF (bevacizumab) to chemotherapy can extend progression-free survival in patients with ovarian cancer,

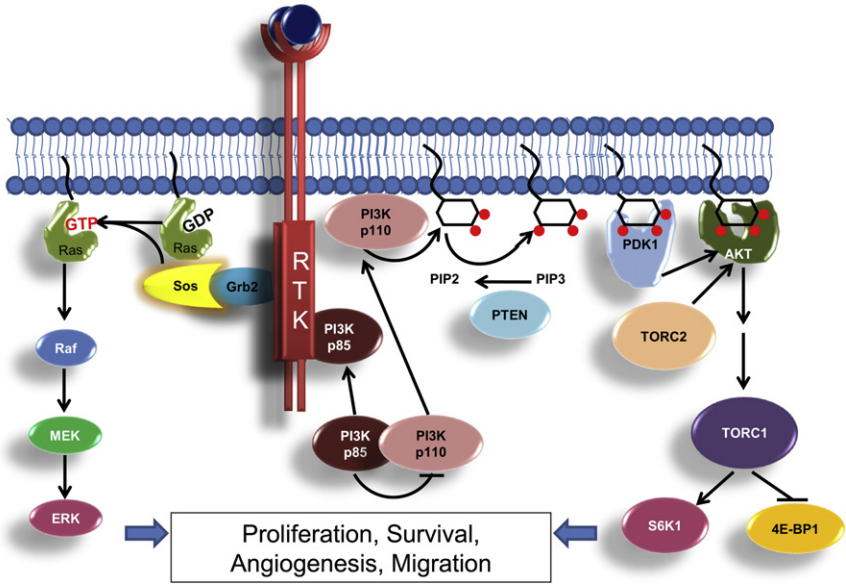

Figure 1.1 Simplified overview of RTK-induced Ras-MAPK and PI3K-mTOR activation: ligand binding to RTK results in receptor dimerization and conformational changes leading to kinase domain activation and trans/cis phosphorylation. Phosphotyrosine sites on the cytoplasmic tail of the receptor recruit phosphotyrosine-binding (SH2 or PTB domain-containing) adapter proteins. These adapters amplify the signal and recruit additional downstream effectors, which activate major proliferative and survival signaling modules (e.g., Ras-MAPK and PI3K-mTOR cascades). For color version of this figure, the reader is referred to the online version of this book.

NSCLC, metastatic clear cell renal carcinoma, and other solid tumors (Bukowski, 2010; Burger et al., 2011; Perren et al., 2011; Planchard, 2011). Unfortunately, not all ligands that are successfully targeted in preclinical models result in tumor shrinkage in patients. For example, the RTK c-MET and its ligand (HGF/SF) are frequently overexpressed in solid tumors (Sierra & Tsao, 2011). Nevertheless, the antibody targeting HGF/SF, rilotumumab, was ineffective in recurrent glioblastoma and renal cell carcinoma (Schoffski et al., 2011; Wen et al., 2011).

3.2. Monoclonal Antibodies *Against RTKs*

Uncontrolled mitogenic signaling can be mediated by activating mutations, amplification of RTKs, or amplification of nonreceptor TKs. Monoclonal antibodies that target RTKs have been used to treat solid tumors, such as cetuximab, the anti-EGFR monoclonal antibody, which is effective in

colorectal and head-and-neck cancers possessing wild-type K-Ras. Acquired resistance to cetuximab was associated with EGFR re-expression due to a trafficking defect and activation of ErbB2, ErbB3, and c-MET (Wheeler et al., 2008). While inhibition of c-MET had no effect, disruption of ErbB2/ErbB3 dimers using another monoclonal antibody resensitized cells to cetuximab.

Trastuzumab, an ErbB2-specific monoclonal antibody, is active against breast cancers overexpressing ErbB2 (Slamon et al., 2001). Both the mechanism of trastuzumab and the mechanisms of acquired resistance have been extensively studied (Pohlmann, Mayer, & Mernaugh, 2009). Resistance can stem from epitope masking (by either Mucin 4 or CD44/hyaluronan polymer complex), mutations in PI3K pathway signaling, and activation of alternative signaling pathways. Co-expression of insulin-like growth factor-1 receptor (IGF1-R) with ErbB2 has been associated with trastuzumab resistance. IGF-1R binds and phosphorylates ErbB2, activating downstream signaling through PI3K. Another RTK, c-Met, is quickly upregulated upon trastuzumab treatment and constitutively activates Akt, leading to trastuzumab resistance (Fiszman & Jasnis, 2011). There is a multitude of additional RTK-specific monoclonal antibodies which are active in the clinic, and many are in different phases of clinical trials.

3.3. Small-Molecule Kinase Inhibitors

3.3.1. Receptor Tyrosine Kinase Inhibitors (TKIs)

Small molecule kinase inhibitors are cell permeable and directly inactivate the intracellular kinase domains of their targets. The vast majority directly binds and blocks the ATP-binding pocket of the kinase. However, some allosterically inhibit their targets. These inhibitors target different types of kinases: RTKs, nonreceptor TKs, serine-threonine kinases, and lipid kinases. Some small-molecule inhibitors target multiple kinases with similar efficiency, whereas monoclonal antibodies are selective for a particular RTK or ligand. There are advantages and disadvantages to multitarget inhibition. Co-targeting several kinases increases the chances of blocking multiple pathways, while decreasing the chances of the tumor cells developing acquired drug resistance. Conversely, these multitarget inhibitors or drug combinations are often more toxic to normal tissues. There are 518 predicted kinases in the human proteome. Although many are not able to drive tumorigenesis on their own, they are capable of regulating cell proliferation, survival, migration, and angiogenesis (Janne et al., 2009). These features suggest that the human kinome represents a constellation of therapeutically relevant targets

for anticancer drug development. Since related kinases share high structural homology within their kinase domains, design and synthesis of specific kinase inhibitors has been challenging. Despite initial skepticism, kinases are highly amenable to targeting by selective small-molecule inhibitors (Janne et al., 2009). The first success of this strategy was with imatinib for CML, approved by the FDA in 2001. Imatinib primarily targets the Bcr-Abl kinase, and results in durable remissions in patients with CML. Imatinib has additional targets, c-Kit and PDGFRα. It has also been approved for gastrointestinal stromal tumors (GIST), where c-Kit is often mutated. Unfortunately, patients with CML are not cured with imatinib, and resistance can arise due to new mutations in Bcr-Abl. Dasatinib and nilotinib, which similarly target Bcr-Abl kinase, have been developed to overcome resistance to imatinib (Erba et al., 2011).

Small-molecule inhibitors of EGFR, gefinitib and erlotinib, are effective in some patients with NSCLC, particularly in patients whose tumors harbor EGFR mutations. EGFR inhibitors suppress key downstream signaling molecules such as ERK, Akt, and Stat3, and subsequently induce apoptosis (Sordella et al., 2004). Continuous culturing of EGFR TKI-sensitive NSCLC cells in the presence of an EGFR inhibitor results in selection of drug-resistant cells. These cultures, along with the biopsies from patients with acquired resistance to an EGFR-TKI, have provided insights into molecular mechanisms of resistance and identified new opportunities for vertical pathway co-targeting to overcome resistance. About 70% of resistant cells manifest a single point mutation in a tyrosine kinase domain of EGFR, T790M. This mutation increases the affinity toward its natural substrate, ATP, and reactivates the kinase by outcompeting the inhibitor (Su et al., 2012). Thus, addition of a downstream inhibitor may overcome the reactivation of EGFR via this mechanism. 20% of instances of acquired resistance may be attributed to the amplification of c-Met which counteracts EGFR inhibition by reactivating redundant proliferative and prosurvival pathways. This provides an opportunity for parallel pathway targeting (Sequist et al., 2011).

Tumors undergo changes during drug exposure, and combined targeting of RTKs and downstream signaling pathways is sometimes necessary to circumvent these adaptive changes. In a recent study, simultaneous EGFR, c-Met, and PDGFR inhibition was necessary to inhibit glioma cell growth. This was partially explained by the fact that EGFR and c-Met both activate PI3K survival signaling. Inhibition of EGFR resulted in compensatory survival signaling by c-Met (Stommel et al., 2007). Systems biology

approaches have been employed to model and predict RTK co-activation networks to analyze acquired resistance data in an attempt to design rational anticancer therapy combinations (Xu & Huang, 2010). Trastuzumab resistance in breast cancer cell lines has been attributed to the formation of a heterotrimer complex containing ErbB2, ErbB3, and IGF-1R, and depletion of either ErbB3 or IGF-1R resensitizes cells to trastuzumab (Huang et al., 2010).

3.3.2. Inhibitors of Signaling Molecules Downstream of Receptor Tyrosine Kinases

Two major signaling pathways that RTKs employ to increase survival and accelerate cell proliferation are the PI3K-mTOR and Ras-MAPK pathways. Both of these pathways are activated by growth factor receptors and result in cell proliferation and survival. These signaling pathways consist of several sequentially activated enzymes, such as protein kinases, lipid kinases, and small G-protein like molecules (Fig. 1.1). Several pathways can be concomitantly induced by a single tyrosine kinase, and transduction of the signal rarely occurs exclusively along the major pathway. Instead, multiple functional regulatory interactions, or points of "cross talk," exist between different pathways. These interactions may be activating or inhibitory. The pathway "cross talk" features diversify the signals transduced by RTKs in terms of duration, localization, lateral signaling (transactivation of other RTKs or pathways), and signal intensity (Fig. 1.2).

While the MAPK and PI3K-mTOR pathways are tightly controlled by RTKs under normal physiological conditions, they are often aberrantly activated by oncogenic RTKs in the course of tumorigenesis or activated by mutations downstream of the RTKs. Indeed, Ras isoforms, PI3K, and Raf are mutated or ectopically activated in many malignant tumors (Maurer et al., 2011; Schubbert et al., 2007; Yuan & Cantley, 2008). Novel inhibitors of MEK, mTOR, and Akt are currently in use in clinical trials or as standard therapy (Benjamin et al., 2011; McCubrey et al., 2010; Pal et al., 2010). In the following sections, we discuss clinically relevant inhibitors of PI3K, mTOR, Akt, Raf, and MEK.

3.3.2.1. PI3K

PI3K is a heterodimer consisting of a p85 inhibitory subunit and a p110 catalytic subunit. It is activated downstream of many RTKs via juxtamembranal recruitment, sequestration of p85, and subsequent release of

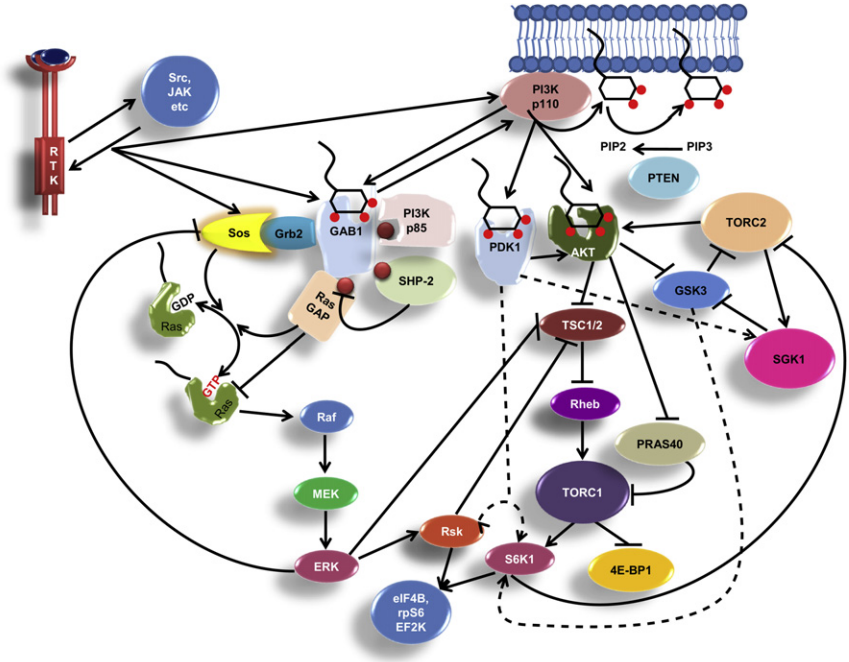

Figure 1.2 Ras-MAPK and PI3K-mTOR activation and cross talk: RTKs can activate intermediary soluble tyrosine kinases such as Src and JAK family members, which in turn can transactivate other RTKs, and/or act directly through recruitment of the adapter proteins (e.g., Shc, Grb2, Gab1, IRS proteins, and others). Several pathways can be concomitantly induced by a single tyrosine kinase. Multiple positive and negative feedback loops, or points of "cross talk," exist between pathways. The pathway "cross talk" features significantly diversify the signals transduced by RTKs in terms of duration, intensity, and regulation. For color version of this figure, the reader is referred to the online version of this book.

p110 (Fig. 1.1). Upon stimulation, PI3K activates a signaling cascade that is depicted in Fig. 1.1.

PIK3CA, one of the isoforms of the PI3K catalytic subunit, is frequently mutated in cancers, including breast (27%), endometrial (23%), colorectal (14%), urinary tract (17%), and ovarian (8%) cancers (Yuan & Cantley, 2008). Several drugs targeting PIK3CA have been developed. Due to kinase domain similarity, many of these inhibitors have dual specificity toward PI3K and mTOR. For instance, PI-103 was developed as a PI3K inhibitor but was also identified as an mTOR inhibitory agent. The poor pharmacokinetics precluded it from entering clinical trials, but the PI-103 backbone served as a blueprint for the development of intermediate PI-540 and PI-620 agents, which possessed better solubility and stability and culminated in the

development of a more potent pan-PI3K inhibitor, GDC-0941 (Raynaud et al., 2009). Preclinical breast cancer models showed that genetic alterations in either PIK3CA or ErbB2 alone or concomitant with PTEN loss are biomarkers of sensitivity to GDC-0941, whereas Ras mutations conferred resistance (O'Brien et al., 2010). The phase I clinical trials with this drug are complete and phase II trials are under way (Salphati et al., 2011).

In vitro data from a recent study indicated that either depletion of PI3K isoforms by RNAi silencing or treatment with BEZ235, a novel dual PI3K/mTOR inhibitor, led to apoptosis in estrogen receptor positive breast cancer cells when accompanied by estrogen deprivation (Crowder et al., 2009). Early phase clinical trials defining the safety of BEZ235 in patients with advanced cancers are ongoing (http://clinicaltrials.gov/ct2/show/NCT00620594, April 22, 2012).

Preclinical data using a pan-PI3K inhibitor, BKM120, shows at least 50-fold higher selectivity toward PI3K compared to other kinases (Maira et al., 2011). *In vitro* data from 353 cell lines indicate that in contrast to Ras and PTEN mutations, PIK3CA mutations are the determinants of sensitivity to this drug. This drug is currently in phase II clinical trials (Bendell et al., 2012).

PIK3KCA activating mutations can coexist with PTEN deletions in subsets of patients with breast, endometrial, and colon cancers (Yuan & Cantley, 2008). Ras mutations coexist with mutant PIK3CA alleles in colorectal cancers, but are mutually exclusive in endometrial and breast cancers. In addition, PIK3CA mutations also coexist with Raf and Ras mutations in a wide variety of advanced cancers (Janku et al., 2011). The latter findings suggest that treatment regimens that coinhibit both the PI3K-mTOR and MAPK pathways might be of therapeutic benefit in such cases.

3.3.2.2. mTOR

mTOR is a molecular hub found in two functionally distinct complexes that sense and incorporate signals from multiple extracellular (e.g., growth factors and oxygen levels) and intracellular (e.g., nutrient availability and energy status) cues and regulate transcription, ribosome biogenesis, translation, cell growth, and metabolism (Petroulakis, Mamane, Le Bacquer, Shahbazian, & Sonenberg, 2006). The best characterized downstream targets of mTOR complex 1 (TORC1) are S6K and 4E-BP1. 4E-BP1 directly regulates translation initiation, while S6K-mediated negative feedback loops are important for regulation of the PI3K-mTOR pathway. For instance, inhibition of mTOR in cancer leads to hyperactivation of cross talk between the PI3K and MAPK cascades (Carracedo et al., 2008). TORC2 has been

less extensively studied but its important role in cell survival, mitosis, and metabolism is highlighted by the phosphorylation and co-activation of Akt, PKC isoforms, and SGK1 (Garcia-Martinez & Alessi, 2008; Ikenoue et al., 2008; Sarbassov et al., 2005). The multiplicity of both positive and negative feedback mechanisms that exist between TORC1, TORC2, and other signaling cascades are depicted in Fig. 1.2. These interactions should be carefully taken in account while developing therapies that target mTOR signaling.

mTOR controlled pathways are deregulated in many types of human cancer (Petroulakis et al., 2006). The prototype mTOR inhibitor, rapamycin, binds the FKBP12 protein and the resultant complex allosterically inhibits TORC1. The derivatives of rapamycin were approved for treatment of advanced renal cell carcinoma and refractory mantle cell lymphoma, and have shown activity in advanced neuroendocrine pancreatic tumors (Yao et al., 2011). Although the mTOR signaling network is often activated in cancer, rapamycin and its analogs have overall not demonstrated impressive anticancer activity. This may be explained by the incomplete mTOR inhibition by allosteric drugs (Choo et al., 2008). The mechanism(s) of resistance may be also dependent on reactivation of molecules normally suppressed by S6K, including PI3K and Akt (see Fig. 1.2). Indeed, combinations of rapamycin with drugs inhibiting either PI3K or Akt seem to increase antiproliferative activity (Benjamin et al., 2011). In addition to allosteric mTOR inhibitors, there are several highly potent ATP-competitive inhibitors of mTOR that target catalytic domains of the kinase in the context of both TORC1 and TORC2. Many of these molecules have recently entered clinical trials in patients with advanced solid tumors, including INK128, AZD8055, AZD2014, OSI027, and TORKi CC223 (Benjamin et al., 2011). A recent study showed that PIK3CA mutant breast cancer cells are sensitive to both allosteric (everolimus) and ATP-competitive (PP242) inhibitors of mTOR, whereas PTEN loss conferred resistance to both drugs (Weigelt, Warne, & Downward, 2011). Another inhibitor of mTOR, OSI-027, is currently in phase I trials after showing activity in xenograft models of different human cancers (Bhagwat et al., 2011).

3.3.2.3. Akt

Activation of Akt kinases requires membrane tethering to the PI3K-generated PIP3 docking site via PH domain and phosphorylation by PDK1. Additionally, Akt requires phosphorylation by TORC2 for full activation (Fig. 1.1) Upon activation, Akt emanates prosurvival signals by inactivating

pro-apoptotic and antiproliferative proteins such as Bad, p27, p53, GSK3, Foxo, TSC2, and others (Crowell et al., 2007). Increased activity and/or higher expression of Akt have been observed in precancerous lesions of lung, prostate, melanoma, colon, cervix, breast, and head-and-neck tissues (Crowell et al., 2007). Drugs directed against Akt isoforms are targeting either the PH domain (e.g., perifosine, PX-316, and SC66) or the kinase domain (e.g., A-443654). In addition, there are Akt1 and Akt2 isoform-specific allosteric inhibitors. One of the problems with perifosine is lack of selectivity toward Akt, since it targets other PH domain-containing proteins as well, which may account for the drug's disappointing results in clinical trials (Argiris et al., 2006; Knowling et al., 2006). The allosteric Akt inhibitor, MK-2206, shows synergism with the mutated B-Raf^{V600E} inhibitor, PLX4032 (vemurafenib), and MEK inhibitor, AZD6244, whereas perifosine surprisingly antagonizes their activity (Liu et al., 2011c).

3.3.2.4. Raf

Raf kinase is activated by Ras in response to mitogenic stimuli and activates MEK and ERK downstream, propagating the proliferative and prosurvival signals (Fig. 1.1). The first Raf isoform, C-Raf, was identified as a viral oncogene. B-Raf is the family member most easily activated by Ras, since it does not require additional phosphorylation/dephosphorylation steps as do A-Raf and C-Raf kinases. Activating mutations in B-Raf, V600E, are relatively frequent in cancers and especially in melanoma (Hong & Han, 2011). Not only does this mutation constitutively activate the MAPK cascade, but it also increases genomic instability. Catalytic activity of normal Raf is exerted by homo- or heterodimer complexes of Raf isoforms. The dimerization is stimulated by Ras and inhibited by Erk1/2 (Rajakulendran, Sahmi, Lefrancois, Sicheri, & Therrien, 2009). C-Raf mutations are less prevalent in human cancers (1%). However, amplification of C-Raf has been identified during development of androgen-independent prostate cancer and in bladder cancer (Edwards et al., 2003; Simon et al., 2001).

Targeting B-Raf^{V600E} by the mutation-specific drug, vemurafenib, has shown excellent single-agent activity in melanoma patients whose tumors harbor B-Raf mutations, with tumor shrinkage in 70–80% of patients. Another mutant B-Raf^{V600E} inhibitor, GSK 2118436, has shown similar activity in melanoma (Maurer et al., 2011). In cancer cells containing wild-type B-Raf, these drugs unexpectedly promote MAPK signaling instead of inhibiting it (Hatzivassiliou et al., 2010). The underlying mechanism was attributed to drug-induced dimerization of B-Raf and C-Raf proteins. In

such a dimer, the presence of only one active kinase is sufficient to drive downstream signaling. Hence, at suboptimal drug concentrations or in the presence of Ras mutations, the resultant signal flux is sufficient to pass the threshold of ERK1/2 reactivation (Poulikakos & Solit, 2011). Resistance observed in relapsed tumors involves switching to other MEK kinases, Ras mutations, or upregulation of RTKs driving alternative mitogenic pathways [(Poulikakos & Rosen, 2011) and Fig. 1.3].

3.3.2.5. MEK

MEK (MAPKK) is a dual (serine/threonine and tyrosine) kinase activated downstream of Raf and upon activation, phosphorylates ERK. Similar to B-Raf inhibitors, MEK inhibitors are active in treating tumors with

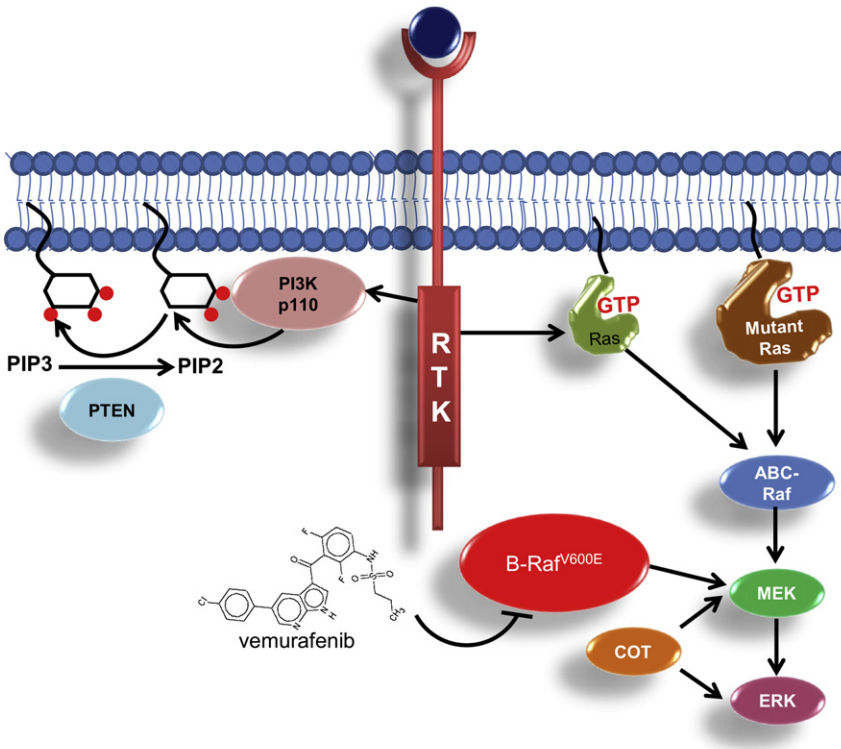

Figure 1.3 Mechanisms of resistance to the B-Raf ^{V600E} inhibitor vemurafenib in melanoma: Acquired resistance to vemurafenib and ERK reactivation can stem from potentiation of signal flux toward ERK via upregulation of C-Raf and signaling through dimerization with other Raf proteins, oncogenic Ras mutation, upregulation of RTKs (e.g., PDGFRβ and IGF-1R), and COT overexpression. For color version of this figure, the reader is referred to the online version of this book.

constitutively active ERK. The broader therapeutic index of inhibitors of mutant B-Raf^{V600E} is attributed to the drug specificity toward the mutated form of the target kinase. However, drug resistance invariably develops and one alternative approach under investigation is co-targeting mutant B-Raf^{V600E} and MEK. Culturing of B-Raf or K-Ras mutant cancer cells in the presence of the MEK inhibitor, AZD6244, results in appearance of resistant clones. Interestingly, in both cases the initially mutated gene, either B-Raf or K-Ras, was amplified. This allowed for increasing the basal level of ERK activation and as a result increased the flux through the ERK pathway sufficiently to reactivate cell proliferation. Increased concentrations of the drug were necessary to cause growth arrest. Vemurafenib treatment in B-Raf^{V600E} mutant melanoma cells has been shown to require almost complete inactivation of ERK in order to elicit growth arrest (Poulikakos & Solit, 2011). Another mode of acquired resistance to MEK inhibitors in K-Ras mutant cells is appearance of activating mutations in PI3K. Additionally, resistant cells may also activate upstream RTKs, such as IGF-1R and PDGFRβ. The latter event not only increases the signal flux through ERK, but also activates MAPK-independent pathways to drive proliferation and survival. Several lines of evidence suggest that the necessity to diminish the signal flux to the possible minimum may require co-targeting Raf and MEK simultaneously, and that such an approach may prove beneficial and nonredundant.

Due to the multiplicity of escape mechanisms in cancer cells, the clinical efficacy of monotherapies based on single agents and targeting individual members of signaling cascades is limited. Combination therapy strategies are discussed in the following.

4. TARGETING OF PARALLEL SIGNALING PATHWAYS

Parallel pathway targeting refers to simultaneous inhibition of more than one signaling cascade responsible for the malignant properties of the tumor. This approach is believed to be beneficial since it may block escape routes and reduce the chances of activating adaptive resistance mechanisms. For instance, many cancers are known to activate RTK networks, and in these cases, inhibition of a dominant RTK will inevitably be compensated by secondary RTKs (Xu & Huang, 2010). Thus, single molecules inhibiting multiple RTKs (or multikinase inhibitors) are showing promising activity in preclinical studies. For example, foretinib, an inhibitor of Axl, c-Met, and

VEGFR, is active in lapatinib (dual EGFR/ErbB2 inhibitor)-refractory breast cancer cells (Liu, Shi, et al., 2011a). A recent report identified simultaneous c-Met and EGFR hyperactivation in malignant mesothelioma and combinations of RTK and mTOR inhibitors were more active in suppressing proliferation of cancer cells than either drug alone (Brevet et al., 2011). Since many RTKs utilize similar signaling networks to drive proliferation and survival, co-targeting RTK-induced downstream signals might enable us to overcome resistance. For example, combining inhibitors of PI3K-mTOR and Ras-MAPK cascades has validated the advantage of combinational therapy; however, this was achieved at the expense of greater toxicity (Shimizu et al., 2012). The first multitarget drug that inhibits both B-Raf and C-Raf along with several tyrosine kinases (e.g., PDGFR, VEGFR, c-Kit), sorafenib, was inactive in melanoma, although it had some activity in renal cell carcinoma and hepatoceulluar carcinoma (Eisen et al., 2006; Escudier et al., 2007; Kane et al., 2009). In addition, this drug shows promising activity in thyroid cancer (Duntas & Bernardini, 2010). Sorafenib is associated with a number of toxicities which are likely due to its multitarget nature (Lamarca et al., 2012).

B-Raf^{V600E} mutant melanoma cells can develop resistance to B-Raf inhibitors as a result of a B-Raf to C-Raf switch. A recent study in melanoma cells showed that resistance mediated by the Raf kinase switch can be overcome by co-targeting MEK and IGF-1R/PI3K (Villanueva et al., 2010). N-Ras–dependent melanomas have been shown to be sensitive to combined targeting of both B-Raf and C-Raf, or B-Raf and PIK3CA simultaneously (Jaiswal et al., 2009). There is an ongoing clinical trial assessing the co-treatment with the MEK inhibitor (AZD6244) and the Akt inhibitor (MK2206) in B-Raf^{V600E} mutant melanomas that failed to respond to selective Raf inhibitors (Nikolaou, Stratigos, Flaherty, & Tsao, 2012). A recent genetic analysis of tumors of 504 patients with various cancers suggested that PIK3CA mutations frequently coexist with RAS and B-Raf mutations (Janku et al., 2011). This notion further substantiates the idea of signaling pathway co-targeting in anticancer therapy. Co-expression of mutated PIK3CA, PTEN, and Ras was also analyzed in a recent paper by Yuan and Cantley (Yuan & Cantley, 2008). By assessing the independent and concomitant abundance of oncogenes in breast, endometrium, and colon cancers, Yuan et al. concluded that while most of the combinations occur coincidently, in some types of cancer the PI3K and Ras-MAPK pathways do not seem to cooperate in tumor formation. However, this does not rule out the possibility of secondary pathway activation as a result of blockade of the primary oncogenic driver pathway.

Another example of an inhibitor that targets several parallel pathways is dasatinib. Dasatinib is over 300-fold more potent than imatinib in inhibiting Bcr-Abl and inhibits most Bcr-Abl secondary or escape mutants. Dasatinib demonstrates more inhibition and less specificity toward Bcr-Abl (Rosenzweig, 2011). Again, off-target interactions of dasatinib (Src, c-Kit, EphrinR, etc.) are most likely responsible for clinical toxicities (McCormack & Keam, 2012). Sunitinib is a multikinase inhibitor targeting RTKs such as PDGFR, VEGFR2, c-Kit, and others. It is approved for renal cell carcinoma and imanitib-resistant GIST. As in the case with other multikinase inhibitors, sunitinib induces variety of toxicities (Gupta & Maitland, 2011; McLellan & Kerr, 2011; Richards et al., 2011; Zhu et al., 2009). Therefore, despite the advantage of multitarget blockade which can potentially overcome acquired resistance, it can be associated with greater toxicities.

5. VERTICAL PATHWAY TARGETING

5.1. Vertical Co-targeting of MAPK Pathway Members

In some cases of acquired drug resistance, tumor cells are selected for amplification of the target itself, mutations in the drug-binding site, and/or overexpression/hyperactivation of upstream or downstream effectors. In such cases targeting several members of the same signaling pathway may prove beneficial since it secures a stronger blockade of the pathway. Another advantage of a vertical pathway targeting approach is the potentially lower toxicity. There are a few paradigms that exemplify this approach. The mutant B-Raf inhibitors vemurafenib and GSK 2118436 show impressive activity in B-Raf^{V600E} mutant melanomas as single agents. Unfortunately, responses are short lived and tumors invariably develop resistance. In many cases, targeted therapies directed against kinases result in appearance of secondary mutations in the "gatekeeper" residues found in the vicinity of the ATP-binding sites of the kinase (Fedorenko, Paraiso, & Smalley, 2011). This escape mechanism prevents drug binding and is seen, for example, in EGFR (T790M) and in Bcr-Abl (T315I). While such mutations for oncogenic B-Raf have been shown *in vitro* (T529 to M, I, or N, with T529N being most resistant), these mutations have not been identified in either melanoma cell lines or tumors with acquired resistance (Whittaker et al., 2010). Instead, B-Raf^{V600E}–dependent melanomas tend to develop resistance through increasing robustness of upstream or downstream signaling. Sequencing of melanomas from 14 patients who

initially responded to vemurafenib but then became resistant has shown that B-Raf^{V600E} was unchanged and maintained drug sensitivity in an *in vitro* kinase assay (Nazarian et al., 2010). However, acquired resistance was attributed to PDGFRβ upregulation or N-Ras oncogenic mutations. Tumor regression with vemurafanib treatment requires near complete inhibition of ERK activity (Bollag et al., 2010). One of the vemurafenibevasion mechanisms employed by melanomas is overexpression of C-Raf and signaling through B-Raf/C-Raf heterodimers (Hatzivassiliou et al., 2010). As demonstrated in a recent study, oncogenic Ras mutations may cooperate with inactivated B-Raf and account for another pathway of MAPK reactivation via C-Raf (Heidorn et al., 2010). Another study identified increased expression of C-Raf and COT kinases as determinants of resistance to B-Raf inhibition in some cases of acquired vemurafenib resistance (Johannessen et al., 2010). Interestingly, MEK inhibition, although reduced, did not abolish ERK phosphorylation in COT overexpressing cells. COT kinase was able to increase ERK phosphorylation not only through MEK activation but also through direct phosphorylation of ERK1. Collectively, these data suggest that although simultaneous co-targeting of oncogenic B-Raf and MEK may prove to be beneficial in B-Raf^{V600E}-addicted melanomas, ERK signaling activation may evolve by additional escape mechanisms (such as COT overexpression) (Poulikakos & Solit, 2011). Raf inhibition in normal cells paradoxically leads to ERK hyperactivation and results in squamous cell carcinomas in some patients, whereas MEK inhibition has the opposite effect on ERK activation in the skin, which is evident by the development of acneiform rashes. These counteracting effects may neutralize treatment-associated toxicities observed with either inhibitor alone (Poulikakos & Solit, 2011). Several clinical phase I/II trials are under way to assess the therapeutical benefits of MEK inhibitor (GSK1120212 or GDC-0973) and B-Raf^{V600E} inhibitor (GSK2118436 or vemurafenib) combinations in patients with melanomas harboring B-Raf^{V600E} mutations (Nikolaou et al., 2012).

Another mechanism of B-Raf^{V600E} inhibitor resistance in melanoma patients is mediated through upregulation of RTKs (such as PDGFRβ and IGF-1R) expression and induction of alternative pathways (Nazarian et al., 2010; Villanueva et al., 2010). In these cases, RTK-specific inhibitors in combination with Raf and MEK inhibitors might resensitize cancer cells to the therapy. Several *in vitro* studies identified Akt, GSK3, and loss of PTEN as factors contributing to sorafenib resistance in B-Raf^{V600E} mutant cancer cells (Chen et al., 2011; Panka et al., 2008; Paraiso et al., 2011).

Consequently, PI3K/Akt/mTOR pathway co-targeting is predicted to sensitize these tumors to B-Raf inhibitors. Nevertheless, the option of co-targeting Raf and MEK in melanoma looks promising. The acquired resistance to vemurafenib is mediated by ERK reactivation through overexpression of wild-type C-Raf, oncogenic Ras mutation, overexpression of RTKs (PDGFRβ and IGF-1R), and COT kinase (Fig. 1.3). By inhibiting mutant B-Raf and MEK or an RTK, B-Raf, and MEK, it might be possible to better inhibit ERK activation.

5.2. Vertical Co-targeting of PI3K-mTOR Pathway Members

Dual PI3K/mTOR inhibitors such as BEZ235 and SAR245409 are currently in phase I/II clinical trials (Holmes, 2011). PI3K hyperactivation was identified as a mechanism responsible for development of resistance to the ErbB2 inhibitor lapatinib in breast cancer cells. Specifically, PTEN loss and PIK3CA mutations were found responsible for the drug resistance. ErbB2 sensitivity could be reestablished by treatment of refractory cells with BEZ235 (Eichhorn et al., 2008). Subeffective low doses of BEZ235 and RAD001 (a rapamycin analog) have been shown to synergistically affect the proliferation and survival of non–small cell lung cancer cells (Xu et al., 2011). Interestingly, at concentrations used, the combination of BEZ235 and RAD001 induced Akt phosphorylation and still killed NSCLC cells *in vitro*. This drug combination also potently attenuated tumor growth in a xenograft mouse model.

Preclinical data suggest that cancer cells employ several mTOR-dependent escape mechanisms to circumvent PI3K-selective inhibition without reactivating PI3K itself. For instance, the PI3K downstream effector, Akt, is co-activated by TORC2 in rapamycin treated cells (Serra et al., 2008). Extensive cross talk exists between PI3K and Ras-MAPK signaling cascades (Fig. 1.2), and ERK/MAPK-mediated mechanisms of TORC1 activation have been described (Mendoza, Er, & Blenis, 2011). For example, ERK- and RSK-mediated phosphorylation events inactivate TSC2, a negative regulator of TORC1. On the other hand, TORC1-specific inhibitors (e.g., rapamycin and its analogs) relieve S6K-dependent negative feedback at the level of IRS1 and rictor phosphorylation and result in more robust PI3K activation. In all of these cases, concomitant inhibition of PI3K and mTOR blocks these mechanisms of resistance. Vertical pathway targeting within the PI3K-mTOR signaling module has been shown to have anticancer activity in multiple studies (Aziz et al., 2010; Cao et al., 2009; Chiarini et al., 2009; Liu et al., 2009; Takeuchi et al., 2005).

5.3. Vertical Co-targeting of RTKs and the PI3K or MAPK Pathway

BKM120, a selective PI3K inhibitor, has shown promising activity in ErbB2 overexpressing breast and gastric cancer cells when combined with trastuzumab in an orthotopic mouse model (Maira et al., 2011). Co-targeting ErbB2 with PI3K/Akt inhibitors may be of tremendous importance, since recent studies have shown that Akt inactivation leads to increased expression and phosphorylation of ErbB3, IGF-1R, and IR in cancer cells (Chandarlapaty et al., 2011). Interestingly, phosphorylation, but not overexpression, of all three receptors was dependent on EGFR/ErbB2 activity.

Inhibition of mTOR activity by rapamycin and its analogs has been associated with disappearance of negative feedback exerted by S6K at the level of IRS1 and rictor phosphorylation (Treins et al., 2010; Um et al., 2004). As a result, PI3K/Akt signaling is activated. Single agent mTOR inhibition has little antitumor effect in most cancers, hence one of the strategies to overcome resistance to mTOR inhibitors is co-targeting upstream RTKs that mediate PI3K activation. IGF-1R employs IRS1 to activate the PI3K/Akt pathway. Moreover, embryonic fibroblasts lacking IGF-1R fail to transform (Sell et al., 1993). Preclinical data suggest that co-targeting IGF-1R and mTOR (using ganitumab and rapamycin) is effective in Ewing's and osteogenic sarcomas (Beltran et al., 2011). The results of a phase I trial combining mTOR and IGF-1R inhibitors (everolimus and figitumumab) in advanced sarcomas and other solid tumors were promising (Quek et al., 2011). Similar to rapamycin, IGF-1R inhibitors were not effective in the clinic as single agents.

A recent study of a murine breast cancer model with mutant PIK3CA showed that one of the genomic alterations responsible for the malignant phenotype was c-Met amplification. Its oncogenic prosurvival action was mediated by endogenous PI3K signaling (Liu et al., 2011b). Resistance of ErbB2-dependent breast cancers treated with trastuzumab is often mediated by aberrant c-Met upregulation and subsequent activation of Akt survival signaling (Fiszman & Jasnis, 2011). Similarly, c-Met amplification rescues lung cancers treated with an EGFR inhibitor (gefitinib) by activating ErbB3, a potent activator of PI3K/Akt pathway (Engelman et al., 2007). Hence, therapies co-targeting c-Met and PI3K might be effective.

Co-targeting MEK and VEGFR2 (with selumetinib and cediranib, respectively) inhibits tumor growth and nearly abolished metastasis in a lung cancer xenograft model. While proliferative effects were efficiently blocked by MEK inhibition alone, antiangiogenic and apoptotic effects were

markedly enhanced when the agents were combined (Takahashi et al., 2012). In addition, selumetinib decreased VEGF production by tumor cells. The VEGF-specific monoclonal antibody bevacizumab is active in several types of cancer; however, its effects are short lived (Waldner & Neurath, 2012). A phase I/II trial combining bevacizumab with the PI3K inhibitor BKM-120 is currently accruing.

6. CONCLUSION

In recent years pharmaceutical companies have developed multiple anticancer agents targeting several important fragile nodes in cancer cells. It is now increasingly apparent that due to escape mechanisms, the use of highly specific tyrosine kinase inhibitors as monotherapies has limited clinical benefit. On the other hand, drug combinations targeting multiple oncogenes or an oncogene and its downstream effector/s, although more effective, can be associated with increased toxicities. Although acquired drug resistance is a perpetual concern while employing any anticancer strategy, finding the right combination of drugs for individual patients might result in maximizing anticancer activity while minimizing toxicity. In this respect targeting tumors with combinations of drugs which inhibit members of the same pathway may be less toxic, while preserving anti-tumor activity. Careful consideration of novel dosing schedules, such as use of intermittent target inhibition strategies, might be beneficial for minimizing toxicities.

Conflict of Interest Statement: HK has served as a consultant to Genentech, Inc., the maker of vemurafenib.

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CHAPTER TWO

Histone Deacetylases (HDACs) as Mediators of Resistance to Apoptosis in Melanoma and as Targets for Combination Therapy with Selective BRAF Inhibitors

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Abstract

HDACs are viewed as enzymes used by cancer cells to inhibit tumor suppressor mechanisms. In particular, we discuss their role as suppressors of apoptosis in melanoma cells and as mediators of resistance to selective BRAF inhibitors. Synergistic increases in apoptosis are seen when pan-HDAC inhibitors are combined with selective BRAF inhibitors. Moreover, cell lines from patients with acquired resistance to Vemurafenib undergo PLX4720 induced apoptosis when combined with pan-HDAC inhibitors. The mechanisms of upregulation of HDACs and the mechanisms involved in HDACi reversal of resistance to apoptosis are as yet poorly understood.

1. INTRODUCTION

1.1. Classes of HDACs

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from lysine residues in the NH2 terminal tails of core histones, resulting in a more closed chromatin structure and repression of gene expression. A number of nonhistone proteins are also targets for HDACs, such as alpha tubulin, heat shock protein (hsp) 90, and hypoxia inducible factors HIF-1α. HDACs 1, 2, 3, and 8 are class I HDACs and are located within the nucleus. In contrast, the class II HDACs 4, 5, 7, and 9 can shuttle between the nucleus and the cytoplasm and may have tissue specific roles (Dokmanovic et al., 2007); for example, HDAC 5 knockdowns have defects in cardiac function. Class IIa HDACs include HDACs 6 and 10, and HDAC 11 is referred to as a class IV

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HDAC. Hypoacetylation has been described as a common property of many cancers (Fraga et al., 2005). Class III HDACs include the Sirtuin (SIRT) family of seven proteins with most interest focused on SIRT1 and 2 (Dokmanovic et al., 2007; Stunkel & Campbell, 2011). These enzymes are dependent on NAD for their activity and do not contain zinc as do the other HDACs. Their dependence on NAD has linked this group of deacetylases to the metabolic activity of cells (Rajendran et al., 2011). Reviews of class I, II, and IV HDACs and of the class III HDACs (Sirtuins) are given in Dokmanovic (Dokmanovic et al., 2007) and Rajendran et al. (Rajendran et al., 2011).

1.2. HDACs in Cancer

Structural mutations in HDACs in cancers are uncommon but upregulation of certain HDACs has been reported in different cancers; for example HDACs 2 and 3 are increased in colon cancer and HDAC 1 in gastric cancer (Bolden et al., 2006; Marks et al., 2001; Stunkel & Campbell, 2011). In neuroblastoma, HDAC 2 was reported to be upregulated by N-Myc and to be targeted to the promoter region of CCNG2 (Cyclin G2) by N-Myc (Kurland & Tansey, 2008; Marshall et al., 2010), thus removing the inhibitory effects of Cyclin G2 on cell division. HDAC 2 was also implicated in downregulation of p21 (Huang et al., 2005). MAGE A2 reduced p53-dependent apoptosis by an HDAC-dependent mechanism in promyelocytic leukemia (Peche et al., 2011). SIRT1 may inhibit p53 activity and is believed to be regulated by both E2F and p53 (Rajendran et al., 2011). HDAC 1, 2, and 3 were associated with high levels of activated NF-kB and a poor prognosis in patients with pancreatic carcinoma (Lehmann et al., 2009). Overexpression of HDACs has long been regarded as instrumental in development of cancer (Marks et al., 2001). Although HDAC expression is commonly upregulated in cancer, suppression of some HDACs has been reported and reduced expression of HDACs 5 and 10 are associated with poor prognosis in lung cancer (Osada et al., 2004).

These studies support the view that HDACs are enzymes used by oncogenes and other proteins to suppress key tumor suppressor mechanisms (Won et al., 2002).

2. HDACS IN MELANOMA

The levels of HDAC expression in melanoma have not been well documented but it was reported that certain proteins can target HDACs to

downregulate gene function. This includes targeting of HDAC 1 by T-box 2 (Tbx2) to the promoter of CDKN1 (p21) (Vance et al., 2005), thereby inhibiting senescence. MAGE-A proteins were shown to target HDAC 3 to p53 and inhibit its transactivating function (Monte et al., 2006). HDAC 4 was reported to downregulate MDA-7/IL-24 production from melanoma cells and thereby removes its inhibitory effect on melanoma cell division (Pan et al., 2010). Recurrent homozygous deletions in HDAC 4 and nonsense mutations in HDAC 3 were also reported by Stark and Hayward (2007).

We examined HDAC expression relative to housekeeping genes in a panel of melanoma lines by real time PCR assays. The cell lines were well characterized for mutations by sequenom assays, Oncocarta version 2 (Lai et al., 2012). As shown in Fig. 2.1, HDAC 1, 2, and 3 were at relatively high levels in all the lines whereas expression of HDAC 4, 8, and 9 were cell-line dependent. Western blot studies with a more restricted panel of antibodies against the HDACs 1, 2, 3, and 8 gave a similar distribution except that HDAC 8 appeared at higher levels perhaps due to differences in protein processing of this particular HDAC (Fig. 2.2A).

The expression of the HDACs did not appear related to the $BRAF^{V600E}$ mutation status but as shown in Fig. 2.2A, levels of HDAC 1, 2, 3, and 8 were higher in NRAS Q61 mutated lines. This is of some interest given that acquisition of mutations in NRAS was a relatively common cause of resistance to selective BRAF inhibitors (Nazarian et al., 2010). The signal

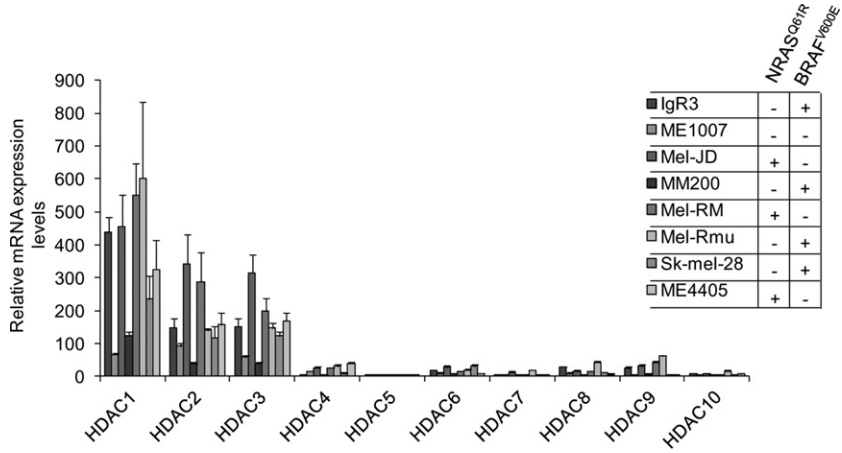

Figure 2.1 ***HDAC mRNA expression levels in human melanoma.*** A panel of eight different human melanoma cell lines were harvested and qRT-PCR was performed and normalized with beta-actin to identify HDAC mRNA levels using Taqman probes.

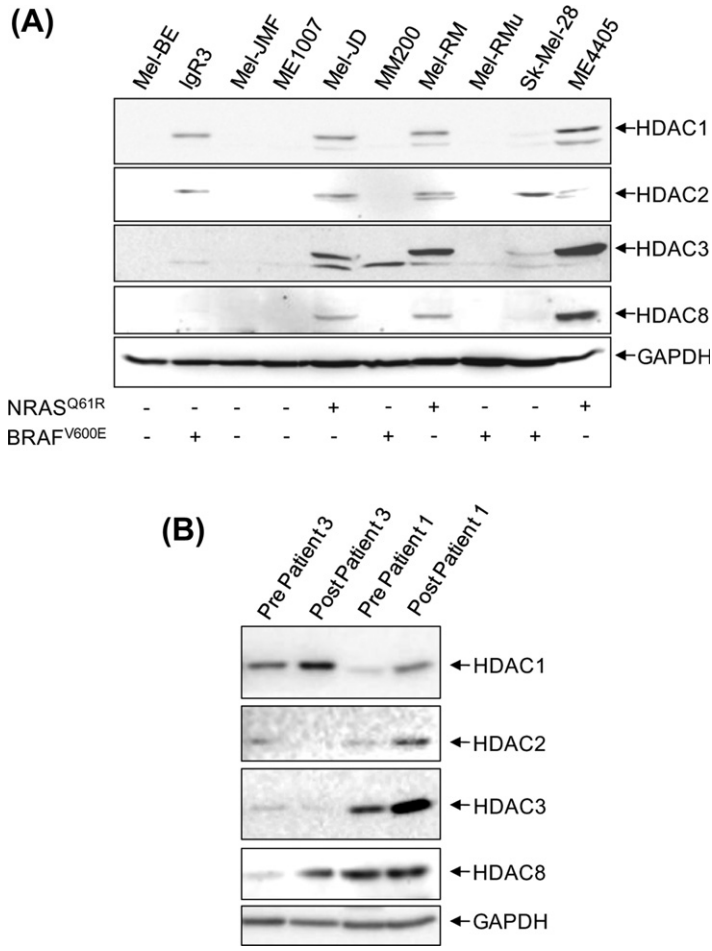

Figure 2.2 ***Class I HDAC protein expression in melanoma and patient cell lines.*** (A) Ten different melanoma cell lines were harvested and whole cell lysates were immunoblotted with indicated antibodies. GAPDH was used to demonstrate equal loading. (B) Melanoma cultures established from patient biopsies (Patient 1 and 3) prior to (pre) and during treatment (post) with PLX4032/Vemurafenib. Whole cell lysates were immunoblotted with indicated antibodies. GAPDH was used to demonstrate equal loading.

pathways involved remain under study but activation of the NF-kB transcription factor appears to be associated with upregulation of HDAC 1, 2, and 3 as also reported in pancreatic cancer (Lehmann et al., 2009). HDACs may inhibit certain NF-kB target genes so that complex feedback loops may be involved (Ashburner et al., 2001; Elsharkawy et al., 2010). In neuroblastoma, N-Myc was linked to overexpression of HDAC 2

(Marshall et al., 2010) and it is possible certain HDACs in melanoma may also be targets of oncogenes. The nature of these is unknown at this time.

3. HDACS AS SUPPRESSORS OF APOPTOSIS

It is well recognized that the ability of cancer cells to suppress apoptosis is a hallmark of malignancy (Bolden et al., 2006; Green & Evan, 2002; Hersey, 2006; Llambi & Green, 2011). HDACs may play a key role in suppressing apoptosis, as shown by previous studies with a pan-HDAC inhibitor, SBHA. As a single agent, SBHA could induce mitochondrial-dependent apoptosis in a small proportion of melanoma lines (Zhang et al., 2004), implicating the HDACs as suppressors of apoptosis. Importantly, SBHA was also shown to induce synergistic increases in TRAIL-induced apoptosis of the melanoma lines that were associated with downregulation of Bcl-XL, Mcl-1, and XIAP and upregulation of BAX, Bak, and Bim (Gillespie et al., 2006). The latter study was the first to suggest that HDAC inhibitors may be best viewed as agents that could facilitate induction of apoptosis by other agents and led to an examination of their effects when combined with selective BRAF inhibitors.

An additional action of HDAC inhibitors related to apoptosis is their ability to inhibit proteasome activity which can trigger apoptosis by effects on a number of proteins such as those in the NF-kB and Akt pathways. It was reported that the protein HR23B was a predictor of sensitivity to this aspect of HDAC inhibitor–induced apoptosis (Fotheringham et al., 2009; Khan et al., 2010). Detailed reviews of HDAC inhibitors, including the Sirtuins, are given in Rodriguez-Paredes and Esteller (Rodriguez-Paredes & Esteller, 2011) and Pan et al. (Pan et al., 2012).

4. HDAC INHIBITORS REVERSE RESISTANCE OF MELANOMA CELLS TO INDUCTION OF APOPTOSIS BY SELECTIVE BRAF INHIBITORS

We have reported elsewhere that selective BRAF inhibitors can induce apoptosis of melanoma albeit at higher concentrations than needed to inhibit cell division. Apoptosis appeared to be mediated by upregulation of the BH3-only protein Bim and production of Bim isoforms, in particular the short form of Bim (Bim_S) that is associated with apoptosis (Jiang et al., 2010a, b). Unfortunately it is now well established that

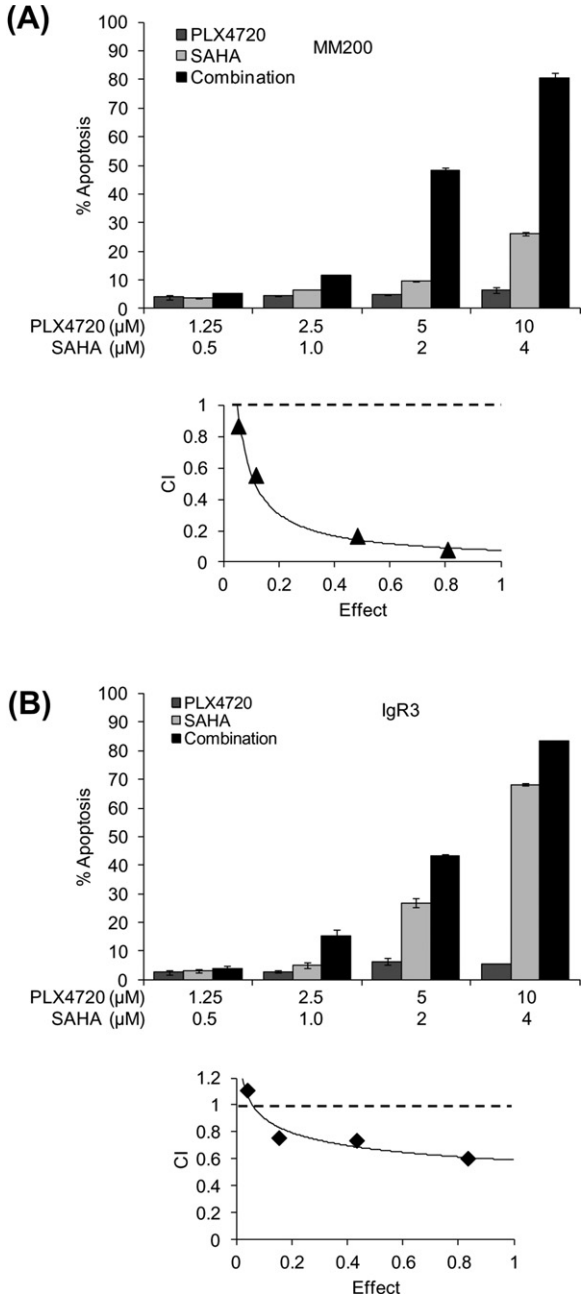

Figure 2.3 ***SAHA sensitizes BRAF^{V600E} melanoma cells to synergistic induction of apoptosis by the selective BRAF inhibitor PLX4720.*** (A, B) Two BRAF^{V600E} melanoma cell lines (MM200 & IgR3) were exposed to single agents of PLX4720 and SAHA and combination at indicated doses. Induction of cell death was measured in cells from

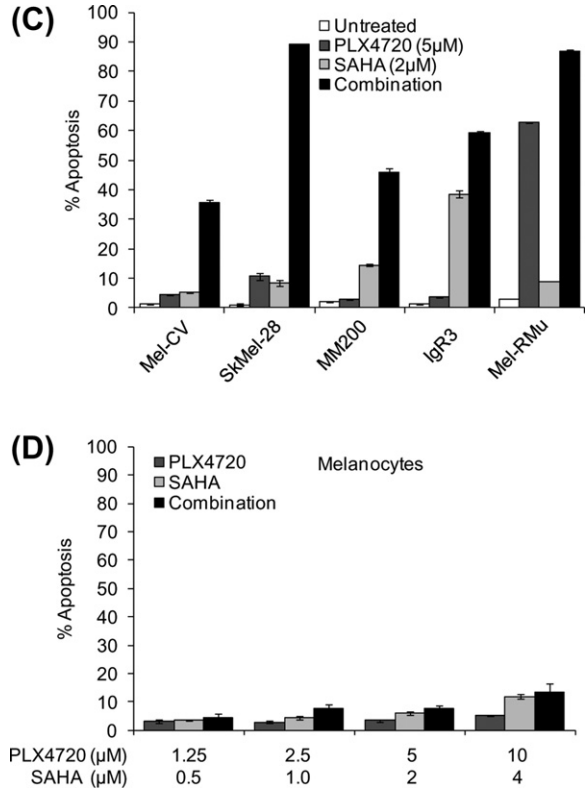

Figure 2.3 (*cont'd*).

prolonged exposure to the selective BRAF inhibitors *in vitro* or in patients can induce resistance to the inhibitors by multiple mechanisms as reviewed by Lai et al. (2012). A possible role for HDACs in such resistance came from studies on the cell lines established from patients undergoing treatment with Vemurafenib (Fig. 2.2B). These showed that HDAC 1, 2, 3,

representative wells after 48 h by the propidium iodide (PI) method. Data shown are the mean ± SE of results from two individual culture wells (top panel). Fa–CI plot generated by the Chou and Talalay method via Calcusyn software is a measure of the synergistic effect between two given drugs at a constant dose ratio with combination index (CI) < 1 indicating strong synergism in respective cell lines (lower panel). (C) Synergistic induction of apoptosis in a panel of $BRAF^{V600E}$ melanoma cell lines in the presence of both BRAF and HDAC inhibitors regardless of sensitivity to individual treatments. (D) Low levels of apoptotic activity induced by PLX4720 and SAHA in melanocytes indicates that drug doses used *in vitro* were not toxic to normal cells.

and 8 were increased in the resistant cell lines compared to that in the pretreatment cell lines. This increase appeared related to activation of NF-kB. Although the resistance was reversed by the pan-HDAC inhibitors it was not reversed by the selective class II HDAC inhibitor MC1568 (Duong et al., 2008) indicating that cytoplasmic events were not key to the reversal of resistance and that intranuclear events were involved (Scognamiglio et al., 2008).

In view of previous studies showing potentiation of TRAIL-induced apoptosis by a pan-HDACi (Gillespie et al., 2006), we tested whether pan-HDACi may have similar effects on apoptosis induced by the selective BRAF inhibitor PLX4720. A panel of $BRAF^{V600E}$ melanoma cells were exposed to PLX4720 alone, SAHA (Vorinostat) alone or both together. As shown in Fig. 2.3A and B, increased drug concentrations at a fixed ratio resulted in marked increases in cell death (top panel) that was synergistic (lower panel), as shown by the Chou and Talalay analysis (Chou & Talalay, 1984). Studies on further cell lines are shown in Fig. 2.3C whereby SAHA alone and PLX4720 alone induced significant cell death in IgR3 and Mel-RMu lines respectively but not in the other $BRAF^{V600E}$ melanomas. However, there was a striking synergistic induction of apoptosis in all lines when both drugs were combined (Fig. 2.3C). Furthermore, there were very low toxicity levels when treating melanocytes at similar drug doses (Fig. 2.3D).

5. REVERSAL OF RESISTANCE TO PLX4720 IS ASSOCIATED WITH CHANGES IN THE BCL-2 FAMILY PROTEINS

The synergistic induction of apoptosis mediated by PLX4720 and SAHA in the Mel-RMu line appeared to be due to upregulation of the BH3-only protein Bim, and downregulation of the antiapoptotic protein Mcl-1. Previous observations indicated that Bim plays a major role in PLX-induced apoptosis in a mitochondrial-dependent manner (Jiang et al., 2010a,b). Knockdown of Bim by siRNA techniques in the Mel-RMu line inhibited cell death induced by PLX4720 as well as the cell death induced by the combination (by >55%), indicating that the synergistic induction of apoptosis may be predominantly regulated via this BH3-only protein (Fig. 2.4A& B). Other factors may be involved such as reduction in Mcl-1 as knockdown of Bim did not significantly reduce apoptosis in the MM200 line (Fig. 2.4C). Studies on the additional anti- and pro-apoptotic proteins involved are continuing.

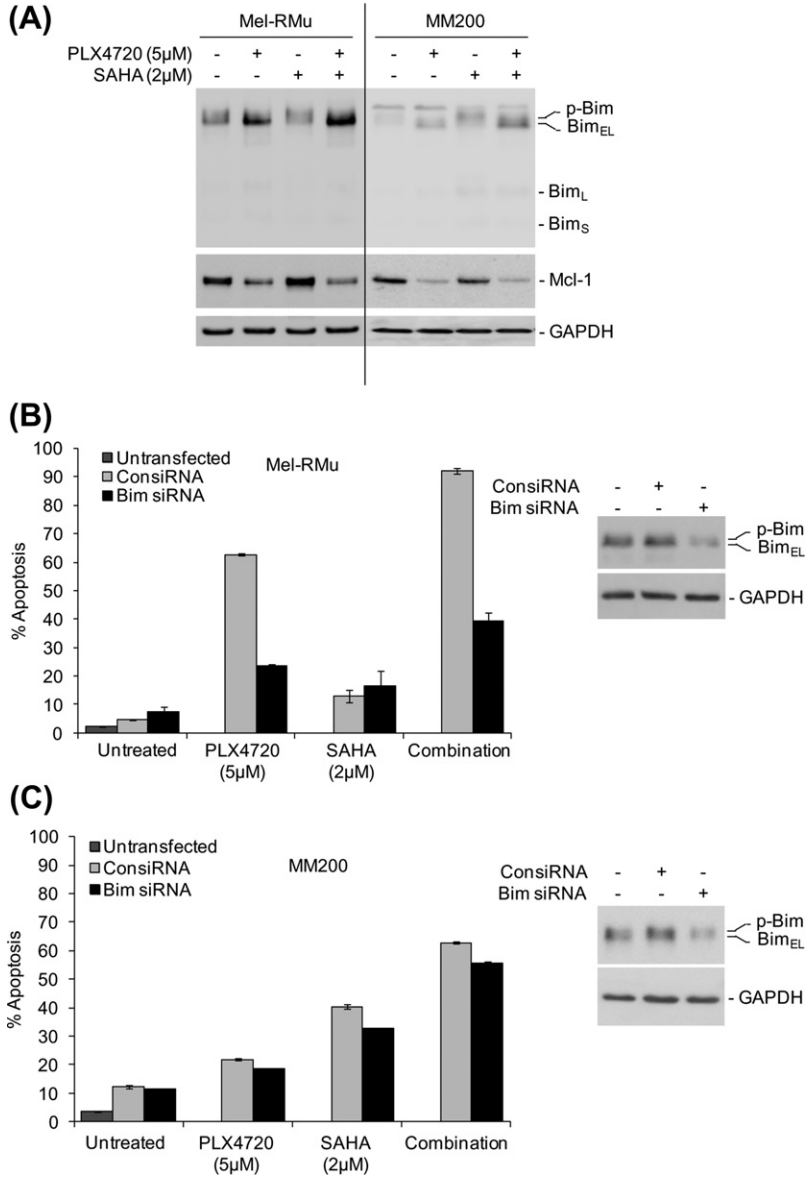

Figure 2.4 ***Influence of Bim in the synergistic induction of apoptosis mediated by PLX4720 and SAHA.*** (A) Changes in Bcl-2 family proteins in the presence of the two compounds were assessed by western blots as described before (Lai et al., 2012). Whole cell lysates from two BRAF^{V600E} melanoma cell lines (Mel-RMu & MM200) were subjected to western blot analysis of Bim, Mcl-1, and GAPDH (loading control). (B, C) Mel-RMu and MM200 cells were transfected with control and Bim siRNA. Twenty-four hours later, these cells were treated with PLX4720, SAHA and the combination, for 48 h. Induction of cell death was measured in cells by the PI method. Whole cell lysates from Mel-RMu and MM200 cells were subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of 2 individual experiments.

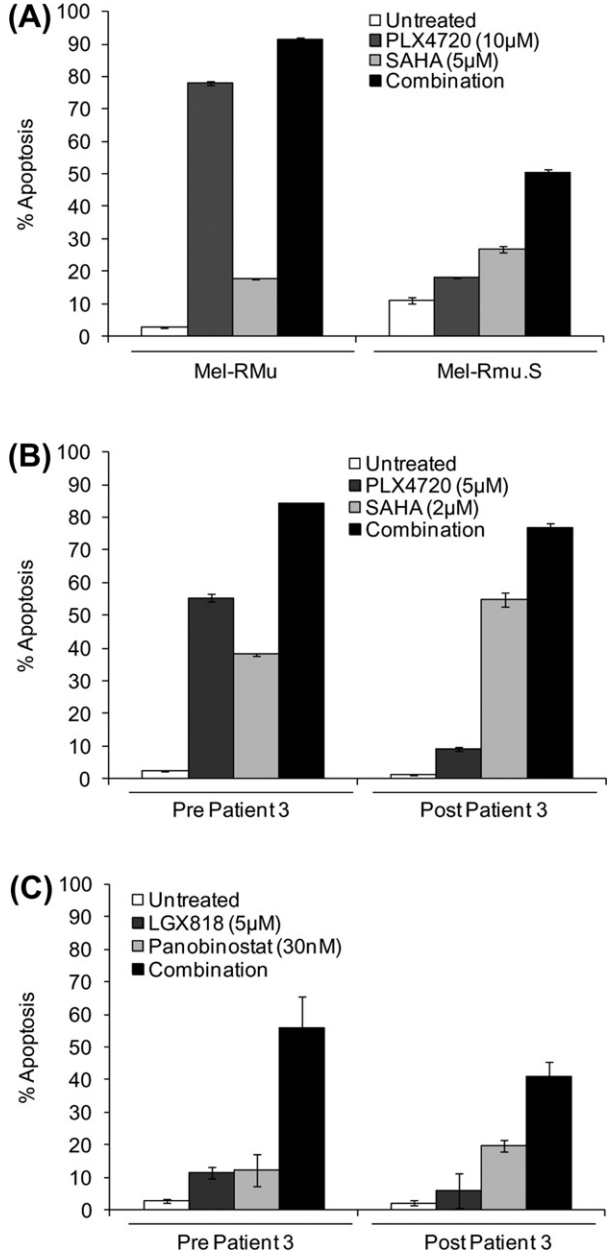

Figure 2.5 ***HDAC inhibitors sensitize BRAF inhibitor-resistant cell lines and fresh melanoma isolates to induction of apoptosis.*** (A) The PLX-sensitive $BRAF^{V600E}$ cell line Mel-RMu was exposed to PLX4720 (10 μM) until a stable resistant population developed over a period of 20 weeks. Culture medium containing PLX4720 was changed every 3 days. These resistant clones depicted as Mel-RMu.S proliferate steadily in

6. PAN-HDAC INHIBITORS REVERSE THE RESISTANCE OF MELANOMA CELL LINES TO SELECTIVE BRAF INHIBITORS: MULTISPECIFIC VERSUS SPECIFIC AGENTS IN REVERSAL OF RESISTANCE TO SELECTIVE BRAF INHIBITORS

We reported elsewhere that melanoma cells grown in the selective BRAF inhibitor PLX4720 became resistant to the inhibitor (Jiang et al., 2010a,b). Figure 2.5A shows that the resistance of a cell line generated in this way was reversed when PLX4720 was combined with the HDAC inhibitor SAHA. To increase the *in vivo* relevance of these findings, melanoma cell lines were established from patients before and after treatment with Vemurafenib at the time of relapse on this treatment as described in Lai et al. (2012). As shown in Fig. 2.5B, treatment with SAHA was able to overcome resistance to PLX4720 and induce a synergistic increase in apoptosis. The synergistic increase was not restricted to the combination of SAHA and PLX4720 as similar treatment with the selective BRAF inhibitor LGX 818 (Novartis) and the pan-HDAC inhibitor panobinostat (LBH589) was able to reverse resistance to apoptosis induced by LGX 818 (Fig. 2.5C).

These studies further suggest that agents targeting multiple pathways may be more effective in overcoming resistance to selective BRAF inhibitors than use of agents that are highly selective for a particular pathway. Other agents in this category include heat shock proteins 90 inhibitors as reported elsewhere (Paraiso et al., 2012) and use of agents which inhibit antiapoptotic proteins such as the BH3 mimetics ABT-737 (Wroblewski et al., submitted).

7. CONCLUSION

The studies described above support the notion that the class 1 HDACs are used by oncogenes and oncogenic pathways to inhibit tumor

PLX4720-containing media, albeit with slow growth rate. Mel-RMu (parent) and Mel-RMu.S (PLX-resistant) were co-treated with PLX4720 and SAHA for 48 h before being subjected to PI analysis. (B) Cell lines were established from patient 3 (described in Lai et al., 2012) before and during treatment with Vemurafenib. The lines were grown in flasks containing fresh DMEM until 70–80% confluent. The cells were exposed to both PLX4720 and SAHA for 48 h before being subjected to PI analysis. (C) Cell lines established from patient 3 pretreatment and during treatment with Vemurafenib (as above) were exposed to combined treatment with the BRAF inhibitor LGX 818 and HDAC inhibitor panobinostat (LBH589). Apoptosis was measured in cells from representative wells after 48 h by the PI method.

suppressor mechanisms and thereby promote survival and progression of melanoma as illustrated in Fig. 2.6. The studies referred to in this chapter have focused particularly on HDAC suppression of apoptosis, which we have previously proposed as a determinant of effective and durable response to selective BRAF inhibitors (Hersey, 2011). In long term established $BRAF^{V600E}$ melanoma lines, marked synergy was evident between the selective BRAF inhibitor PLX4720 and the pan-HDAC inhibitor SAHA (Vorinostat). This was seen at concentrations below the effective clinical doses and may point to acceptable toxicity profiles in patients. Importantly there was no evidence of apoptosis in normal cultured melanocytes.

To further investigate the clinical relevance of the results, the studies were repeated in pairs of freshly isolated cell lines established from patients treated with Vemurafenib before and after development of resistance to the drug. Once again, impressive synergy in induction of apoptosis was seen. This was evident not only with the combination of PLX4720 and SAHA but also with the combination of a relatively new selective BRAF inhibitor LGX 818 and another pan-HDAC inhibitor panobinostat (LBH589). In the latter studies, LGX 818 appeared to induce less apoptosis but nevertheless the combination was still able to induce significant levels of apoptosis at dose levels of both drugs well below clinically toxic levels.

The mechanisms underlying synergism with HDAC inhibitors are likely to be complex and different for different melanoma cell lines. This was evident in the Bim knockdown studies where there was a clear indication in one cell line (Mel-RMu) that Bim was the major mediator of apoptosis whereas in another cell line knockdown of Bim had very little effect. FOXP3 is known to be an important regulator of Bim expression. It is believed to form stable complexes with certain HDACs which could

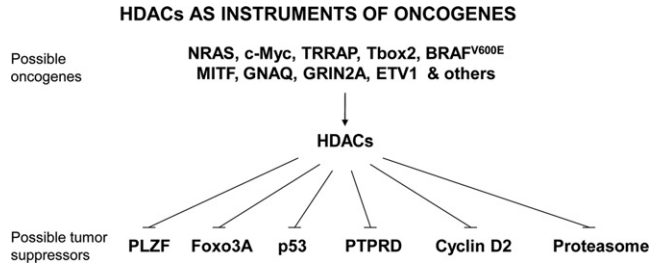

Figure 2.6 HDACs as instruments of oncogene-mediated inhibition of tumor suppressors. The oncogenes and tumor suppressors referred to are reviewed in Walia et al. (2012) and Dutton-Regester et al. (2012). The term "tumor suppressor" is used in a broad sense rather than that used by Haber and Harlow (1997).

influence the expression of Bim (Zhang et al., 2012). HDACs may also have profound effects on immune responses which remain an important topic of ongoing research (Suliman et al., 2012). A number of other possible mechanisms remain to be explored such as involvement of p53-dependent apoptosis. Previous studies have shown that MAGE A2 proteins can target HDAC 3 to p53 and inhibit its transactivation function (Monte et al., 2006). In addition, the MAGE A, B, and C proteins can form complexes with KAP1 and thereby suppress p53 function (Yang et al., 2007). Studies showing that HDACi may have an important role as inhibitors of proteasome also raise questions as to whether this role may be important in inducing apoptosis (Fotheringham et al., 2009).

Previous expression array data (Peart et al., 2005) from studies on Jurkat and CEM cells treated with SAHA or depsipeptide identified a number of changes in pro- and antiapoptotic genes such as APAF1 and BAK and TNF family receptors and ligands. BH3-only family proteins were not among these changes so that there may be a different set of changes in melanoma cells. Similar studies on non–small cell lung cancer cells identified nine genes that were associated with sensitivity to TSA and SAHA. These were not known to be directly involved in apoptosis but to influence p53 and other functions such as the proteasome (Miyanaga et al., 2008).

A number of additional questions remain unanswered. One is whether the upregulation of the HDACs in melanoma is due to direct targeting by oncogenes as reported for upregulation of HDAC 2 by N-Myc in neuroblastoma (Marshall et al., 2010). An increasing number of possible oncogenes are being described in melanoma, such as ETV1 (Walia et al., 2012). cMyc and cMET remain possible candidates in some melanoma and may be linked to aberrant activation of NF-kB (Thu et al., 2011). However, these oncogenes were not detected in studies on genes amplified or deleted in melanoma (Dutton-Regester et al., 2012). In ocular melanoma, loss of the BRCA1 associated protein-1 (BAP1) is thought to result in failure to deubiquinate histone H2A and thereby change chromatin structure that possibly can be reversed by HDAC inhibitors (Harbour, 2012).

Furthermore, it remains unknown whether specific HDACs may be more important than others in suppression of apoptosis. Studies in colon, pancreatic, and lung carcinoma showed that HDAC 8 was needed for the expression of p53 (Yan et al., 2012). HDAC 5 and 9 were reported to be upregulated in high risk medulloblastoma and were possible markers of risk (Milde et al., 2010). HDAC 1 and 2 were highly expressed in renal cell carcinoma but HDAC 3 was less frequently expressed (Fritzsche et al., 2008).

It is not clear from any of these studies whether HDAC expression is predictive of response to certain HDACi. As more selective HDAC inhibitors become available, these questions will have additional importance (Pan et al., 2012). The present studies on melanoma cell lines provide possible snapshots of what may be happening in the tumors of patients and provide a rationale for combining BRAF inhibitors with HDAC inhibitors in patients failing treatment with selective BRAF inhibitors. It is also possible that there may be a role for testing newly described inhibitors of bromodomain proteins to downregulate some oncogenes such as cMyc and in modulation of gene expression as reviewed elsewhere (Arrowsmith et al., 2012).

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Conflict of Interest Statement: The authors have no conflicts of interest to declare.

ABBREVIATIONS

CDKN1 cyclin-dependent kinase 1
HDAC histone deacetylases
HIF hypoxia inducible factor
Hsp90 heat shock protein family 90
Sel BRAF selective BRAF inhibitors
SIRT1 Sirtuin 1
Tbx2 T-box 2

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CHAPTER THREE

Targeting the Tumor Stroma as a Novel Treatment Strategy for Breast Cancer: Shifting from the Neoplastic Cell-Centric to a Stroma-Centric Paradigm

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Abstract

The lack of targeted therapy for women with triple negative breast cancer demands a "think-outside-the-box" approach in search of novel treatment strategies. Although cancer drug development traditionally focused on targeting the tumor cell cycle, emphasis has recently shifted toward the tumor microenvironment for novel therapeutic and prevention strategies. The tumor microenvironment is a dynamic composite of cells broadly categorized as immune cells and nonimmune cells within a scaffold of extracellular matrix, where tumor cells thrive. Among the various nonimmune cell types, cancer stromal cells have emerged as critical players in promoting tumor proliferation, neovascularization, invasion, and metastasis as well as interacting with immune cells to tilt the equilibrium toward a tolerogenic environment that favors the tumor cells. In view of recent work that demonstrated that the depletion of fibroblast activation protein (FAP) expressing tumor stromal cells resulted in stunted tumor growth and improved response to tumor vaccination, the tumor microenvironment is, therefore, fertile ground for development of novel therapy with the potential of augmenting existing treatment and prevention options. In this review, we will focus on current evidence supporting the role of cancer associated fibroblasts (CAFs), with a special focus on FAP^+ stromal cells, in promoting tumor growth. The role of CAFs in promoting an immunosuppressive environment, which may accelerate tumor progression, will be discussed with the hope that therapeutics developed to target the "generic" tumor microenvironment may be effective for malignancies such as triple negative breast cancer, for which targeted therapy is not available to date, in the future.

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1. INTRODUCTION

Robust evidence has underscored the role of cancer associated fibroblasts (CAFs), the most abundant nonimmune cell type in the tumor microenvironment, in tumor progression. Much less is known, however, regarding their role in promoting an immunosuppressive environment that abrogates spontaneous immune pressure against cancer progression, thus allowing tumor cells to thrive. In this review, we will summarize the role that CAFs play in the tumor microenvironment, highlighting new research that suggested that stromal cells, especially those that express fibroblast activation protein (FAP) may partner with immune cells to promote a tolerogenic microenvironment within the tumor proper. We will also discuss novel therapeutic avenues based on this emerging evidence, which can be developed to augment current limited therapeutic options for malignancies such as triple negative breast cancer which lacks effective targeted therapy.

1.1. Triple Negative Breast Cancer: An "Orphan" Breast Cancer Subtype Without Effective Targeted Therapy

Of the 230,480 breast cancers diagnosed in 2011, ~20% will be triple negative breast carcinomas (TNBC) (2011 ACS Cancer Facts and Figures). TNBC, as the name implies, is defined by the lack of expression of estrogen (ER), progesterone (PR) or Her2-neu (Her2) receptors for which targeted therapeutics such as endocrine therapy or trastuzumab are ineffective. TNBC also has less favorable prognosis compared to other breast cancer subtypes (Dent et al., 2007). The lack of targeted therapy for this more aggressive breast cancer is particularly challenging in the recurrent and metastatic setting when conventional chemotherapy combinations have been exhausted. It is imperative to find better treatment options for this "orphaned" breast cancer subtype which lacks effective targeted therapy to date. In addition, the incidence of TNBC is higher in African American women, with a significantly higher risk of relapse (Carey et al., 2006) and therefore represents a major target in the national effort to correct cancer health disparities.

1.2. Tumor Microenvironment: An Undercharted Territory for Drug Development

Most of the effort in the development of cancer therapeutics in the past few decades has been focused on targeting epithelial cancer cells. Drugs that target the tumor stroma has not been in the forefront except for

bevacizumab, a monoclonal antibody which targets vascular endothelial growth factor (VEGF) and inhibits tumor angiogenesis (Presta et al., 1997). Although there is evidence that bevacizumab can slow disease progression, survival was not prolonged. Bevacizumab was disapproved by the FDA in November 2011 for use to treat metastatic breast cancer (Lenzer, 2011).

Ongoing clinical trials targeting TNBC such as those that utilize combination drug therapies such as Poly (ADP-ribose) polymerase (PARP) inhibitors and conventional chemotherapeutic agents are aimed at targeting the cancer epithelial cells. Newer drugs that may potentially target the tumor microenvironment such as fresolimumab, a monoclonal antibody that binds to TGF beta, are currently being tested in clinical trials (clinicaltrials.gov). As Notch signaling pathways are known to be upregulated in TNBC (Clementz et al., 2011), a gamma-secretase inhibitor, R04929097, an inhibitor of Notch signaling, is being evaluated in women with TNBC. There is some evidence to suggest that this novel drug may mediate its action via the tumor microenvironment (Rehman & Wang, 2006; Shao et al., 2011; Steg et al., 2011).

The paucity of therapy targeting the tumor microenvironment highlights the need to develop novel drugs that target this compartment so as to augment our current treatment options for breast cancer. Tumor stroma is composed of various nonimmune cells such as fibroblasts, endothelial cells, pericytes, and immune cells in a scaffold of extracellular matrix (ECM) (Egeblad et al., 2005; Hanahan & Weinberg, 2011; Hu & Polyak, 2008; Liotta & Kohn, 2001; Neesse et al., 2011; Pietras & Ostman, 2010; Tlsty, 2008). Each component in the tumor stroma plays a crucial role in tumor progression and hence could be targeted in cancer therapy. In addition, certain chemotherapeutic agents elicit an immunogenic death in tumor cells that boost antitumor immune responses, which are ultimately responsible for their sustained therapeutic benefit (reviewed in (Zitvogel et al., 2011)). In contrast, multiple immunotherapeutic approaches have so far failed to improve survival in a clinical setting due, in part, to an incomplete understanding of the mechanisms of immunosuppression within the tumor microenvironment. As we begin to appreciate the delicate balancing act between tumor promoting/tolerogenic and tumor suppression played by immune cells within the tumor microenvironment, novel therapeutics that inhibit specific immunosuppressive pathways within immune cells have started to yield exciting and promising results (review in Pardoll & Drake, 2012).

Recent studies suggested that drugs that act on the tumor microenvironment by directly modulating immune environment or indirectly by

altering the composition of the extracellular matrix in the tumor stroma may have inhibitory effects on tumor cell proliferation and/or augment therapeutic effects of standard chemotherapy agents (Neesse et al., 2011). Whether these results can be translated into novel strategies in the treatment of TNBC remain to be seen.

1.3. CAF Heterogeneity in the Tumor Microenvironment: Friend or Foe

Evidence suggests that fibroblasts are heterogeneous and have distinct phenotypes which are organ specific (Chang et al., 2002). Fibroblast heterogeneity has been described in normal tissues of lung, skin, sclera, and orbit (Baglole et al., 2005). Fibroblasts are spindle-shaped cells that constitute the main cellular fabric of the stroma in the breast cancer microenvironment and contribute to its structural integrity. Within the tumor microenvironment, fibroblast heterogeneity has also been reported in mouse breast and pancreatic tumor models (Sugimoto et al., 2006).

It is well established that fibroblasts derived from normal breast stroma have distinct gene-expression profile compared with fibroblasts derived from breast carcinomas (Allinen et al., 2004). Immunohistochemistry analyses on tissue sections also demonstrated distinct differences between the stroma of normal or malignant breast tissues. For instance, most invasive human breast cancers show a modest number of myofibroblasts, which abundantly express α-smooth muscle actin. In addition, myofibroblasts share phenotypic attributes with "activated fibroblasts" present in areas of inflammation and wound healing (Orimo & Weinberg, 2007).

Heterogeneity within the breast cancer stroma was indeed noted in a recent study comparing the gene-expression profiles between morphologically normal versus tumor stroma from 53 invasive breast cancer tissue samples. A stromal gene signature was identified as an independent prognostic variable in this study (Finak et al., 2008). In addition, a unique stromal signature was observed in breast cancer from women with African American descent compared with European American descent (Martin et al., 2009). These studies suggest that heterogeneity exists within the breast cancer stroma that may further stratify the current tumor classification which is largely based on the receptor status on tumor epithelial cells. The potential heterogeneity in cancer-associated fibroblasts within the tumor microenvironment offers a unique opportunity to develop novel therapies for breast

cancer treatment, possibly tailoring to specific cancer subtypes such as triple negative breast cancer.

1.4. CAF Markers as "Druggable" Targets?

CAFs are the predominant stromal cells found within the tumor microenvironment. These cells are no longer considered passive bystanders in tumorigenesis (Hanahan & Weinberg, 2011). CAFs have long been known to promote tumor growth (Camps et al., 1990; Gleave et al., 1991; Hayward et al., 2001; Olumi et al., 1999; Picard et al., 1986). CAFs are also major producers of ECM and play an essential role in ECM remodeling. As we begin to understand the role of CAFs in tumor progression, CAFs are likely to interact with their neighboring partners and distant sites (e.g., bone marrow) via multiple autocrine and paracrine signaling pathways which are, in part, mediated through remodeled ECM/integrin signaling (Levental et al., 2009; Orimo et al., 2005; Paszek et al., 2005; Weaver et al., 1997). Thus, CAFs may be ideal targets for drug development especially given their genetic stability unlike their tumor cell counterparts. However, CAF-targeted drug development has been hampered by the lack of a robust and "druggable" CAF marker. Several CAF markers, namely α-smooth muscle actin (SMA), platelet-derived growth factor receptor (PDGFR) and fibroblast-specific protein (FSP-1, also known as S100A4), have been extensively studied and reviewed previously (Orimo & Weinberg, 2007; Pietras & Ostman, 2010; Sugimoto et al., 2006).

Of the various better-known CAF markers, PDGFR is the only molecule expressed on cell surface rendering it an ideal drug target. PDGFR is also abundantly expressed in pericytes, which are contractile cells that are intimately associated with endothelial cells. The mechanism of action of PDGFR in stromal cells, which include CAFs and pericytes, has been extensively reviewed elsewhere (Pietras & Ostman, 2010; Pietras et al., 2008). Briefly, the recruitment of pericytes and formation of neovascularization within the tumor microenvironment are intimately dependent on PDGF-B, a ligand for PDGFR (Abramsson et al., 2003). Imatinib, a small molecule tyrosine kinase inhibitor, which has been shown to inhibit PDGFR, was able to slow cervical cancer progression in a mouse tumor model (Pietras et al., 2008). However, results from clinical trials have been disappointing. In clinical trials involving women with metastatic breast cancer, imatinib as a single agent was poorly tolerated

and appeared to have no clinical activity (Cristofanilli et al., 2008; Yardley et al., 2009).

The role of FSP-1, or S100A4, in tumor progression has also been extensively studied (Egeblad et al., 2005; Grum-Schwensen et al., 2010; Tarabykina et al., 2007). Unlike PDGFR, FSP-1 is an intracellular protein. Therefore, its utility as a CAF marker and drug target is less ideal. There is evidence suggesting that depletion of FSP-1 may result in an immune environment which is less tolerogenic for tumor proliferation and metastasis, suggesting a role of FSP-1 in tumor metastasis (Grum-Schwensen et al., 2010). Whether FSP-1 is a therapeutic target in CAF is yet to be determined.

Although SMA expression has been the canonical marker for "activated" fibroblasts (Orimo & Weinberg, 2007), our own work showed that SMA is expressed heterogeneously within the tumor stroma of triple negative breast cancer and other breast cancer subtypes (Tchou, unpublished data). As reported in earlier studies (Park et al., 1999; Rettig et al., 1993), we found that FAP, a membrane-bound serine protease, is a robust stromal cell marker in all breast cancer subtypes, including TNBC. FAP is a homodimeric integral membrane serine protease that belongs to the S9 dipetidyl peptidase (DPP) family (Garin-Chesa et al., 1990; Kelly, 2005). FAP is unique amongst this family of DPPs in that, in addition to DPP acitivity, FAP also exhibits endopeptidase activity (Aertgeerts et al., 2005; Aggarwal et al., 2008; Edosada et al., 2006; Park et al., 1999). FAP is expressed selectively in the stroma of >90% of epithelial cancers, including breast cancer (Rettig et al., 1993). FAP is also expressed in scars and in areas that are undergoing active stromal remodeling.

As FAP is a cell surface marker and appears to be expressed robustly in CAFs but less so in normal stromal fibroblasts, FAP is, therefore, a potentially ideal CAF marker and drug target (Kelly, 2005; Park et al., 1999; Santos et al., 2009). There is now unequivocal evidence supporting the role of FAP in tumor progression. Briefly, abrogating FAP activity, by genetic deletion or pharmacological inhibition, resulted in markedly reduced tumor burden in mouse tumor models for lung and colon cancer (Santos et al., 2009). Collagen content and organization were also perturbed in the tumors that developed in the absence of FAP activity. A concomitant decrease in microvessel density was observed. Moreover, the ablation of FAP expressing cells in a recent study resulted in stunted tumor growth and enhanced response to tumor treatment by tumor vaccination in mouse tumor models (Kraman et al., 2010). As FAP expression is ubiquitous in the

stroma of all breast cancer subtypes (Tchou, unpublished data), strategies targeting FAP expressing cells will likely be applicable for triple negative breast cancer and other breast cancer subtypes and possibly tumors of other organ sites.

2. EXPLOITING THE MULTIFACETED ROLES OF CAF IN TUMOR PROGRESSION IN DEVELOPMENT OF NOVEL THERAPEUTIC STRATEGIES FOR TNBC

2.1. The Role of CAF in Remodeling ECM

The pioneer work of Mina Bissell and others has shifted the paradigm in understanding how studying cells in a three-dimensional architectural context yields the most biologically relevant information (Petersen et al., 1992; Weaver et al., 1997). Recent work has further shed light into our understanding that the cancer epithelial cells depend on their stromal cells and extracellular matrix as co-conspirators (Provenzano et al., 2009; Tlsty, 2008). The composition and organization of ECM is critical to homeotstasis. Under normal conditions, the ECM is largely comprised of specific isoforms of collagen and various ECM proteins, such as proteoglycans and glycosaminoglycans, which promote cellular quiescence in a defined tissue architecture. In cancer, the ECM undergoes an intricately orchestrated process referred to as remodeling. Recent work has shed light into our understanding that cancer epithelial cells depend on surrounding stromal cells and extracellular matrix to proliferate, invade, and evade immune attack (reviewed in Provenzano et al., 2009; Tlsty, 2008).

These new experimental systems have thus allowed the identification of the crucial role of the composition and organization of ECM in tissue homeostasis. Under normal conditions, the ECM is largely comprised of specific isoforms of collagen and various ECM proteins such as proteoglycans and glycosaminoglycans that promote cellular quiescence in a defined tissue architecture (Larsen et al., 2006). The distinctive stiffness of tumor tissues is likely imparted by the ECM, with collagen as one of the major components. CAFs are the major cell type contributing to the generation of ECM because they synthesize and secrete components such as collagens, fibronectin, and proteoglycans. In addition, CAFs also produce degrading proteases that mediate an intricately orchestrated process referred to as ECM remodeling. ECM modulates tumor progression at multiple levels. For instance, a higher collagen density could promote cell proliferation and invasion via activation

of the Ras-MAPK (mitogen-activated protein kinase) signaling pathway (Provenzano et al., 2009). The contribution of a dense ECM to malignant progression has been further illustrated in one study in which transgenic mice deficient in MMP-1 developed larger mouse mammary tumor as a result of increased stromal collagen density (Provenzano et al., 2008). In addition, microscopic evaluation of collagen organization within the tumor stroma using either second harmonic generation in two photon microscopy or birefringence in polarized light microscopy showed that the tumor stroma harbors thicker collagen fibrils in a highly organized fashion (Kakkad et al. 2010; Nadiarnykh et al., 2010). When this highly organized collagen network was disrupted either by directly inhibiting FAP using FAP-specific inhibitors or by genetically ablating FAP expressing cells, tumors were smaller and developed much more slowly (Kraman et al., 2010; Santos et al., 2009).

The role of ECM in the progression of other tumors such as pancreatic adenocarcinoma, which is characterized by a densely fibrotic tumor stroma, has also been recently underscored by Vonderheide and colleagues (Beatty et al., 2011). The authors found that *in vivo* activation of macrophages using CD40 agonists augmented chemotherapy effects in both pancreatic cancer patients and a preclinical model. Importantly, these effects were associated with collagen depletion within the tumor stroma. Whether the relationship between collagen density alteration within the tumor stroma and tumor regression is causal remains to be established. However, this seminal observation highlights the importance of ECM in tumor progression. The availability of clinical grade reagents guarantees further investigation of the potential of this approach in other settings such as breast cancer, which also orchestrate an abundant desmoplastic reaction within the tumor.

Another role for ECM in promoting tumor progression is via integrins which mediate cell attachment with ECM to provide traction necessary for cell motility and invasion. In addition, ECM and integrins collaborate to regulate gene expression associated with cell growth, differentiation, and survival; all of which are deregulated during cancer progression (Comoglio & Trusolino, 2005; Paszek et al., 2005).

The ECM with its highly organized collagen fibrils around tumor cells has long been thought to create a physical barrier for tumor invasion or metastasis but recent studies have challenged this concept (Provenzano et al., 2008). Nevertheless, ECM does act as a barrier for chemotherapeutic agents preventing these cancer killing agents to achieve therapeutic levels within the tumor microenvironment (reviewed in Neesse et al., 2011).

Therefore, the arena to develop new drugs that can alter the tumor ECM directly or indirectly remains wide open (Kraman et al., 2010; Santos et al., 2009).

2.2. Role of CAF in Promoting Tumor Proliferation, Angiogenesis, and Metastasis

CAFs are known to accelerate tumor cell proliferation *in vitro*, while normal fibroblasts from healthy tissues inhibit it (reviewed in Pietras & Ostman, 2010). Most importantly, the contribution of CAFs as active co-conspirators in accelerated tumor progression has been supported in multiple studies (Camps et al., 1990; Gleave et al., 1991; Hayward et al., 2001; Olumi et al., 1999; Picard et al., 1986). The mechanistic role of CAFs in tumor progression was further highlighted by the work of Bhowmick and coworkers (Bhowmick et al., 2004). In this study, $TGFBR2^{flox/flox}$ transgenic mice were crossed with fibroblast-specific protein (FSP)-cre mice resulting in mice with TGFBR2 knock-out specifically in cells that expressed FSP, that is fibroblasts. These C57BL/6 mice spontaneously developed squamous cell carcinoma in the fore stomach and prostate suggesting that the lack of TGFBR2 mediated signaling in CAFs drive tumor progression. The effects of TGF beta/TGFBR2 paracrine signaling between epithelium and stroma were mediated by hepatocyte growth factor. Abrogating the TGF beta/TGFBR2 signaling axis in these knock-out mice resulted in upregulation of HGF, which promoted tumor cell proliferation via the HGF/c-met pathway.

More recent work by Orimo and colleagues demonstrated that CAFs mediate their effects partly via the secretion of stromal cell-derived factor-1 (SDF-1), also known as CXCL12, which acts via CXCL12/CXCR4 signaling by recruiting endothelial progenitor cells derived from the bone marrow to migrate into the tumor microenvironment to form new tumor microvasculature, thus promoting angiogenesis (Orimo et al., 2005). In this study, primary CAFs derived from human breast tumors promoted the growth of MCF-7-ras breast cancer cells when these tumor cells were co-injected with CAFs into immunocompromised mice (Orimo et al., 2005). Interestingly, the effect of CAFs on the degree of tumor growth promotion was variable dependent on the origin of these CAFs, suggesting an inter-patient heterogeneity. These studies therefore highlight both the opportunity and the need for tailored therapies that may target the tumor microenvironment with patient-specific characteristics.

The use of 3-D coculture models has further illustrated the contribution of CAFs to microvessel formation by endothelial cells (Noma et al., 2008). In this 3-D system where esophageal carcinoma cells were embedded in a collagen I matrix, Noma and colleagues identified a new mechanism whereby VEGF produced by CAFs in response to TGF-β derived from esophageal carcinoma cells promoted microvessel formation from human umbilical endothelial cells that were embedded in the 3-D culture. Interruption of this and other pathways (e.g., the PDGF paracrine signaling pathway) between cancer epithelial cells and CAFs, or between CAFs and endothelial progenitor cells, may offer alternative therapeutic targets which disrupt tumor neovascularization critical in sustaining tumor cell growth (Pietras et al., 2008). As mentioned previously, results from clinical trials evaluating drugs that target tumor angiogenesis such as bevacizumab (targeting VEGF pathways) and imatinib (targeting the PDGFR pathway) have been disappointing (Cristofanilli et al., 2008; Lenzer, 2011).

In another recent study using a 3-D coculture, the role of fibroblasts in promoting the invasion of squamous cell carcinoma cells into ECM was further emphasized (Gaggioli et al., 2007). In this study, integrin α3, integrin α5, and Rho, were implicated in promoting a fibroblast-led collective invasion of SCC, suggesting a role for CAFs in directly leading the invasion of cancer cells into the surrounding ECM. A more recent paper using a mouse breast tumor model (4T1) further supported this notion *in vivo* (Liao et al., 2009). In this study, CAFs were depleted using a DNA vaccine targeting FAP. After treating tumor bearing mice with the DNA vaccine, the tumors grew slower and the number of metastasis was smaller in the treated mice highlighting the essential role of CAF in tumor proliferation and metastasis.

Although preclinical studies have shown that FAP-specific inhibitors can inhibit tumor growth, clinical trials evaluating FAP-specific inhibitors have yielded disappointing results (Narra et al., 2007). Recent work suggested that the membrane-bound dipeptidase, FAP, or FAP expressing cells (i.e., CAFs), play a critical role in the tumor microenvironment by promoting tumor proliferation, angiogenesis, and metastasis (Kraman et al., 2010; Liao et al., 2009; Santos et al., 2009). Future studies are needed to dissect whether the role of FAP in tumor promotion depends on its protein function or on the function of the cells that express FAP. Results reported by Kraman and coworkers supported the latter.

2.3. Role of CAFs in Promoting Immunosuppression in the Tumor Microenvironment

Until very recently, a role for CAFs in modulating the function of immune cells in the tumor microenvironment was under-investigated. One of the first studies that provide insight into the role of cancer-associated fibroblast in modulating the immune environment within the tumor came from the work of Liao (Liao et al., 2009). The authors noted that depleting CAF, using a DNA vaccine that targeted FAP, reversed the polarization of the immune microenvironment from a Th2 to Th1 skewing. As a result, a decreased number of TAMs, myeloid-derived suppressive cells, and so on, were noted in the 4T1 mouse breast tumors formed in these vaccinated mice. In addition, a significant decrease in metastasis was noted when the DNA vaccine was administered in conjunction with the chemotherapeutic agent doxorubicine, further highlighting the role of CAFs in tumor progression by (1) modulating the response to chemotherapy and (2) modulating metastatic potential of 4T1 cancer cells.

More recently, a seminal study by Kraman et al. has generated significant excitement in the field by conclusively highlighting for the first time the potential of targeting the immunosuppressive activity of CAFs (Kraman et al., 2010). Using a new transgenic mouse model that allows specific depletion of FAP(+) cells, where FAP was primarily expressed by CAFs and only expressed by a negligible proportion of tumor cells in the described mouse tumor model, the authors achieved immunological control of established tumors of different histological origins. Importantly, tumor rejection was completely dependent on the cytokines TNF-α and INF-γ. Because INF-γ was only produced by lymphocytes (T cells, NK cells, and NKT cells), these results imply that, somehow, CAFs prevent the antitumor activity of INF-γ–producing immune cells.

Despite the therapeutic potential of targeting the immunosuppressive activity of CAFs, the precise mechanisms employed by CAFs to paralyze antitumor immunity remain unknown. It is possible that CAFs abrogate the activity of tumor-reactive T cells and/or NK cells directly, through the secretion of tolerogenic cytokines such as IL-10 or TGFβ. Alternatively, CAFs could express membrane-bound tolerogenic mediators such as butyrophilins (Smith et al., 2010), which have been recently demonstrated to dampen the activity of activated T cells (Cubillos-Ruiz et al., 2010). Finally, CAFs could promote the recruitment of other immunosuppressive leukocytes (e.g., macrophages) by secreting chemokines or creating

a proteolytic milieu, so that these cells would be directly responsible for the tolerogenic activity. In any case, these recent findings on FAP make it a potentially appealing drug target for breast cancer. Firstly, because FAP is robustly expressed in the stroma of all breast cancer subtypes including triple negative breast cancer (Tchou, unpublished observation). Secondly and more importantly, because FAP expression appears to be restricted to cancer tissues, while normal tissue only expresses minimal level of FAP mainly in the stroma around the terminal lobular alveolar units (Tchou, unpublished observation).

3. FUTURE CAF TARGETING STRATEGIES

As commented above, the tumor microenvironment is a largely uncharted territory for drug development. While development of new chemotherapeutic drugs targeting tumor cells is primarily done *in vitro* through molecular screening, the effect of targeting tumor microenvironmental components, including CAFs, can only be evaluated *in vivo*. Recent optimization of antibodies blocking various immunosuppressive signals has underscored that relevant mouse tumor models in immunocompetent hosts, when they are thoughtfully chosen, will best reflect therapeutic potential in humans and can be used to predict side effects (Pardoll & Drake, 2012). There is, therefore, an urgent need to develop and coordinate a synergistic research model where preclinical studies using animal models will complement clinical studies in humans.

If relevant preclinical models are available to recapitulate the specific expression of FAP in CAFs in human tumors, new avenues for the design of therapeutic interventions could be opened. For instance, FAP (and therefore CAFs) could be targeted with humanized antibodies, the repertoire of which is rapidly growing in the therapeutic arsenal. Alternatively, dendritic cell-based or classical vaccination strategies could be designed to target FAP. Specific targeting of CAFs would eliminate their immunosuppressive activity and could be combined with other synergistic immunotherapeutic interventions, such as T cell adoptive transfer. In addition, autologous T cells could be engineered to target FAP and ablate CAFs, which could also be combined with other immune- or chemotherapeutic approaches.

Depletion of CAFs would potentially not only unleash spontaneous anti-tumor immunity, but could also ablate their tumor promoting effects. Because CAFs are crucial for the production of ECM, the protective effect of collagen

on tumor cells would be also affected. Fibroblasts therefore offer a common targetable connection between virtually all mechanisms that drive malignant progression in the tumor microenvironment, rather than merely providing structural support. The next years should therefore see the development of an array of new interventions targeting this crucial tumorigenic component.

4. CONCLUSION

The lack of efficacy of therapeutics that target the tumor microenvironment highlights the need to understand why and how we can develop more effective drugs that target this tumor compartment so as to augment our current treatment options for breast cancer. Newer drugs targeting the immune cells within the tumor microenvironment have shown promise in clinical trials against several tumors (Pardoll & Drake, 2012). However, there is no established clinical intervention targeting CAFs, the main cellular component of stroma. Evidence now suggests that CAFs, with their effects on antitumor immunity, tumor growth, and malignant dissemination provide fertile ground for drug development. As FAP expression is ubiquitous in the stroma of all breast cancer subtypes, strategies focusing on FAP expressing cells will likely be applicable for triple negative breast cancer and other breast cancer subtypes and possibly tumors of other organ sites.

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ABBREVIATIONS

Cre Cre recombinase
Flox flanked by LoxP
INF γ interferon gamma
NK cells natural killer cells
PDGFR platelet derived growth factor receptor
TGFBR2 TGFβ 2 receptor
Th1 helper T cell response
TNF Tumor necrosis factor

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CHAPTER FOUR

Targeting the Metabolic Microenvironment of Tumors

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Abstract

The observation of aerobic glycolysis by tumor cells in 1924 by Otto Warburg, and subsequent innovation of imaging glucose uptake by tumors in patients with PET-CT, has incited a renewed interest in the altered metabolism of tumors. As tumors grow *in situ*, a fraction of it is further away from their blood supply, leading to decreased oxygen concentrations (hypoxia), which induces the hypoxia response pathways of HIF1α, mTOR, and UPR. In normal tissues, these responses mitigate hypoxic stress and induce neoangiogenesis. In tumors, these pathways are dysregulated and lead to decreased perfusion and exacerbation of hypoxia as a result of immature and chaotic blood vessels. Hypoxia selects for a glycolytic phenotype and resultant acidification of the tumor microenvironment, facilitated by upregulation of proton transporters. Acidification selects for enhanced metastatic potential and reduced drug efficacy through ion trapping. In this review, we provide a comprehensive summary of preclinical and clinical drugs under development for targeting aerobic glycolysis, acidosis, hypoxia and hypoxia response pathways. Hypoxia and acidosis can be manipulated, providing further therapeutic benefit for cancers that feature these common phenotypes.

1. INTRODUCTION

Otto Warburg first described an increased rate of aerobic glycolysis followed by lactic acid fermentation in cancer cells in 1924, later termed the Warburg Effect (Warburg et al., 1927). Almost a century of research has confirmed Warburg's initial observation, solidifying increased glycolytic flux as a common cancer phenotype (Hanahan & Weinberg, 2011). Increased expression of glycolytic genes are observed in ~70% of human cancers (Altenberg & Greulich, 2004). Warburg had hypothesized the metabolic shift away from oxidative phosphorylation was due to mitochondrial dysfunction, yet this has not been substantiated (Warburg, 1956). While interest in cancer

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metabolism peaked in the middle part of the twentieth century, interest waned with the advent of molecular biological techniques in the 1970s. In 1976, Sidney Weinhouse famously declared that ***"Since our perspectives have broadened over the years, the burning issues of glycolysis and respiration in cancer now flicker only dimly"*** (Weinhouse, 1976). The development of ^{18}F-fluorodeoxyglucose (^{18}FDG)-PET imaging to visualize increased glucose uptake in tumors and metastasis has rekindled interest in cancer metabolism, and is commonly used clinically for diagnosis and disease monitoring (Kelloff et al., 2005). An important characteristic of the tumor microenvironment commonly found in cancers and a selection force for the glycolytic phenotype is hypoxia. Tumor hypoxia can be transient or chronic either spatially or temporally, leading to significant heterogeneity and stress. Hypoxia is a challenge clinically due to its correlation with poor prognosis and association with resistance to chemotherapy and radiation therapy (Dewhirst et al., 2008).

We have previously proposed a series of microenvironment barriers that must be overcome for a tumor to develop during carcinogenesis (Gatenby & Gillies, 2008; Gillies et al., 2008). As carcinogenesis begins, inadequate growth promotion and loss of contact with the basement membrane are encountered first, which are commonly overcome by developing an insensitivity to anti-growth signals and self-sufficiency in growth signals—two Hallmarks of Cancer defined by Hanahan and Weinberg (Hanahan & Weinberg, 2011). As *in situ* cancers grow further away from the vasculature and beyond the diffusion limit of oxygen, the available concentration of oxygen is reduced, leading to hypoxic conditions. In locally invasive and metastatic lesions, hypoxia is exacerbated when neoangiogenesis creates a chaotic and immature vasculature network resulting in inconsistent oxygen delivery (Gillies et al., 1999). Cancer cells upregulate glycolysis to maintain energy production in the absence of oxygen (The *Pasteur Effect*), eventually becoming the preferred energy production pathway even during reoxygenation (The *Warburg Effect*). Aerobic glycolysis is accompanied by lactic acid fermentation, creating significant amounts of free protons (H^+) which are shuttled to the extracellular tumor microenvironment to maintain intracellular pH (pHi) at physiological levels. Increasing amounts of H^+ being pumped into the extracellular space creates an acidic microenvironment, which is known to select for cells with enhanced metastatic potential as well as provide resistance to chemotherapy (Moellering et al., 2008; Raghunand & Gillies, 2000; Rofstad et al., 2006; Schlappack et al., 1991; Wojtkowiak et al., 2011).

The tumor microenvironmental characteristics described earlier are heterogeneous within a tumor and are found in virtually all human solid

tumors. Furthermore, while there are common metabolic phenotypes, these can arise by a multitude of genetic changes, otherwise known as the "functional equivalence principle" (Gillies et al., 2008). Hence, targeting the causes and consequences of the tumor microenvironment is an effective way to reach a large population of patients and inhibition can potentially overcome tumor growth and metastasis. In this review, we describe techniques used clinically for imaging the tumor metabolic microenvironment, as well as developmental drugs to target various aspects of tumor metabolism. Finally, we detail methods that are currently being investigated preclinically and clinically to manipulate the tumor microenvironment for therapeutic benefit.

2. IMAGING THE TUMOR MICROENVIRONMENT

Imaging approaches to characterize the metabolic microenvironment of tumors provide useful biomarkers for diagnosis and monitoring therapy response. In the future, it is expected that imaging will be able to be the most beneficial therapy for a particular patient. In the next section, we will detail some of the most common MRS, MRI, and PET clinical imaging methods of imaging tumor pH and hypoxia (for more detailed review see Hashim et al., 2011; Pacheco-Torres et al., 2011).

2.1. MRS and MRI

Magnetic resonance spectroscopy (MRS) imaging techniques depend on differences in chemical shifts of either endogenous or exogenous nuclear MR-active compounds based on pH-dependent or -independent resonances (Gillies & Morse, 2005). pH measurements with ^{31}P-MRS can compare the chemical shifts of endogenous inorganic phosphate (P_i) to measure pHi with that of exogenous 3-aminopropyl phosphonate (3-APP) to measure extracellular pH (pHe) (Shepherd & Kahn, 1999). Hyperpolarized ^{13}C bicarbonate enters into a Henderson-Hasselbalch equilibrium which can be used to spatially image a tumor pHe (Gallagher et al., 2008). While imaging with hyperpolarized ^{13}C bicarbonate is more sensitive than imaging with 3-APP, the main limitation lies with the rapid (within 1–2 min) decrease in hyperpolarization of bicarbonate. An alternative magnetic resonance imaging (MRI) technique is to use pH-dependent relaxation, such as gadolinium-DOTA-4AmP^{5-} in mixture with dysprosium-DOTP^{5-} (Garcia-Martin et al., 2006; Raghunand et al., 2003).

2.2. PET

Positron emission tomography (PET) imaging of tumors with ^{18}F-2-deoxyglucose (FDG) has had the most impact clinically in diagnosis, analysis of cancer staging, and monitoring response to therapy (Kelloff et al., 2005). FDG is taken up *via* glucose transporters (GLUT1 or GLUT3) and is phosphorylated by hexokinase, effectively trapping FDG in the cytoplasm unable to be further metabolized. PET imaging measures the annihilation reaction between a positron released from FDG during decay with a neighboring electron. Computer analysis of the signals received from annihilation reactions can reconstruct the location and quantity of positron-emitting radionucleotides, giving an accurate description of a tumor and metastasis.

A number of PET tracers for hypoxia have been developed. ^{18}F-fluoromisonidazole (FMISO) has been the most widely developed and used to image hypoxia in tumors (Valk et al., 1992). FMISO is a nitroimidazole derivative which enters cells through passive diffusion and undergoes a reduction reaction. Once reduced, FMISO becomes trapped and concentrated in cells in the absence of oxygen, allowing for PET imaging to detect regions of hypoxia within a tumor. FMISO has been studied extensively, and is available through an IND for detection of hypoxia in patients on clinical trials. Clinical studies suggest that uptake of FMISO by a tumor is predictive of its resistance to treatment radiation therapy (Thorwarth et al., 2006).

Electron paramagnetic resonance imaging (EPRI), an imaging technique similar to nuclear magnetic resonance, measures the interactions between molecular oxygen and a nontoxic stable radical tracer (Matsumoto et al., 2010). EPRI is able to measure the partial oxygen pressure (pO_2) of tumors without radioisotopes and is capable of measuring dynamic pO_2 changes, allowing for the measurement of intermittent hypoxia in tumors (Bennewith et al., 2002). Although it has only been applied preclinically, EPRI is able to measure tumor hypoxia quickly generating 3-dimensional pO_2 maps from data obtained during imaging.

3. TARGETING GLUCOSE METABOLISM

Aerobic glycolysis has long been known to be a common hallmark of solid tumors. This metabolic switch has been proposed to provide an advantage to growing tumors by allowing adaptation to low oxygen environments. This leads to increased acidification of the local tumor

microenvironment, allowing for evasion of the immune system and increased metastatic potential (Gillies et al., 2008; Kroemer & Pouyssegur, 2008). In the next section, we describe drugs that are in preclinical or clinical studies that target glucose metabolism of tumors (Fig. 4.1).

3.1. Targeting Glucose Transporters

Glucose, a major carbon source for cells, is a 6-carbon ring structure converted to pyruvate canonically along the Embden-Meyerhof glycolytic pathway. Entry of glucose into cells occurs by facilitated diffusion through a family of 14 membrane-bound proteins called glucose transporters (GLUTs). GLUT1, the founding member of the GLUT family, was isolated from erythrocytes in 1977 (Kasahara & Hinkle, 1977). Upregulation of GLUT1 and GLUT3 expression has been described in many cancers, and may be a key step in tumor progression. Increased expression of GLUTs correlate with poor prognosis and short survival of patients with ovarian,

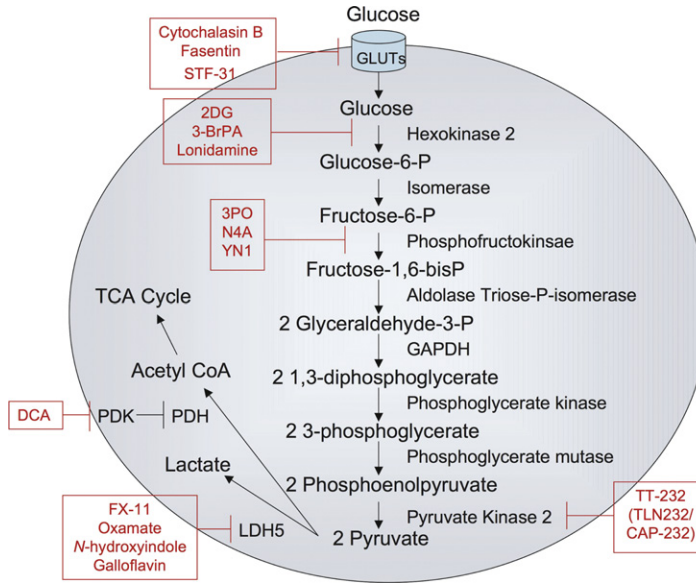

Figure 4.1 ***Inhibitors of glucose metabolism.*** The figure depicts the glycolytic pathway from glucose entry into cells through production of pyruvate, which is converted either to lactate or to acetyl-coA for entry into the TCA cycle. Movement of metabolic intermediates through the pathway is designated by arrows. Enzymes in the glycolytic pathway are placed next to the arrow leading from their substrate to their product. Inhibitors of glycolytic enzymes or glucose transporters appear in boxes. For color version of this figure, the reader is referred to the online version of this book.

breast, and squamous cell carcinomas (Ayala et al., 2010; Cantuaria et al., 2001; Pinheiro et al., 2011). GLUT1 ($K_m = 6.9$ mM) and GLUT3 ($K_m = 1.8$ mM) each have a high affinity for glucose, and are thought to be the main transport mechanisms for glucose into cells (Burant & Bell, 1992; Gould et al., 1991; Shepherd & Kahn, 1999). Importantly, Hatanaka showed in 1974 that glucose uptake by cells is a rate-limiting step in glycolysis. Subsequent work by other groups determined that transformed cells with increased expression of GLUTs at the plasma membrane is a strong independent prognostic indicator for FDG uptake and glucose consumption (Birnbaum et al., 1987; Bos et al., 2002; Flier et al., 1987; Hatanaka, 1974).

Increased expression of GLUT1 and GLUT3 during tumor progression allows for unregulated metabolism of glucose, making it an intriguing therapeutic target. Recent research described the cytotoxic and chemosensitizing properties of anti-GLUT1 antibodies in numerous lung and breast cancer cell lines reconfirming the importance of glucose uptake for survival (Rastogi et al., 2007). Decades of research have resulted in the discovery of many other GLUT inhibitors, including Cytochalasin B and select tyrosine kinase inhibitors (Taverna & Langdon, 1973; Vera et al., 2001).

High-throughput screening for drugs capable of sensitizing cells that evade FAS ligand-induced apoptosis have identified fasentin, a small molecule inhibitor that binds to the intracellular channel of GLUT1, reducing glucose transport (Schimmer et al., 2006). Further studies uncovered altered expression of genes involved in glucose metabolism following treatment of FAS-resistant prostate and leukemia cells with fasentin and FAS ligand (Wood et al., 2008). Ultimately, fasentin alone was unable to induce cell death in FAS-ligand resistant cells, despite a rapid, albeit, partial reduction in glucose uptake following fasentin treatment.

Renal cell carcinoma (RCC), known for harboring inactivating mutations in the von Hippel-Lindau (VHL) ubiquitin ligase gene, was identified as a candidate for chemical synthetic lethality screening for GLUT inhibitors (Chan et al., 2011). VHL mutations often coincide with a reorganized metabolic profile, wherein the tumor becomes highly glycolytic and relies on high levels of GLUT1 expression. One class of compounds, led by STF-31, caused necrotic cell death in RCC cells lacking functional VHL. *In silico* modeling revealed a potential docking site for STF-31 located in the central channel of GLUT1, and further functional studies confirmed inhibition of GLUT1 by STF-31. FDG-PET scans confirm reduced glucose uptake in RCC tumors treated with STF-31, corresponding with retarded tumor

growth. Lack of toxicities resulting from treatment with STF-31 encourages further research into its therapeutic potential and widespread efficacy in other tumors overexpressing GLUT1.

3.2. Targeting Hexokinase

As glucose enters the cystol, hexokinase phosphorylates the sixth carbon, effectively trapping glucose intracellularly and priming it for catabolism. Hexokinase-2 is frequently overexpressed in cancers, overcoming silencing methylation found on its promoter in normal tissues (Goel et al., 2003). Expression of hexokinase is transcriptionally regulated by both p53 and hypoxia-inducible factor 1α (HIF1α) (Mathupala et al., 1997). Glucose analogs, specifically 2-deoxyglucose, can be radiolabeled to image tumors with increased glucose uptake (^{18}FDG), and have also been studied as inhibitors of glycolysis (Kurtoglu et al., 2007; Lampidis et al., 2006). These analogs enter cells normally through GLUT1 or GLUT3 transporters and are phosphorylated by hexokinase. As with glucose, the 6-phospho form of these analogs are unable to exit cells, and are feedback inhibitors of hexokinase activity. However, unlike glucose, the phosphorylated glucose analogs are unable to be rapidly catabolized through the remainder of the glycolytic pathway, that is, phosphofructokinase, and can build up to high levels intracellularly, where they prevent further glucose metabolism. Although there have been some successes using deoxyglucose *in vitro* and in animal models as a glycolytic inhibitor, clinical successes have not extended past utilization as an imaging contrast agent to visualize tumors or as a radio-sensitizing agent (Ramirez-Peinado et al., 2011; Song et al., 1976).

3-bromopyruvate (3-BrPA) has been identified as a potent inhibitor of glycolysis through its promiscuous inhibition of hexokinase-2 as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 3-BrPA has been widely studied as an alkylating agent, but its first anticancer properties were identified in 2001 as an inhibitor of hexokinase-2 (Ko et al., 2001; Meloche et al., 1972). Selectivity appears to depend on its uptake by overexpressed monocarboxylate transporter, SLC5A8 (Thangaraju et al., 2009). In addition to its use as a single agent, recent research has focused on combining 3-BrPA with other chemotherapies to overcome ATP-requiring multidrug resistance (MDR) mechanisms. Nakano et al. used 3-BrPA to sensitize MDR-expressing tumors to daunorubicin or doxorubicin treatment (Nakano et al., 2011). Similar work by Zhou et al. confirms that intracellular ATP is

essential for drug resistance, and that disruption of cellular energy levels through inhibition of hexokinase-2 by 3-BrPA resensitized MDR cells to therapy (Zhou et al., 2012).

Lonidamine was first identified as an inhibitor of aerobic glycolysis through inhibition of hexokinase-2 in tumor cells in 1981 (Floridi & Lehninger, 1983; Floridi et al., 1981). As with 3-BrPA, inhibition of hexokinase-2 by lonidamine induced apoptosis (Brawer, 2005). Lonidamine acts as a single agent and has been extensively studied as a treatment for MDR (Li et al., 2002; Ravagnan et al., 1999). Already approved for use as an anticancer chemotherapy in Europe, phase II clinical trials began in the United States in 2005 treating patients with benign prostatic hyperplasia (BPH) (Brawer, 2005; Ditonno et al., 2005). Despite reports of some cancer patients receiving 40 times the dose than patients in the US trial, and indications that prostate volumes were reduced during treatment, the US phase II trial was terminated due to liver toxicities and no subsequent trials have begun (Ditonno et al., 2005; Milane et al., 2011b). In an effort to harness the therapeutic efficacy of lonidamine against MDR and reduce toxicities due to dosage, Milane et al. have developed epidermal growth factor receptor (EGFR)-targeted nanoparticles encapsulating lonidamine and paclitaxel (Milane et al., 2011a,b). Orthotopic MDR-positive breast cancer xenografts treated with targeted drug-containing nanoparticles showed reduced tumor growth compared to treatment with blank nanoparticles. Transient weight losses were observed in all groups. Liver toxicities were highest in animals treated with soluble paclitaxel alone or soluble paclitaxel + lonidamine, and were less severe when drugs were bound to nanoparticles. Hematologic analyses also revealed reduced toxicity following treatment with drug combinations encapsulated within nanoparticles. Overall, lonidamine is a promising hexokinase-2 inhibitor that may show clinical benefit either alone or in combination with other chemotherapies.

3.3. Targeting Phosphofructokinases

Phosphofructokinase-1 (PFK-1) catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate in a rate-limiting step in the glycolytic pathway. Regulation of PFK-1 activity is reduced as a result of oncogene activation, such as Ras or Src, through elevated levels of fructose-2,6-bisphosphate—a physiologic activator of PFK-1 (Bosca et al., 1986; Kole et al., 1991). Phosphofructokinase-2 (PFK-2), as well as the p53 target TIGAR, is a regulator of the steady-state level of intracellular

fructose-2,6-bisphosphate, and the PFKFB3 isozyme has been identified to be overexpressed in leukemias and solid tumors (Atsumi et al., 2002; Bensaad et al., 2006). Small molecule inhibitors targeting the substrate-binding domain of PFKFB3 have been identified as antineoplastic agents (Clem et al., 2008). *In vitro* inhibition of recombinant PFKFB3 revealed 3PO (3-(3-Pyridinyl)-1-(4-Pyridinyl)-2-Propen-1-one) as a lead compound that inhibits PFKFB3 but does not affect activity of PFK-1. 3PO was further shown to inhibit normal cell cycling in several solid tumor and hematologic cell lines further inhibiting tumor growth in xenograft models of lung, breast, and leukemia by suppression of glycolytic flux (Clem et al., 2008).

To improve upon clinical limitations of 3PO, such as solubility and high preclinical doses, Akter et al. has engineered nanoparticle drug delivery systems for 3PO (Akter et al., 2011, 2012). Encapsulating 3PO within a hydrophilic shell through conjugation to block copolymers improved 3PO bioavailability. 3PO conjugated block copolymers were also engineered with a hydrazone bond that is cleaved in acidic conditions ($pH < 7.0$) to preferentially target acidic tumor microenvironments. *In vitro* experiments with 3PO containing micelles resulted in significant cell death across several cell lines providing encouragement for future work in preclinical models.

In a separate study, N4A and YN1 were identified to be competitive inhibitors of PFKFB3 (Seo et al., 2011). While treatment of cells with these novel compounds resulted in decreased glycolytic flux followed by cell death, selectivity of the drugs was not ideal, and further optimization of the drug scaffold is currently underway.

3.4. Targeting Pyruvate Kinase M2

Pyruvate kinase (PK) catalyzes the transfer of a phosphate from phosphoenolpyruvate to ADP in the final step of aerobic glycolysis, resulting in one molecule each of ATP and pyruvate. Of the four pyruvate kinase isoforms, PKM1 is expressed in most tissues. PKM2 is a splice variant of PKM1 that is primarily expressed in embryonic development, but is also reported to be the main isoform expressed in tumors (Christofk et al., 2008). PKM2 expression has been associated with the Warburg Effect, carcinogenesis, and tumor growth. Due to increased expression of PKM2, cancer patients typically have higher levels of PKM2 in plasma and saliva, and this is being investigated in a clinical trial to determine if salivary levels of PKM2 can be used as a biomarker for malignancy (NCT01130584).

TT-232 (TLN-232/CAP-232) is a somatostatin structural analog that has been shown to significantly reduce tumor growth in murine models, and has entered clinical trials for refractory metastatic RCC and melanoma (NCT00422786 and NCT00735332). TT-232 has antiinflammatory effects through its interaction with somatostatin receptor 4 (SSTR4), a G protein-coupled receptor, and antitumor effects mediated through its inhibition of PKM2 (Elekes et al., 2008; Stetak et al., 2007). Unlike somatostatin, TT-232 is able to exhibit antitumor effects without the antisecretory activity that is required for somatostatin's efficacy in neuroendocrine tumors and pancreatitis (Greenberg et al., 2000). In addition to inhibition of PKM2, treatment of cells with TT-232 inhibits proliferation, induces cell cycle arrest, and initiates apoptosis (Stetak et al., 2001; Vantus et al., 2001). Phase I clinical trials of TT-232 were successfully completed without significant adverse events, allowing entry into phase II trials.

3.5. Targeting Pyruvate Dehydrogenase Kinase

Following the conversion of phosphoenolpyruvate to pyruvate by PK, further oxidation of pyruvate is enabled by mitochondrial pyruvate dehydrogenase (PDH), which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, which can then enter the tricarboxylic acid (TCA) cycle. PDH is negatively regulated at three serine phosphorylation sites by pyruvate dehydrogenase kinase (PDK), which shifts glucose from oxidative to glycolytic metabolism (Holness & Sugden, 2003).

Dichloroacetate (DCA) has been used clinically over the past several decades for the treatment of lactic acidosis and mitochondrial disorders (Stacpoole et al., 1988). DCA is an inexpensive, orally available drug that targets PDK (Bowker-Kinley et al., 1998; Knoechel et al., 2006; Stacpoole, 1989), and has recently been shown to have anticancer effects both *in vitro* and *in vivo* (Bonnet et al., 2007; Wong et al., 2008; Xie et al., 2011). The Michelakis group hypothesized that inhibition of PDK with DCA could shift glucose metabolism from glycolytic to oxidative, eliminating excessive lactic acid production observed in cancer cells (Bonnet et al., 2007). Indeed, treatment of lung, glioblastoma, and breast cancer cells reversed cell metabolism from glycolytic to oxidative; and in doing so increased ROS production, decreased mitochondrial membrane potential, and sensitized cells to apoptosis. *In vivo* rodent studies demonstrated the antitumor properties of DCA by reducing overall tumor volumes and inducing apoptosis in a lung cancer xenograft model (Bonnet et al., 2007). Further preclinical studies have

shown DCA to have similar proapoptotic effects on endometrial cancer cells as well as sensitizing prostate cancer cells to radiation therapy (Cao et al., 2008; Wong et al., 2008). Numerous clinical trials are currently recruiting, or underway, to administer DCA as a single agent, or in combination with other chemotherapies or radiation, in a wide range of cancers. The first published data from clinical trials with DCA as an anticancer therapy was recently published (Michelakis et al., 2010). Resected glioblastoma tissue from 49 patients treated with DCA confirmed mitochondrial depolarization *in vivo*. Five patients with either newly diagnosed or recurrent glioblastoma were placed on a treatment regimen of DCA with standard therapies, temozolomide (TMZ) and radiation therapy, after surgical tumor debulking. During a 15-month follow-up, toxicities were moderate, with peripheral neuropathy being the only toxicity noted with ~80% of patients remaining clinically stable 15 month after the onset of therapy.

3.6. Targeting Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate. LDH is a tetrameric protein made from two different (heart and muscle) subunits. LDH5 (a.k.a. LDH-A or M_4) is usually expressed in muscle tissue and has a low K_m for pyruvate, while LDH1 (a.k.a. H_4) is more ubiquitously expressed and has a lower K_m for lactate. During the redox reaction of pyruvate to lactate, NADH is oxidized to NAD^+, replenishing intracellular levels of NAD^+ and allowing glycolysis to become self-sufficient. LDH5 subunits are transcriptionally regulated by HIF1α and hence levels of LDH5 are increased in HIF1α–positive cancers (Firth et al., 1995; Semenza et al., 1996). Recently, LDH5 has been shown to be important for tumor initiation, although the exact mechanism is currently unclear (Fantin et al., 2006; Goldman et al., 1964; Xie et al., 2009).

Gossypol, a cotton seed extract, has been studied as an antifertility drug that inhibits sperm LDH, and further experimentation has revealed cross inhibition of gossypol analogs to LDH5 (Kim et al., 2009). More recent gossypol analog studies focusing on 8-deoxyhemigossylic derivates that target the NADH and pyruvate binding sites of LDH identified 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid, or FX11, as a preferential inhibitor of LDH5 (Yu et al., 2001). Treatment of human lymphoma cells, P493, with FX11 correlated with knockdown of LDH5 by siRNA by increasing oxygen consumption, ROS production, decreasing ATP levels, and cell death (Le et al., 2010). Similar results were

observed in RCC and breast cell lines, with the sensitivity to FX11 being highest in cells with a more glycolytic phenotype. *In vivo* studies also indicated that FX11 inhibits both carcinogenesis and tumor progression of lymphoma and pancreatic tumors (Le et al., 2010). It was notable that these treatments were not myelosuppressive or toxic, despite the presence of LDH-A in normal tissues. Although a promising candidate drug to target the glycolytic phenotype of tumors, FX11 is not yet in clinical trials.

The most recent research for novel LDH5 inhibitors began in an attempt to fabricate a drug suitable for entry into the clinic. From this research, a series of *N*-hydroxyindole-based inhibitors were generated to have specificity for LDH5 over LDH1 (Granchi et al., 2011). *In vitro* experiments showed promising K_i values in the low micromolar range for some of the compounds synthesized. Additionally, cellular assays resulted in reduced lactate production and retarded cellular proliferation. Virtual screening of the National Cancer Institute (NCI) Diversity Set by another group identified galloflavin as a novel LDH inhibitor (Kim et al., 2009). Galloflavin was further characterized and shown to bind preferentially to free enzyme without blocking either the pyruvate or NADH binding sites. Enzymatic assays using purified LDH1 and LDH5 showed that galloflavin acts as an inhibitor of both isoforms. Cellular assays confirmed *in vivo* activity of galloflavin with reduced lactate production, a reduction of cellular ATP levels, and decreased cellular proliferation. Preliminary murine experiments suggest that galloflavin could be a well tolerated drug that should be developed further.

4. TARGETING HYPOXIA

Hypoxia is another common phenotype of solid tumors. As tumors grow, proangiogenic factors stimulate new vessel growth within a tumor. However, these new vessels tend to be immature and chaotic, and hence lead to poor perfusion (Gillies et al., 1999). Tumors found to contain hypoxic regions typically respond poorly to therapy in the clinic (Dewhirst et al., 2008). Hypoxia can be difficult to target due to its spatial and temporal heterogeneity within tumors and the fact that hypoxic volumes are the most poorly perfused. Nonetheless, successful approaches to target hypoxia have been developed, and some of these are in clinical trials. These approaches can be broadly described as (1) targeting hypoxia response pathways; (2) drugs that require hypoxia for

their activity and thus efficacy and (3) methods to manipulate hypoxia to our advantage to increase efficacy of hypoxia-activated prodrugs (Table 4.1).

4.1. Targeting Hypoxia Response Pathways

Tumors typically have lower oxygen concentrations (pO_2) than levels detected in normal tissue (Hockel & Vaupel, 2001). As a tumor grows outward, away from blood vessels, the ability to receive oxygen from diffusion through tissue diminishes quickly leading to diffusion-limited (or chronic) hypoxia. Additionally, perfusion-limited (or acute) hypoxia can

Table 4.1 Drugs Targeting Hypoxia or Hypoxia Response Pathways

Drug	Target	Stage of Development
Topotecan	Topo I/HIF1α expression	FDA approved (ovarian, cervical, SCLC)
EZN-2968	HIF1α expression	Phase I/Pilot study
PX-478	HIF1α expression/ protein stability	Phase I
Rapamycin	mTOR	FDA approved for non-oncogenic indications
CCI779 (temsirolimus)	mTOR	FDA approved (renal cell carcinoma, mantle cell lymphoma)
RAD001 (everolimus)	mTOR	FDA approved (renal cell carcinoma, pancreatic neuroendocrine tumors & non-oncogenic indications)
Metformin	AMPK/mTOR/cell cycle	FDA approved for non-oncogenic indications
Bortezomib (PS-341)	Proteasome/UPR	FDA approved (mantle cell lymphoma, multiple myeloma)
STF-083010	IRE1/UPR	Preclinical
Salicaldehydes	IRE1/UPR	Preclinical
Tirapazamine (TPZ)	Hypoxia	Clinical trials completed
TH-302	Hypoxia	Phase I–III
Banoxantrone (AQ4N)	Hypoxia	Phase I
Apaziquone (E09)	Hypoxia	Phase I–III
PR-104	Hypoxia	Phase I–II

result from variable blood flow through chaotic and immature vessels that are characteristic of tumors. Hypoxia can be a significant source of stress for cancer cells and several survival and response pathways have been identified that allows cancer cells to overcome oxygen stress.

4.1.1. Targeting the HIF1α Pathway

Modulation of the hypoxia response in cells is orchestrated by transcription factors, hypoxia inducible transcription factors, HIF1α and/or HIF2α. Under normoxic conditions, HIF1α is inactivated *via* proteasomal degradation, regulated by the VHL ubiquitin ligase (Jaakkola et al., 2001; Ohh et al., 2000). In response to hypoxia, HIF1α is not degraded and the resulting stabilized protein will heterodimerize with HIF1β (a.k.a. the aryl hydrocarbon receptor nuclear translocator, ARNT) and activate promoters containing hypoxia response elements (HREs). Transcriptional targets of HIF1α can be found in glycolytic, angiogenic, survival, and migration pathways (Semenza, 2003). Constitutive HIF1α stabilization has been observed in many cancers and is correlated with aggressive disease, poor prognosis, and drug resistance, making HIF1α an attractive drug target (Birner et al., 2000; Bos et al., 2003; Giatromanolaki et al., 2001; Osada et al., 2007). This is an active area of research and there are numerous investigational drugs aimed at inhibiting HIF1α with a number of approaches: for example, targeting HIF1α mRNA expression, protein translation, protein stability, and transcriptional activity. Following, we illustrate some of these approaches. More exhaustive discussion of this subject can be found at (Vaupel, 2004).

Topotecan is an FDA approved drug that is indicated for ovarian, cervical cancers, and small cell lung carcinoma. The primary mechanism of action is through inhibition of topoisomerase I which induces genotoxic stress through DNA double strand breaks (Hsiang et al., 1985). Screening of the NCI Diversity Set of chemical compounds for small molecule inhibitors led to the discovery of a second mechanism of topotecan activity through inhibition of HIF1α expression (Rapisarda et al., 2002). Further topotecan studies confirmed inhibition of HIF1α expression, concluding that translation of HIF1α is inhibited in a topoisomerase 1-dependent mechanism by topotecan (Rapisarda & Uranchimeg et al., 2004a). Tumor xenograft models treated with topotecan have decreased HIF1α levels, diminished angiogenesis, and reduced tumor growth (Rapisarda & Zalek et al., 2004b). Furthermore, patients treated with topotecan had low to undetectable levels of HIFα in tumor biopsies, correlating with decreased levels of vascular endothelial growth factor (VEGF) and GLUT1 (Kummar et al., 2011).

Seven of 10 patients treated with topotecan to receive dynamic contrast enhanced (DCE)-MRI exhibited decreased blood flow and permeability through their tumors after one treatment.

Abolishing expression of HIF1α has been shown to be an effective way to inhibit tumor growth, inspiring the development of methods to target mRNA expression of HIF1α as an alternative to targeting HIF1α stability. An antisense oligonucleotide designed to inhibit HIF1α expression has moved into clinical trials (Li et al., 2005). EZN-2968 was developed by Enzon Pharmaceuticals, Inc. using locked nucleic acid (LNA) oligonucleotide technology to reduce HIF1α expression (Greenberger et al., 2008; Vester & Wengel, 2004). EZN-2968 was confirmed to selectively inhibit HIF1α mRNA expression *in vitro*, resulting in a lasting decrease in HIF1α protein levels, followed by a reduction in expression of HIF1α target genes. EZN-2968 also showed activity in a tumor xenograft model by repressing tumor growth. Phase 1 clinical studies treating hematologic patients with EZN-2968 have recently concluded (NCT00466583) and have been followed by a pilot trial that is currently recruiting patients with liver metastasis (NCT01120288).

PX-478 is an orally available small molecule that has been shown to inhibit HIF1α activity by reducing HIF1α levels (Welsh et al., 2004). Tumor xenograft experiments using a variety of tumor cell lines showed that treatment with PX-478 reduced tumor growth or tumor regression which correlated with decreased levels of HIF1α and its target genes GLUT1 and VEGF. The half life of PX-478 in murine plasma is short at 50 min, although concentrations capable of inhibiting HIF1α expression can be found for 8 h. Imaging of tumor xenografts with DCE and diffusion-weighted (DW)-MRI showed that treatment with PX-478 reduced tumor blood vessel permeability within 2 h of treatment and returned to normal 48 h after treatment (Jordan et al., 2005). Mechanistic studies have revealed that PX-478 may have multiple mechanisms of action in the inhibition of HIF1α by hindering both transcription and stability of HIF1α protein (Koh et al., 2008). PX-478 can also contribute to clinical efficacy by acting as a radiosensitizer in prostate cancer cell lines and in *in vivo* tumor models (Palayoor et al., 2008; Schwartz et al., 2009). Recently, phase I clinical trials investigating the safety and preliminary efficacy of PX-478 in patients with advanced solid tumor or lymphomas were completed (NCT00522652). Results from the phase I trial, presented at the 2010 ASCO Annual meeting, showed stable disease (SD) in ~40% of participants with mild toxicities (Tibes et al., 2010).

4.1.2. Targeting mTOR

The mammalian target of rapamycin (mTOR) is a kinase that is activated during cell stresses, including nutrient and energy depletion, triggering a signaling cascade regulating metabolism and many cell survival mechanisms (Dazert & Hall, 2011; Jung et al., 2010). mTORC1, a subunit of a complex nucleated by mTOR, has been shown to be important for tumorigenesis following activation of AKT (Skeen et al., 2006). Exposure to hypoxia in normal cells promotes activation of the tuberous sclerosis protein 1 complex (TSC1/2), which in turn negatively regulates the mTOR complex (Liu et al., 2006). Additional evidence indicates that inhibition of the mTOR complex due to hypoxia can be accomplished through interaction with promyelocytic leukemia (PML) tumor suppressor or disruption of mTORC1 binding to RHEB (Bernardi et al., 2006; Li et al., 2007). It is hypothesized that hypoxia-mediated inhibition of mTOR is a selective mechanism for mutations that are beneficial for cell growth in hostile environments (Graeber et al., 1996). Alternatively, constitutively active mTOR has been observed in advanced breast cancer. In addition, loss of mTOR repressors, such as PTEN and TSC1/2, can result in unregulated mTOR activity (Connolly et al., 2006; Kaper et al., 2006). While the exact role mTOR plays in carcinogenesis is not fully understood, mTOR inhibitors have been successful on the bench, and have moved into the clinic.

Rapamycin, a metabolite isolated from bacteria, was first identified in the 1970s to be a powerful antifungal drug (Vezina et al., 1975). Rapamycin was quickly determined to have antitumor activity, and was discovered to selectively target mTOR allosterically in the early 1990s (Heitman et al., 1991; Houchens et al., 1983). Rapamycin also has potent immunosuppressive activity and is approved for transplant patients to prevent organ rejection as well as antirestenosis after heart surgery due to its antiangiogenic properties, but is not an approved medication for the treatment of cancer. Analogs of rapamycin, or "rapalogs," are constantly being designed to be more specific to mTOR and have better pharmacologic properties and have been successful in the clinic. Currently, CCI779, or temsirolimus, is approved for treatment of RCC and mantle cell lymphoma, and is being investigated clinically for the treatment of other cancers, such as leukemia, non–small cell lung cancer (NSCLC), and breast cancer (Hess et al., 2009; Hudes et al., 2007; Rini, 2008). RAD001, or everolimus, has been approved for RCC and pancreatic neuroendocrine tumors, as well as an antirejection medication following organ transplant (Gabardi & Baroletti, 2010; Motzer

et al., 2008). In addition to single agent drugs, rapalogs are being investigated in coordination with drugs that target other signaling pathways to improve efficacy, such as PI3K or AKT (Ayral-Kaloustian et al., 2010; Cirstea et al., 2010; Ikezoe et al., 2007).

The antidiabetic drug metformin and its analogs buformin and phenformin have recently been identified as having potential anticancer activity. Metformin reduces blood glucose levels through decreasing hepatic gluconeogenesis and activation of AMPK (AMP-activated protein kinase) and is commonly used clinically for the treatment of type 2 diabetes (Hundal et al., 2000; Stumvoll et al., 1995; Zhou et al., 2001). AMPK can regulate activity of mTOR through activation of TSC1/2 (Inoki et al., 2003). Studies of diabetic patients receiving metformin revealed significantly reduced cancer risk compared to cohorts receiving other diabetic medications (Bowker et al., 2006; Evans et al., 2005). *In vitro* studies later confirmed that metformin represses growth of breast cancer cells through an AMPK-dependent signaling and inhibition of mTOR mechanism (Dowling et al., 2007; Zakikhani et al., 2006). Metformin treatment seems to inhibit other cellular processes such as the cell cycle through reduction of cyclin D1 and diminishing the transcription of GRP78, an estrogen receptor chaperone protein that is elevated in cancers and involved in Unfolded Protein Response (UPR) signaling (Ben Sahra et al., 2008; Saito et al., 2009). Metformin is currently being investigated clinically to determine if it is best used as a treatment or a preventative medication.

4.1.3. Targeting UPR

Hypoxia inhibits the ability of the endoplasmic reticulum (ER) to properly fold and organize proteins. The UPR is activated in the ER under hypoxia stress, which functions to maintain ER homeostasis or initiate apoptosis. Three proteins found at the ER membrane, PERK (PKR-like ER kinase), IRE1 (inositol requiring 1), and ATF6 (activating transcription factor 6), act independently to signal stresses leading to UPR activation (Koumenis et al., 2002). Response by the UPR to hypoxia is important for tumor growth, and aberrant UPR signaling due to the absence of PERK or IRE1 results in increased regions of hypoxia and reduced growth rates (Bi et al., 2005; Romero-Ramirez et al., 2004). Activation of the UPR response results in both reduction of translation and inhibition of protein maturation pathways as well as a detoxification process known as ER-associated degradation

(ERAD) and induction of autophagy (Rouschop et al., 2010). In addition to activation of UPR in response to hypoxia, other cellular stresses often found in solid tumors can lead to UPR activation. Such stresses include calcium homeostasis, redox status, and glucose depravation, making UPR an important cellular response mechanism in cancer, and also an attractive pathway to target clinically.

The ERAD response to cellular stresses is activated by the UPR and results in priming misfolded proteins to be shuttled out to the cytoplasm for proteasomal degradation (Travers et al., 2000). Blocking the ERAD response through proteasome inhibitors like bortezomib (PS-341) has been a successful strategy for tumors with high ER stress such as multiple myeloma (Lee et al., 2003; Nawrocki et al., 2005). Recent research suggests that hypoxia sensitizes cells to ER stress resulting from bortezomib treatment, leading authors to suggest pairing bortezomib with normoxia targeting drugs to improve therapeutic response (Fels et al., 2008). Such combinations have been investigated in murine models, and have shown to repress tumor growth when bortezomib was used in coordination with a HDAC6 specific inhibitor, ACY-1215, in a multiple myeloma model (Santo et al., 2012). Clinical trials are also ongoing, investigating the efficacy of combining bortezomib treatment with other chemotherapies, such as mitoxantrone (topoisomerase II inhibitor), mapatumumab (antibody specific for TRAIL death receptor), and vorinostat (HDAC inhibitor).

IRE1 has two enzymatic domains, a kinase domain and an endonuclease domain (Dong et al., 2001; Nock et al., 2001). Crystal structures have shown that IRE1 dimerizes in a juxtaposed configuration that allows for autophosphorylation resulting in increased endonuclease activity (Han et al., 2009; Korennykh et al., 2009). Screening for potential inhibitors of IRE1 using a cell-based reporter system identified STF-083010 (Papandreou et al., 2011). Treatment of multiple myeloma cells with ER stresses resulted in mRNA cleavage of XBP1 by IRE1, which was abrogated with treatment of STF-083010 (Back et al., 2006). STF-083010 was shown to selectively inhibit the endonuclease activity of IRE1 without affecting kinase activity. Although *in vivo* antitumorigenic responses were observed, more research will need to be performed to optimize an IRE1 inhibitor using STF-083010 as a scaffold. Another high-throughput screening search found salicylaldimine analogs to be inhibitors of IRE1 (Volkmann et al., 2011). Similar to STF-083010, salicaldehydes inhibit IRE1 endonuclease activity *in vitro* and

in vivo, increasing the interest to develop more potent and selective inhibitors targeting IRE1.

4.2. Using Hypoxia to Our Advantage

4.2.1. Use of Bioreductive Drugs

Bioreductive prodrugs are a class of drugs that are inert in tissues with normal pO_2 but are able to undergo chemical reduction in tissues with severe hypoxia to release cytotoxic warheads, selectively targeting cancer cells within hypoxic regions. In general, there are five different chemical scaffolds that have been used to generate bioreductive prodrugs (nitro groups, quinones, aromatic *N*-oxides, aliphatic *N*-oxides, and transition metals), all of which are able to be reduced in the absence of oxygen. One of the earliest reports of the use of bioreductive quinones to selectively target hypoxia is the use of mitomycin C in the 1960s (Iyer & Szybalski, 1964; Schwartz et al., 1963). During the last half century, bioreductive drugs scaffolds have been improved upon making them more selective and potent in hypoxic tumors.

Tirapazamine, or TPZ, is one of the most advanced bioreductive drugs through the clinical trials process. TPZ is built off of an aromatic *N*-oxide bioreductive scaffold (Zeman et al., 1986). During hypoxia, TPZ undergoes an intracellular one-electron reduction to a radical anion, then further converted to either a hydroxyl radical or an oxidizing radical, ultimately resulting in DNA damage (Anderson et al., 2003; Baker et al., 1988; Zagorevskii et al., 2003). TPZ creates DNA interstrand cross-links which stall replication forks and induce DNA breaks that require homologous recombination repair (Evans et al., 2008). TPZ has been extensively studied clinically in combination with cisplatin and radiation in patients with squamous cell carcinoma, head and neck cancer, and lung cancer with moderate to inconclusive results (Le et al., 2004; Rischin et al., 2005; Rischin et al., 2010; von Pawel et al., 2000). Further analysis showed that TPZ was being metabolized too quickly, and was not effectively penetrating tumor tissues (Hicks et al., 1998; Kyle & Minchinton, 1999). Consequently, TPZ analogs are currently being developed with the goal of improving drug solubility, cytotoxicity, selectivity, and tissue penetration characteristics (Hicks et al., 2010).

TH-302 is built upon a scaffold of a 2-nitroimidazole and is a nitrogen mustard prodrug that is selectively reduced under hypoxia ($<0.5\%$ O_2) (Duan et al., 2008). As TH-302 is reduced, the prodrug splits and releases its

cytotoxic warhead, bromo-isophosphoramide mustard (Br-IPM). As Br-IPM is released into hypoxic tissue, it cross-links with DNA, killing cells in the hypoxia compartment as well as neighboring cells with its bystander effect (Sun et al., 2012; Zhang et al., 2005). TH-302 was shown to have efficacy *in vitro* and *in vivo* in a wide subset of cancer cell lines and xenografts and was further found to have favorable drug-like properties and pharmacokinetic profiles (Duan et al., 2008; Hu et al., 2010; Meng et al., 2012). TH-302 entered phase I clinical trials as a single agent drug in patients with advanced solid tumors and has also been tested in combination with doxorubicin in patients with advanced soft tissue sarcoma, gemcitabine in patients with pancreatic cancer; docetaxel for patients with prostate or lung cancers (Ganjoo et al., 2011; Weiss et al., 2011). TH-302 was generally well tolerated, but some patients experienced skin and mucosal dose-limiting toxicities. Recently, phase I/II clinical trials of TH-302 as a single agent concluded with SD or better detected across a number of cancer types. Current clinical trials are investigating the efficacy of TH-302 as a single agent or in combination therapy for cancers, including melanoma, multiple myeloma, RCC, pancreatic carcinoma, and phase III trials have begun in patients with sarcoma.

Banoxantrone, or AQ4N, is a N-oxide bioreductive prodrug that was developed to selectively target hypoxic regions of tumors (Smith et al., 1997). The reduction under hypoxia releases a cytotoxic alkylaminoanthraquinone metabolite (AQ4) which induces DNA damage through inhibition of topoisomerase II. AQ4N has been shown to be efficacious in murine models of breast cancer when combined with chemotherapy or radiation therapy (Gallagher et al., 2001; Patterson et al., 2000; Williams et al., 2009). Phase I clinical trials have investigated the activity of AQ4N either as a single agent or in combination with radiation therapy (Papadopoulos et al., 2008; Steward et al., 2007). AQ4N was well tolerated by patients and is now being tested in clinical trials to evaluate the efficacy of AQ4N (NCT00394628, NCT00109356, and NCT00090727).

Two other bioreductive drugs, apaziquone (E09) and PR-104, have been successful on the bench top and have moved into clinical studies (Hendricksen et al., 2009; McKeage et al., 2011). While bioreductive drugs have been especially successful in preclinical studies, and have shown some success in the clinic, no bioreductive prodrug has been approved by the FDA to date. Current research is aimed at improving bioreductive prodrug selectivity, stability, and cytotoxicity. Additionally, research is ongoing to

develop bioreductive prodrugs that are non-genotoxic and instead target other cellular processes. For example, 2-nitroimidazole-5-ylmethyl is a 2-nitroimidazole that releases 5-bromoisoquinoline after reduction, targeting poly(ADP-ribose) polymerase 1 (PARP1) (Parveen et al., 1999).

4.2.2. Manipulating Hypoxia

While the data from hypoxia activated prodrugs (HAPs) in the clinic are promising, it can be reasoned that they may be more efficacious if tumor hypoxia can be selectively and transiently increased at the time of treatment. Thus, inducing hypoxia in tumors can be an efficient way of increasing the efficacy of drugs that target hypoxia. There are a number of mechanisms available with which to exacerbate tumor hypoxia selectively, including metabolically (e.g., pyruvate or DCA), or by reducing oxygen delivery (e.g., antiangiogenic agents or vasodilators).

It has recently been shown that tumor hypoxia can be increased following intravenous injection of pyruvate (Saito et al., 2011), whose mechanism of action may involve inducing cells to increase respiration (Kauppinen & Nicholls, 1986). EPRI, a spectroscopic imaging technique that measures *in vivo* oxygen concentrations, of tumors in mice following an intravenous injection of hyperpolarized ^{13}C pyruvate revealed a significant decrease in tumor oxygenation that reached a maximum at 1 h, and returned to normal within 5 h (Saito et al., 2011). Knowledge of a tumors oxygenation status is important for treatment plans, as pyruvate-induced hypoxia reduced the ability of radiotherapy to kill cancer cells even after tumor oxygenation had returned to normal levels. DCA, an inhibitor of PDK, has also been reported to initiate a metabolic switch in cancer cells from glycolysis to oxidative phosphorylation (Xie et al., 2011). Induction of oxidative phosphorylation by DCA increased reactive oxygen species, pH, and apoptotic proteins in HeLa cells. Additionally, the metabolic switch observed after DCA treatment correlated with an increased sensitivity of HeLa cells to cisplatin, suggesting that manipulation of a tumors metabolism may be therapeutically successful.

Tumor oxygenation can also be manipulated by controlling oxygen delivery with antiangiogenic or antivascular agents. Angiogenesis is a common phenotype ("Hallmark") of cancer that is regulated by HIF1α signaling (Hanahan & Weinberg, 2011). Tumors support an induction of angiogenesis by producing angiogenic growth factors such as VEGF and platelet-derived growth factor (PDGF). Several antiangiogenic inhibitors that target the immature angiogenic vasculature have been approved,

including sorafenib, a VEGFR and PDGFR inhibitor, avastin (bevacizumab), an antibody targeting VEGF, and sunitinib, a VEGFR and PDGFR inhibitor (Chung et al., 2010). Although resistance to anti-angiogenic drugs has become a major obstacle in clinical cancer treatment (Mitchell & Bryan, 2010), their use to acutely increase hypoxia in combination with HAPs has not yet been published. Alternatively, there are agents, such as combretastatin, that will target mature vessels, and these are also known to increase tumor hypoxia (Dachs et al., 2006). Another characteristic of the immature tumor vasculature is a lack of tone. Thus, vasodilators, such as hydralazine, induce a systemic drop in blood pressure, which is not matched by the tumor vasculature, causing a transient decrease in perfusion within the tumor (Sonveaux, 2008a). This "steal" phenomenon has been demonstrated using Doppler Ultrasound to measure decreased tumor blood flow (Horsman et al., 1992). The decrease in perfusion leads to increases in acidosis and hypoxia; both have been shown using pH electrodes or MRS, for acidosis and pO_2 electrodes for hypoxia (Adachi & Tannock, 1999; Belfi et al., 1994; Nordsmark et al., 1996; Okunieff et al., 1988).

5. TARGETING ACIDOSIS

The microenvironment of solid tumors is known to be more acidic (pH 6.5–6.9) than the physiological pH of normal tissue (pH 7.2–7.5), which can be attributed to a tumor's increased glycolytic flux and poor vasculature perfusion (Griffiths, 1991; Wike-Hooley et al., 1984). Acidic microenvironments have been shown to increase the invasiveness of a tumor, leading to increased metastasis (Moellering et al., 2008; Rofstad, 2000; Rofstad et al., 2006). In this section, we will describe drugs that target acidosis in tumors and systematic approaches to reduce acidosis in the tumor microenvironment (Fig. 4.2).

5.1. Targeting Proton Transport

Metabolically produced hydrogen ions (acid) can be exported from cells by a variety of mechanisms including, *inter alia*, sodium-hydrogen exchange (NHE), anion exchangers (AEs), vacuolar ATPases (V-ATPases), and membrane-bound carbonic anhydrases (CAs) (Neri & Supuran, 2011). NHE and AE are ubiquitously expressed and have proven to be poor anticancer drug targets, either through inefficacy or through toxicity, and

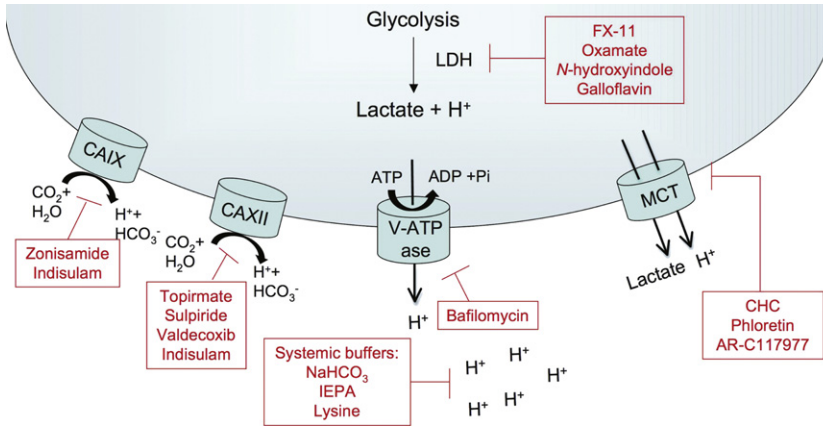

Figure 4.2 ***Proteins that contribute to tumor acidosis and their inhibitors.*** The figure depicts proteins and transporters that contribute to extracellular acidosis in a tumor due to increased lactate production from increased glycolytic flux. Included are CAIX and CAXII, carbonic anhydrases that catalyze the interconversion between carbon dioxide and water to bicarbonate and protons; and V-ATPases and MCTs, which allow transport of H^+ into the extracellular environment. Inhibitors of the proteins that contribute to tumor acidosis appear in boxes. For color version of this figure, the reader is referred to the online version of this book.

these have been reviewed (Grinstein et al., 1989). Following, we will discuss some of the newer, less well-explored members of this class of transporters.

CAs are metalloenzymes that catalyze the interconversion of carbon dioxide and water to bicarbonate and protons. Mammalian carbonic anhydrases (α-CAs) can be cystolic, mitochondrial, secreted, or membrane-bound. The primary function of mammalian CAs is to maintain the acid–base balance of cells, tissue, and blood. As aerobic glycolysis becomes the primary means of energy production for a tumor cell, the ability to regulate physiological pHi becomes paramount to maintain cellular processes such as proliferation as well as inhibition of apoptosis (Shen et al., 2006; Tiseo et al., 2009). CAIX and CAXII are two transmembrane CAs that have been identified to be associated with tumor progression and metastasis (Aulitzky et al., 1989; Fantin et al., 2006). As a transcriptional target of HIF1α, CAIX expression is upregulated in hypoxic tissue and has been shown to be a poor prognostic marker in several cancer types, including breast cancer (Lou et al., 2011). CAXII is also overexpressed in tumors and is associated with disease progression and response to therapy (Supuran, 2008; Tureci et al., 1998). As carbon dioxide is hydrated, HCO_3^- is moved intracellularly to maintain pHi while protons are pumped into the extracellular environment of a tumor,

decreasing the pHe (Shepherd & Kahn, 1999) promoting an aggressive metastatic environment (Jaakkola et al., 2001; Xie et al., 2009). Members of α-CA require zinc for activity, making them susceptible to inhibition by sulfonamides, which coordinates with the zinc ion found in the active sites of CAs. Sulfonamide analogs, such as topiramate, sulpiride, and valdecoxib, have been shown to potently inhibit CAXII, while zonisamide has been identified to be an effective inhibitor of CAIX (Greenberger et al., 2008; Li et al., 2005). Perhaps the most studied sulfonamide analog, indisulam, has high affinity for CAIX and CAXII, in addition to seven other CAs (Greenberger et al., 2008; Li et al., 2005). Indisulam inhibits CAIX in nanomolar quantities and shows efficacy against tumor xenografts *in vivo*. In addition to CAIX inhibition, indisulam induced sequelae, such as disruption of the G1/G2 phases of the cell cycle and expression changes of genes related to cell adhesion, cell signaling, and altered glucose metabolism (Owa et al., 1999; Rapisarda et al., 2002; Rapisarda & Zalek et al., 2004b; Vester & Wengel, 2004).

Clinical trials for the treatment of solid tumors with indisulam have been ongoing for the past decade. Five phase I clinical trials have been conducted focusing on optimizing the dosing regimen of indisulam to patients with solid tumors (Birner et al., 2000; Bos et al., 2003; Giatromanolaki et al., 2001; Kummar et al., 2011; Welsh et al., 2004). Fatigue and mucositis were noted as adverse events during the trial, and reversible neutropenia and thrombocytopenia were dose-limiting toxicities. Phase II trials have been completed on patients with platinum-pretreated NSCLC in a multicenter study (Jordan et al., 2005). While some patients experienced a positive response to indisulam, the effect was not long term. Objective responses to indisulam therapy were not achieved during this trial, which may be attributed to inherent difficulties of being a second-line therapy to platinum-pretreated NSCLC (Koh et al., 2008). Further trials are being conducted using indisulam as both a single agent and as combination therapy for different tumor types.

Another membrane-bound transporter involved with acidification of the tumor microenvironment is V-ATPase (Palayoor et al., 2008; Schwartz et al., 2009). In tumor cells, V-ATPases can prevent intracellular acidification by transporting protons into lysosomal compartments that are released into extracellular space, or by directly pumping protons into the tumor microenvironment (Skeen et al., 2006). In addition to promoting tumor metastasis by acidifying the tumor microenvironment, overexpression of V-ATPases following chemotherapy treatment appears to be a drug resistance mechanism (Li et al., 2007; Liu et al., 2006). In 1988, bafilomycins were identified

to be potent inhibitors of V-ATPases (Bernardi et al., 2006). Since this discovery, several generations of V-ATPase inhibitors have been developed and investigated and can be classified into five families of V-ATPase inhibitors (Perez-Sayans et al., 2009). While targeting V-ATPases is desirable as an anticancer target to reduce metastatic potential and drug resistance, clinical relevance is unknown due to likely toxicities (Bi et al., 2005; Connolly et al., 2006; Kaper et al., 2006; Koumenis et al., 2002; Luciani et al., 2004).

The monocarboxylate transporter 1, MCT1, a membrane-bound transporter is required for lactate (coupled with a proton) to move across the plasma membrane. MCT1 has been documented to have dysregulated expression in colorectal, breast, and cervical carcinomas (Asada et al., 2003; Pinheiro et al., 2008a; Pinheiro et al., 2008b). Inhibition of MCT1 reduces intracellular pH and induces apoptosis, making it an attractive target for antitumorigenic therapy (Sonveaux et al., 2008b). Several small molecule inhibitors of MCT1 have been identified including α-cyano-4-hydroxycinnamate (CHC), phloretin, and AR-C117977 (Bueno et al., 2007; Sonveaux et al., 2008b). Currently, no MCT1 inhibitors are being investigated clinically.

5.2. Manipulating Tumor Microenvironment pH

Orally distributed systemic buffers have been shown to be an effective way to increase pHe of a tumor (Silva et al., 2009). Continuous oral delivery of sodium bicarbonate to tumor bearing mice have been shown to increase selectively the pHe of a tumor and are effective at reducing the rate and size of metastasis, without changing the volume of the primary tumor (Jahde et al., 1990; Robey et al., 2009). In addition to reducing metastasis, buffering with sodium bicarbonate increased breast tumors sensitivity to doxorubicin and mitoxantrone, chemotherapies known to be ineffective in acidic tumor environments (Jahde et al., 1990; Raghunand et al., 2001; Wojtkowiak et al., 2011). A similar reduction in metastasis was achieved using orally available imidazole(IEPA) or lysine buffers in murine experimental metastasis models (Ibrahim Hashim, Cornnell, et al., 2011; Ibrahim Hashim, Wojtkowiak, et al., 2011).

6. MANIPULATING THE MICROENVIRONMENT FOR THERAPEUTIC BENEFIT

Combination therapy has been a long-standing strategy for the treatment of cancer patients. Drug resistance to single agent regimens is a major obstacle

in the clinic and combination therapy aims to target more of a heterogeneous tumor, reducing the ability of a tumor to develop resistance. The commonality of phenotypic characteristics of the tumor microenvironment between patients encourages the targeting of the microenvironment in combination with other cytotoxic chemotherapies. In the earlier sections, we detailed a number of approaches to target the tumor metabolic phenotype as well as describing strategies to manipulate hypoxia (exacerbation of hypoxia metabolically or by reducing oxygen delivery) and acidosis (buffer therapy) for therapeutic benefit. In this section, we will describe additional combination therapies that manipulate the metabolic or physiologic phenotype of cancers.

2DG, the glucose analog hexokinase inhibitor, has been unsuccessful as a single agent chemotherapy in the clinic, but has recently been of interest as a sensitizer of cancer cells to other chemotherapies or radiation therapy (Coleman et al., 2008; Lin et al., 2003; Simons et al., 2007; Zhang & Aft, 2009). Targeting metabolic pathways or DNA integrity through ionizing radiation (IR) or treatment with drugs like metformin in coordination with 2DG treatment can lead to significant antitumor effects (Ben Sahra et al., 2010; Cheong et al., 2011). Clinical studies have verified that cotreatment of 2DG with IR is safe for patients, and reduced toxicity associated with IR in some patients (Mohanti et al., 1996; Singh et al., 2005). Preclinical studies using 2DG as a sensitizer are promising; however, clinical studies investigating the efficacy need to be completed before 2DG sensitizing treatment becomes routine.

VEGF inhibitors, and antiangiogenic inhibitors in general, have similarly unintended effects on the tumor microenvironment, resulting in normalization of the tumor vasculature. Vascular normalization, first described by Rakesh K. Jain, is a maturation of existing immature vessels within a tumor when neoangiogenesis is inhibited (Goel et al., 2011; Jain, 2001, 2005). Vascular maturation results in better oxygen delivery and tumor perfusion, relieving interstitial tumor pressure which is hypothesized to provide better drug delivery to patients and reduce resistance to chemotherapy (Jain, 2005). Treatment of tumor bearing mice with VEGF inhibitor DC101 resulted in tumor vascular remodeling, where vasculature became nonleaky and more organized (Tong et al., 2004). Further studies have been conducted to study the timing of vascular normalization with optimal sensitivity to radiation treatment (Matsumoto et al., 2011; Winkler et al., 2004). Vascular normalization has been observed in patients with nonmetastatic rectal adenocarcinoma receiving bevacizumab (Willett et al., 2004, 2009, 2010).

Table 4.2 Clinical Trials[a]

Drug	Clinicaltrials.gov Identifier	Site	Phase	Sponsor
Biomarker study	NCT01130584	Salivary levels of PKM2	Observational	National University Hospital, Singapore
TT-232	NCT00422786	Renal cell carcinoma	II	Thallion Pharmaceuticals
TT-232	NCT00735332	Melanoma	II	Thallion Pharmaceuticals
EZN-2968	NCT00466583	Carcinoma/lymphoma	I	Enzon Pharmaceuticals, Inc.
EZN-2968	NCT01120288	Neoplasms/liver metastases	I	National Cancer Institute
PX-478	NCT00522652	Advanced solid tumors/ lymphoma	I	Oncothyreon Inc.
AQ4N	NCT00394628	Glioblastoma multiforme	Ib/IIa	Novacea
AQ4N	NCT00109356	Lymphoma/leukemia	I/II	Novacea
AQ4N	NCT00090727	Solid tumors/non-Hodgkin's lymphoma	I	Novacea

[a]Table describes clinical trials mentioned in review. Additional trials can be found at www.clinicaltrials.gov.

Although tumor reduction was not observed, microvessel density and vascular permeability decreased and histological analysis confirmed the presence of mature vasculature within tumors. Preclinical and clinical studies have provided support for the vascular normalization hypothesis; however, more studies need to be completed to fully optimize the normalization window to improve efficacy of this treatment.

7. CONCLUSION

Initially a barrier during carcinogenesis, the tumor microenvironment during the later stages of carcinogenesis provides an advantage for a tumor to outcompete normal tissue, becoming more aggressive and metastatic. Additionally, common characteristics of a tumor microenvironment provide a haven of protection for a tumor against chemotherapies. The immature and chaotic vasculature that exacerbates hypoxia within a tumor also provides minimal perfusion through a tumor for effective drug therapy, and extracellular acidosis due to preferential metabolism through aerobic glycolysis creates an environment that effectively traps weakly basic drugs from moving intracellularly. Extensive research has been focused on targeting the tumor microenvironment, providing clinicians with chemotherapies that target the glycolytic pathway, acidosis, hypoxia, and hypoxia response pathways (Table 4.2). Manipulation of the tumor microenvironment has been an effective strategy for the treatment of a wide range of patients and will continue to be an important area of drug discovery in the future.

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NON-STANDARD ABBREVIATIONS

FDG ^{18}F-2-deoxyglucose
2DG 2-deoxyglucose
3-APP 3-aminopropyl phosphonate
3-BrPA 3-bromopyruvate

3PO 3-(3-Pyridinyl)-1-(4-Pyridinyl)-2-Propen-1-one
ALT alanine aminotransferase
AMPK AMP-activated protein kinase
AQ4 alkylaminoanthraquinone
ATF6 activating transcription factor 6
BPH benign prostatic hyperplasia
Br-IPM bromo-isophosphoramide mustard
CA carbonic anhydrase
CHC α-cyano-4-hydroxycinnamate
DCA dichloroacetate
DCE-MRI dynamic contrast enhanced MRI
DW-MRI diffusion-weighted MRI
EGFR epidermal growth factor receptor
EPRI Electron paramagnetic resonance imaging
ER endoplasmic reticulum
ERAD ER-associated degradation
FMISO ^{18}F-fluoromisonidazole
FX11 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid
GAPdH glyceraldehyde-3-phosphate dehydrogenase
GLUT glucose transporters
HIF1α hypoxia inducible factor 1α
HRE hypoxia response element
IEPA imidazole
IR ionizing radiation
IRE1 inositol requiring 1
LDH lactate dehydrogenase
LNA locked nucleic acid
MCT1 monocarboxylate transporter 1
MDR multidrug resistance
MRS magnetic resonance spectroscopy
mTOR mammalian target of rapamycin
PARP1 poly(ADP-ribose) polymerase 1
PDGF platelet-derived growth factor
PDH pyruvate dehydrogenase
PDK pyruvate dehydrogenase kinase
PERK PKR-like ER kinase
PET positron emission tomography
PFK-1 phosphofructokinase-1
pHe extracellular pH
pHi intracellular pH
P_i inorganic phosphate
PK pyruvate kinase
PML promyelocytic leukemia tumor suppressor
pO_2 partial oxygen pressure
RCC renal cell carcinoma
SD stable disease
SSTR4 somatostatin receptor 4

TCA tricarboxylic acid cycle
TMZ temozolomide
TPZ tirapazamine
TSC1/2 tuberous sclerosis protein 1 complex
UPR unfolded protein response
VEGF vascular endothelial growth factor
VHL von Hippel-Lindau

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CHAPTER FIVE

Targeted Therapy for Brain Metastases

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Abstract

The prevention and treatment of brain metastases is an increasingly important challenge in oncology. Improved understanding of the molecular pathogenesis of a number of cancers has led to the development of highly active targeted therapies for patients with specific oncogenic events. Such therapies include EGFR inhibitors for lung cancer, HER2/neu inhibitors for breast cancer, and BRAF inhibitors for melanoma. This review will discuss the development of these targeted therapy approaches, existing data about their role in the management of brain metastasis, and opportunities and challenges for future research in this critical area.

1. INTRODUCTION

In 2010 it was estimated that more than 200,000 cancer patients would be diagnosed with brain metastases (Maher et al., 2009). Historically, brain metastases were often detected in the setting of disease progression at multiple metastatic sites. However, with the development of increasingly effective systemic therapies, brain metastases are now often being diagnosed as one of the initial sites of relapse in patients who are otherwise free of disease, or as the only site of progression while other metastases remained controlled. Such relapses underscore the need to develop a specific understanding of this disease entity as a precursor to the development of potential site-specific therapies. However, the development of systemic therapies for brain metastases is complicated by a number of clinical factors, including the following:

- The presence of the blood–brain barrier, which can reduce the penetration of molecules into the CNS
- Both tumor growth and treatment side effects (i.e., hemorrhage, edema, bystander necrosis) in the CNS can cause significant and rapid decreases in the quality of life

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- Limitations on the ability to sample CSF and/or tumor tissue in order to interrogate pharmacokinetics, pharmacodynamics, and mechanisms of resistance
- The frequent exclusion of patients with brain metastases from clinical trials

Across multiple tumor types, clinical outcomes in patients with brain metastases remain quite poor. The median survival of patients treated with aggressive therapies is generally 4–12 months, which is only a slight increase over the prognosis of 2–3 months that is expected with supportive care alone (Eichler & Loeffler, 2007). The majority of brain metastases are attributable to lung and breast cancer, which is not surprising as these are the two most common tumor types. However, the third most common source of brain metastases is melanoma. Although melanoma is the most aggressive form of skin cancer, it is a relatively uncommon disease; thus, it stands out as a disease in which the risk of CNS metastasis is extremely high. In contrast, colon cancer is extremely common, but it rarely is complicated by the development of brain metastases.

The treatment of many cancers, including lung, breast, and melanoma, has been changed dramatically by the development of personalized targeted therapy approaches (Davies et al., 2006). The successful development of targeted therapies (Table 5.1) depends first upon the identification of an activated target which the tumor cells depend upon. After the identification of such targets, the clinical exploitation of this information depends upon the development of agents that are able to inhibit the target and/or its effectors at clinically tolerable doses. While the initial development of targeted therapy strategies was limited by a relative dearth of therapeutic agents, the current challenge for oncologists is to prioritize agents for testing when there are many candidates available against a given target. As there are growing examples of the critical nature of the degree of target inhibition, differences in pharmacokinetic properties and/or drug delivery methods are critical issues, particularly in the development of systemic therapies for brain metastases.

Table 5.1 Molecular Targets and Targeted Therapies

Cancer	Molecular Target	Agents
Non–small cell lung	*EGFR* mutation EML4-ALK translocation	Gefitinib, erlotinib Crizotinib
Breast cancer	*HER2/neu* amplification	Trastuzumab, lapatinib
Melanoma	*BRAF* mutation	Vemurafenib, dabrafenib
Renal cell carcinoma	*VHL* inactivation	Sorafenib, sunitinib, pazopanib

Finally, while overcoming these challenges has resulted in targeted therapies with dramatic clinical activity in genetically selected patient populations, virtually all patients respond only for a limited period of time before the tumors become resistant. The achievement of durable clinical benefit requires an understanding of the mechanisms that underlie resistance in order to develop rational and effective strategies to prevent and/or overcome them.

As stated above, lung cancer, breast cancer, and melanoma are the three most common causes of brain metastasis. In recent years, oncogenic events have been identified and successfully targeted in each of these tumor types. While the initial clinical development of these agents generally excluded patients with brain metastases, research in this area is accelerating due to the growing appreciation of the need to develop therapies for this disease entity. In order to facilitate future research with these and other new agents, we will summarize existing research regarding the ability of these targeted therapies to prevent and treat brain metastases.

2. LUNG CANCER

2.1. Background

Lung cancer is the leading cause of cancer-related deaths. In 2008, an estimated 2.4 million new cases of lung cancer were diagnosed, and approximately 1.4 million patients died from this disease, worldwide (Jemal, Bray, et al., 2011; Siegel et al., 2011). The majority (~85%) of lung cancers are classified as non–small cell lung cancers (NSCLC), while the remainder are small cell lung cancers (SCLC). NSCLC is further divided into several histologically defined subtypes, including squamous cell, adenocarcinoma, large cell, and mixed histology tumors.

The clinical management of NSCLC is primarily defined by the clinical stage of disease (Ettinger et al., 2010). Patients with localized early-stage tumors (i.e., stages I and II) are treated with surgical resection of the primary tumor, or alternatively radiation therapy (XRT). Adjuvant chemotherapy is appropriate for some of these patients. Patients with locally advanced disease (stage IIIA/B) may be treated with surgery as the primary modality with adjuvant treatment, but often are managed with a combination of chemotherapy and radiation. For patients with stage IV disease, chemotherapy is the standard of care for most patients, generally with platinum-containing regimens. Recently, oncogenic events have been identified in NSCLC that

have rapidly led to development and approval of targeted therapies for this aggressive disease.

2.2. EGFR

2.2.1. Mutations in EGFR

The epidermal growth factor receptor (EGFR; also called HER1, or ErbB1) is a receptor tyrosine kinase. In normal cells, the binding of various ligands to the extracellular domain of the EGFR results in formation of catalytically activated homo- and heterodimers of the molecule. These active dimers undergo autophosphorylation of key residues that can be used as markers of EGFR activity. The activated EGFR also phosphorylates a variety of substrates to activate several important intracellular signaling cascades, including the RAS-RAF-MEK-ERK, PI3K-AKT, JAK-STAT, SRC, and FAK pathways. Through these and other substrates, activation of the EGFR contributes to the growth and survival of many types of cancer, including NSCLC. Protein-based studies have demonstrated that >50% of NSCLC show evidence of EGFR activation. However, the development of effective targeted therapy strategies has been closely tied to the identification of genetic abnormalities in the *EGFR* gene.

Gefitinib is a small molecule inhibitor of the EGFR. In early phase clinical testing, treatment with gefitinib produced dramatic clinical responses and benefit in a small number of NSCLC patients. Detailed molecular analysis of these patients led to the discovery that most of the responding patients had somatic mutations in the *EGFR* gene (Lynch, 2004; Paez, 2004). Subsequent studies have found that *EGFR* mutations are associated with several clinical features of NSCLC patients, including adenocarcinoma histology, being a nonsmoker, and female gender (Shigematsu et al., 2005). The rates of *EGFR* mutations also vary by ethnicity, with a prevalence of 30–50% in NSCLC patients in Eastern Asia, as compared to ~10% in North America and Europe. The most common mutations in *EGFR* are the L858R substitution in exon 21 and short deletions in exon 19, which together represent ~90% of the mutations reported in NSCLC patients. The L858R mutation affects the tyrosine kinase activation loop, while the deletions in exon 19 affect the region of the ATP-binding site of the catalytic domain of the protein. The deletions in exon 19 result in structural changes that increase the binding affinity of small molecule EGFR inhibitors. Multiple studies have demonstrated that the presence of the L858R substitution or exon 19 deletions correlate with increased clinical benefit

from small molecule inhibitors of the EGFR kinase domain; however, a similar correlation is not seen with mutations in exons 18 and 20, which represent the remaining 10% of reported *EGFR* mutations.

2.2.2. EGFR: Targeted Therapies

Gefitinib was the first EGFR tyrosine kinase inhibitor (TKI) to demonstrate clinical efficacy in patients. It gained initial conditional approval from the United States FDA for the treatment of NSCLC based on promising results in phase I and phase II clinical trials (Fukuoka et al., 2003; Ranson et al., 2002; Twombly, 2002). However, it failed to improve outcomes significantly in a randomized phase III trial (Thatcher et al., 2005). Its use in the United States was subsequently restricted to those patients who were already taking and receiving benefit from gefitinib, or in clinical trials (Blackhall et al., 2006). However, gefitinib continues to be used in other countries in Asia and Europe for patients with EGFR mutations. Erlotinib is a more potent small molecule inhibitor of EGFR's kinase activity (Wang et al., 2012).

Erlotinib is a structurally unrelated EGFR TKI. Erlotinib gained regulatory approval as a result of the BR.21 trial, which demonstrated that the use of the agent in previously treated stage IIIB and IV NSCLC patients resulted in statistically significant improvements in overall response rate (8.9% vs. <1%), median progression-free survival (2.2 vs. 1.8 months) and overall survival (6.7 vs. 4.7 months) (Shepherd et al., 2005). Increased clinical responsiveness and duration of disease control in the BR.21 trial correlated with adenocarcinoma histology, female gender, a history of being a never-smoker, and Asian ethnicity. Univariate analysis of the correlation of survival to the molecular characteristics of the patients enrolled in the trial also demonstrated significant benefit for erlotinib treatment in patients with any expression of the EGFR (by immunohistochemistry) or amplification or polysomy of the *EGFR* gene, which were detected in 57% (184 of 327) and 44% (56 of 127) of the evaluable patients (Tsao et al., 2005). Mutations in *EGFR* were detected in 23% of the patients, but only 47% of those were L858R or exon 19 deletions. Interestingly, neither the presence of those common mutations nor "any" *EGFR* mutation correlated significantly with clinical benefit from erlotinib, although nonsignificant trends for benefit were observed for patients with both classes of mutations. On multivariate analysis, only the correlation of EGFR expression with response rate remained significant among the molecular correlates. More recently, a randomized phase III trial in previously untreated NSCLC patients with either L858R or exon 19 deletion mutations in *EGFR* demonstrated that

treatment with erlotinib resulted in significant improvements in overall response rate (83% vs. 36%) and progression-free survival (median 13.1 months vs. 4.6 months) as compared to treatment with carboplatin and gemcitabine (Zhou et al., 2011).

Although EGFR inhibitors generally result in dramatic responses in patients with exon 19 deletions and L858R mutations, these responses are almost always transient, with most patients developing resistance within 1 year of the start of treatment. The development of resistance to EGFR inhibitors is frequently associated with the development of secondary mutation in *EGFR*, most commonly T790M, which accounts for ~50% of the secondary resistance mutations. Short insertions in exon 20 of *EGFR* have also been identified at the time of resistance. In addition to mutations affecting *EGFR*, resistance may be caused by the amplification of the *c-MET* gene, or by mutations in *KRAS* (Engelman et al., 2007; Kobayashi et al., 2005).

In addition to TKIs, the EGFR can be inhibited by blocking antibodies. Cetuximab is a chimeric monoclonal antibody that has gained FDA approval in both colon cancer and head and neck squamous cell carcinoma (HNSCC). The addition of cetuximab to chemotherapy has been shown to slightly improve clinical response rates and overall survival in phase III clinical trials in metastatic NSCLC patients (Lynch et al., 2010; Pirker et al., 2009). Interestingly, there does not appear to be a correlation between the presence of activating EGFR mutations and clinical benefit from cetuximab (Khambata-Ford et al., 2010), but there appears to be a correlation with increased expression of EGFR (Pirker et al., 2012). Cetuximab also failed to induce any clinical responses in a cohort of patients with activating mutations who had progressed on EGFR TKI therapy (Neal et al., 2010).

2.2.3. EGFR and Brain Metastasis

It is estimated that 20–40% of patients with NSCLC will eventually develop brain involvement (Ceresoli, 2012). Similar to other diseases, the outcomes in these patients are quite poor. Patients treated with supportive care generally have a median survival of 3 months or less, which is improved only slightly (4–6 months) with active interventions. The impressive clinical activity of EGFR kinase inhibitors supported the rationale to investigate the role of this target in brain metastases from NSCLC. One potential confounder in the development of this clinical approach is the possibility of discordance in *EGFR* mutation status between primary tumors and metastases. One study ($n = 336$) of primary tumors and metastases identified

a discordance rate of 22.5% for *KRAS* mutations, and 32.5% for *EGFR* copy number analysis (by FISH), but no *EGFR* mutations were identified in any of the tumors included in the study (Monaco et al., 2010). A smaller study of 25 patients in which 5 patients were identified with *EGFR* mutations in their primary tumors showed a complete lack of concordance with the metastases in that study (Kalikaki et al., 2008). The finding, however, contrasts with more recent studies that have demonstrated continued presence of the same *EGFR* mutation in progressing metastases that was originally identified in the primary tumors of patients, and with the general pattern of relatively uniform responses to EGFR inhibitors. Limited data is available specifically for NSCLC brain metastases, but the data that exists suggests high concordance. One study of 19 patients with resected NSCLC brain metastases detected mutations in 12 (63%) of the brain tumors. The same mutation was identified in the primary tumors from the six patients with materials available for testing, and exact concordance was also detected in two patients in whom a second brain metastasis had also been resected (Matsumoto et al., 2006). A larger study of 55 patients with matching primary tumors and brain metastases evaluated *EGFR* mutation status, copy number, and protein expression (Sun et al., 2009). Only 1 of the 42 patients evaluable for mutations in both lesions had an *EGFR* mutation, which was identical in the primary tumor and the brain metastasis. *EGFR* copy number showed a relatively high concordance of 84% overall, which was slightly higher for synchronous lesions (11/11, 100%) than for metachronous primaries and metastases (34/44, 77%). While there was no significant difference in total EGFR protein expression levels (IHC) between the matching tumors, the brain metastases did demonstrate higher expression of phosphorylated (activated) EGFR ($p < 0.0001$).

The high rate of *EGFR* mutations identified in the cohort of 19 NSCLC brain metastases described above raised the question of whether this genetic event correlates with an increased risk of brain metastasis. A retrospective study of 117 NSCLC adenocarcinoma patients in Korea reported that the presence of a mutation in the *EGFR* correlated with a lower risk of disease recurrence (Lee et al., 2009). However, the patients with *EGFR* mutations demonstrated a trend for increased risk of having isolated brain metastasis as the first site of disease recurrence after surgery (24% vs. 9%, $p = 0.15$).

Retrospective studies have also tried to address if treatment with EGFR TKIs can reduce the risk of brain metastasis. A study of 100 NSCLC patients with *EGFR* mutations (51% exon 19 deletion, 33% L858R) who were treated with either erlotinib or gefitinib as their first systemic therapy examined the risk

and timing of brain metastasis formation (Heon et al., 2010). Nineteen of the patients had brain metastases diagnosed prior to the EGFR TKI treatment, seventeen of whom received CNS-directed therapies. The median time to any disease progression for the full cohort of patients was 13.1 months. After a median follow-up of 42 months, 28% of the patients had developed new brain metastases, or progression of existing metastases; the median time to new brain metastasis or progression was 19 months. Among the patients with no evidence of brain involvement at the start of treatment ($n = 81$), the 1- and 2-year risk of CNS involvement was 6% and 13%, which compares favorably to the rates reported in unselected patients treated with chemotherapy(Ceresoli et al., 2002) . In the *EGFR*-mutant patients with preexisting brain metastases, the rates were 11% and 47%, respectively. Ten patients in the trial had CNS involvement as one of their initial sites of disease progression, with five patients having the brain as their only site of progression. Underscoring the clinical significance of failure in the CNS, while the median survival for the full cohort from the start of EGFR inhibitors was 33.1 months, the median survival after the diagnosis of brain involvement was just over 5 months. There was a significant difference in the rate of progression in the brain based on the specific *EGFR* mutation that was present. Patients with the L858R metastasis had the lowest 2-year rate of brain metastasis (3%), whereas higher rates were observed with exon 19 deletions [21%, Hazard Ratio (HR) 2.7] or any other mutation (38%, HR 5.7).

Several trials have also tested the efficacy of EGFR inhibitors in patients with parenchymal brain metastases, and support the specific benefit of these agents in patients with *EGFR* mutations. A retrospective study of 69 patients with NSCLC brain metastases treated with erlotinib reported a response rate of 82.4% in the patients with *EGFR* mutations ($n = 17$) and 0% in those with a wild-type or unknown ($n = 52$) gene sequence. Significant differences in progression-free (median 11.7 vs. 5.8 months) and overall survival (12.9 vs. 3.1 months) were also observed (Porta et al., 2011). Recently, a prospective study of the efficacy of EGFR inhibitors in NSCLC patients with previously untreated brain metastases and confirmed mutations in exons 19 or 21 in *EGFR* has been reported in abstract form (Kim, Flaherty et al., 2011; Kim, Kim, et al., 2011; Kim, Lee, et al., 2011). All patients were treated with either gefitinib ($n = 17$) or erlotinib ($n = 6$). The overall response rate was 70%. The median progression-free survival was 6.6 months, with a significant difference observed between patients with exon 19 (14 months) vs. exon 21 (4.6 months, $p = 0.03$) mutations, although there was no significant difference in overall survival between the two groups of patients.

There is mixed data about the mechanisms of resistance to EGFR inhibitors in NSCLC brain metastases. It is possible that resistance may emerge due to incomplete inhibition of the EGFR, due to decreased penetration of the blood–brain barrier. Comparative analysis of blood and CSF levels of erlotinib in four patients with CNS metastases demonstrated ~5% penetration of the drug into the CSF (Togashi et al., 2010), which is approximately twice the penetration that has been reported with gefitinib (Jamal-Hanjani & Spicer, 2011). Individual case reports also support this hypothesis. For example, the case of a patient with a known exon 19 deletion in *EGFR* has been described (Jackman et al., 2006). This patient had a cell line established from a metastasis that had an IC_{50} of 10–50 nmol for gefitinib *in vitro*. The patient initially had a good response to combined treatment with paclitaxel, carboplatin, and gefitinib, which was given at a dose of 250 mg daily. Unfortunately, the patient developed new brain metastases despite continued control of extracranial disease. The patient was treated with whole-brain XRT in combination with gefitinib at the same dose. The patient had progressive neurologic symptoms, and imaging demonstrated new leptomeningeal involvement, which was confirmed by CSF cytology. The patient's chemotherapy regimen was changed, and the dose of gefitinib was increased to 500 mg daily. In the setting of persistent leptomeningeal involvement and worsening symptoms, the levels of gefitinib in the CSF were measured, and revealed that the levels (6.2 nM on one sampling, 18 nM on another) were below the concentrations required to inhibit the growth of the cell line that had been established from the patient's tumor. The patient was subsequently treated with single-agent gefitinib, initially at a dose of 750 mg daily, then increased to 1000 mg daily. With this increase, the detected levels of gefitinib in the CSF exceeded 40 nM; concurrently, cytological examination of CSF revealed an absence of malignant cells, and the patient's clinical condition and radiographic findings improved markedly. Eventually, the dose of gefitinib was reduced to 500 mg daily due to side effects; subsequently, the CSF cytology reverted to positive. The dose of gefitinib was increased again, but the patient continued to deteriorate, and eventually died. Postmortem sampling of the patient's progressing metastases from the lung, liver, and intestines demonstrated the presence of a secondary *EGFR*-T790M mutation, but this mutation was not present in the DNA from the sampled brain metastasis (which still retained the initial exon 19 deletion). Another patient has been reported with a similar discordance, with a T790M mutation identified in a progressing extracranial metastasis but

not in the brain metastasis from the same patient (Balak et al., 2006). However, other cases have been reported in which mutations typically associated with secondary resistance to EGFR inhibitors have been identified in resected progressing brain metastases (Heon et al., 2010).

2.3. EML4-ALK

Another promising target in the management of NSCLC is in the EML4-ALK fusion protein. This protein results from an inversion event on chromosome 2, which was initially reported in 2007 (Soda et al., 2007). This chromosomal abnormality results in a fusion of the *EML4* (echinoderm microtubule-associated protein-like 4) and *ALK* (anaplastic lymphoma kinase) genes to produce a novel protein that is constitutively active and oncogenic. This genetic event is detected in approximately 4% of NSCLCs, and it is essentially mutually exclusive with activating *EGFR* and *KRAS* mutations. Clinically, the EML4-ALK fusion is associated with younger age, male gender, never or light smoking history, and adenocarcinoma histology.

Crizotinib is a small molecule inhibitor of ALK and c-MET tyrosine kinases. In preclinical studies, crizotinib demonstrated selective inhibitory activity in cell lines with activating alterations in the *ALK* gene (McDermott et al., 2008). In a phase I clinical trial of 82 patients with *ALK* rearrangements, treatment with crizotinib resulted in a clinical response rate of 57%, a disease control rate of 87% at 2 months, and an estimated 6-month progression-free survival rate of 72% (Kwak et al., 2010). While this trial did include a substantial number of patients with brain metastases, all of those lesions were treated with CNS-directed therapies (i.e., whole-brain XRT) prior to the start of crizotinib, and to date the rate of intracranial responses have not been reported (Shaw et al., 2011). However, a meeting abstract has reported that among sixteen patients who were continued on crizotinib after initial progression, four (25%) had evidence of progression in the CNS, including three patients with progression in the CNS alone (Camidge et al., 2011). Additional reports are expected in the near future to further address the potential role of crizotinib in the treatment of NSCLC brain metastases with the EML4-ALK fusion.

3. BREAST CANCER

The most common cancer in women is breast cancer. In 2008, an estimated 1.38 million cases of breast cancer were diagnosed in women

worldwide, which represented 23% of newly diagnosed cancer in women that year, and 458,400 women died from this disease (Siegel et al., 2011). Breast cancer historically has been grouped into three categories: hormone receptor positive (HRBC), HER2/neu amplified (HER2), and "triple negative" (TNBC) breast cancer (no expression of estrogen or progesterone receptors and no amplification of *HER2/neu*). Approximately 70% of breast cancers express the estrogen receptor (ER) and/or the progesterone receptor (PR), making HRBC the most common form of breast cancer. Patients with localized or locally advanced breast cancer are treated with surgical resection, generally with adjuvant therapies used to reduce the risk of disease recurrence, including XRT, chemotherapy, and/or antiestrogens (Carlson et al., 2009). Antiestrogens are generally the primary therapeutic modality used if the cancer recurs systemically. As a class, HRBC have a relatively good prognosis. TNBC, which make up ~10% of patients, are managed similarly, with the exception that there is no role for antiestrogens. TNBC have a worse prognosis than the HRBC. HER2 cancers represent a subset of tumors that historically had a very poor prognosis. However, the natural history of this tumor subtype has changed with the development of effective targeted therapies against HER2.

3.1. HER2

3.1.1. HER2/neu Amplification

The HER2 breast cancers (~20%) are characterized by amplification of the *HER2/neu* gene, which is also known as ErbB2. Like EGFR, HER2 is a member of the ErbB family of cell surface receptors. In contrast to the EGFR, the HER2 protein does not have a catalytic kinase domain. HER2 triggers intracellular signaling by forming heterodimers with other ErbB family members that have catalytic activity. Activation of HER2 results in activation of multiple intracellular kinase signaling cascades, including the RAS-RAF-MEK-ERK and PI3K-AKT pathways. The diagnosis of HER2 breast cancer may be made either by demonstration of 3+ staining intensity by certified immunohistochemistry, or by FISH assay that demonstrates 6 or more copies of the *HER2/neu* gene per cell, or a ratio of *HER2/neu* to chromosome 17 greater than 2.2. Tumors with IHC scores of 0 or 1+ may be regarded as negative for HER2; those with 2+ IHC require FISH analysis for classification. Similarly, *HER2/neu* copy number of ≤ 4 or *HER2/neu*:chromosome 17 ratio of less than 1.8 are considered negative; tumors with 4–6 copies or a ratio of 1.8:2.2 are considered borderline (Carlson et al., 2006).

3.1.2. HER2/neu: Targeted Therapies

Trastuzumab is a humanized murine IgG monoclonal antibody that binds to the extracellular domain of HER2. Trastuzumab has limited activity as a single agent in breast cancer. However, it has demonstrated significant clinical benefit when combined with chemotherapy. This was demonstrated initially in women with metastatic breast cancer with high expression of HER2 protein or amplification of the *HER2/neu* gene. The addition of trastuzumab to chemotherapy significantly improved clinical response rates (50% vs. 32%), time to progression (median 7.4 vs. 4.6 months), and survival (median 25.1 vs. 20 months) (Slamon et al., 2001). The most significant toxicity of trastuzumab is decreased cardiac function, which is generally reversible. Importantly, analysis of the clinical outcomes with trastuzumab supports that the clinical benefit is largely restricted to patients with increased copies of the *HER2/neu* gene compared to those with increased protein expression but normal gene copy number (Mass et al., 2005). Subsequent clinical trials demonstrated that trastuzumab is also a remarkably effective adjuvant therapy when given in combination with chemotherapy in women with locally advanced breast cancer, achieving an approximately 50% reduction in events (recurrent cancer, second primary cancer, or death) in three large clinical trials (Piccart-Gebhart et al., 2005; Romond et al., 2005).

Lapatinib is a small molecular TKI that inhibits both HER2 and the EGFR. Similar to trastuzumab, lapatinib has demonstrated significant activity in combination with chemotherapy. Lapatinib gained regulatory approval based on demonstrated efficacy when combined with the chemotherapy agent capecitabine in HER2-positive breast cancer patients who had progressed on a trastuzumab-containing regimen (Cameron et al., 2008; Geyer et al., 2006). The addition of lapatinib resulted in significant improvements in the clinical response rate (24% vs. 14%) and progression-free survival (median 6.2 vs. 4.3 months), and a trend for improved overall survival (HR 0.78, $p = 0.18$). Analysis of progression-free survival in this trial demonstrated a significant benefit for lapatinib in patients with *HER2/neu* gene amplification by FISH (HR 0.47, $p < 0.0001$), but not in patients with diploid gene copy number (HR 0.89, $p = 0.79$) (Cameron et al., 2008). There was no significant correlation between EGFR expression levels and progression-free survival. Of note, single-agent lapatinib therapy (without chemotherapy) in patients previously treated with trastuzumab is only 5% (Blackwell et al., 2009).

3.1.3. HER2/neu and Brain Metastasis

The overall risk of brain metastasis in patients with advanced breast cancer is 10–15%. This estimate is largely based on the rate of symptomatic brain metastasis, but up to 30% involvement has been reported in autopsy series (Lin & Winer, 2007). A number of clinical characteristics correlate with an increased risk of brain metastasis, including younger age and African American ethnicity. Multiple lines of evidence also link HER2 to brain metastasis.

Early retrospective trials in the trastuzumab era reported that 25–40% of advanced HER2 breast cancer patient developed brain metastasis (Lin & Winer, 2007). In addition to these rates of symptom-based detection, one study in which asymptomatic HER2 patients were screened identified brain involvement in 34% (Niwinska et al., 2007). Supporting a causative role for HER2 in brain metastasis formation, preclinical studies demonstrated that enforced expression of HER2 in a human breast cancer cell line resulted in the formation of larger brain metastases in a mouse model (Palmieri et al., 2007). This study also found that the levels of HER2 mRNA in brain metastases were five times as high as the levels in HER2-amplified primary tumors. More recently, a quantitative analysis of HER2 protein expression in trastuzumab-treated breast cancer patients reported that increased HER2 expression significantly correlated with decreased time to brain metastasis (Duchnowska et al., 2012). Finally, a retrospective study of breast cancer patients enrolled in adjuvant therapy trials before the development of trastuzumab found that the HER2 patients had a significantly higher 10-year incidence of CNS metastasis (6.8% vs. 3.5%, $p < 0.01$) (Pestalozzi et al., 2006).

The high rate of brain metastasis observed in contemporary HER2 breast cancer patients is also likely due in part to the fact that the brain is a sanctuary site for tumor growth in patients treated with trastuzumab. The size of the trastuzumab antibody structure results in poor penetration of the blood–brain barrier. Analysis of CSF in HER2 breast cancer patients with brain metastases demonstrated that the ratio of trastuzumab levels in the serum to the CSF was 420:1 (Stemmler et al., 2007). Treatment with XRT improved penetration somewhat (76:1), and penetration was higher in patients with concomitant leptomeningeal involvement (49:1). The lack of efficacy of trastuzumab to specifically prevent brain metastasis is supported by the results of the NSABP B-31 adjuvant therapy trial, which demonstrated that the overall rate of CNS metastases did not differ between the patients who were

or were not treated with trastuzumab ($p = 0.35$), despite the fact that trastuzumab markedly reduced the overall event rate (Lin & Winer, 2007).

There are reports that have described successful treatment of leptomeningeal disease from HER2 breast cancer by the intrathecal delivery of trastuzumab (Oliveira et al., 2011; Perissinotti & Reeves, 2010; Stemmler et al., 2006). However, most efforts in the development of treatments for HER2 breast cancer brain metastasis have focused on lapatinib, which has a much smaller molecular weight (<1 kDa). In the initial study that demonstrated the efficacy of lapatinib in HER2 breast cancer patients who had previously progressed on trastuzumab-containing regimens, there was a decreased incidence of the brain as a site of initial progression on the lapatinib arm ($p = 0.045$). Larger studies are ongoing to determine if initial treatment of metastatic HER2 breast cancer with lapatinib will reduce the risk of initial relapse in the CNS (Metro & Fabi, 2012). Two phase II trials have been reported studying the efficacy of single-agent lapatinib in HER2 patients previously treated with trastuzumab and brain XRT. The trials reported relatively modest response rates of 2.5% and 6%, although one study reported a response rate of 20% in patients who entered an optional extension phase of combined treatment with lapatinib and capecitabine (Lin et al., 2008; Lin et al., 2009). A randomized trial comparing the efficacy of lapatinib in combination with capecitabine or with topotecan in HER2 breast cancer patients with progressive CNS disease after trastuzumab and XRT was stopped early due to significant toxicity with the topotecan combination, which also failed to achieve any clinical responses. A promising clinical response rate of 38% was observed with the combination of lapatinib and capecitabine (Lin et al., 2011).

4. MELANOMA

Over 3 million skin cancers are diagnosed every year. The most common skin cancers are basal cell carcinomas and squamous cell carcinomas, which together represent more than 90% of cases. Melanoma is the third most common skin cancer, and will be diagnosed in an estimated 70,230 patients in the United States in 2012 (Siegel et al., 2011). While melanoma represents less than 5% of the skin cancers that are diagnosed, it is the cause of more than 70% of skin cancer related deaths. The age-adjusted incidence of melanoma increased more than 200% from 1975 to 2008, accompanied by a 60% increase in annual mortality (Hall et al., 1999; Howe et al., 2001;

Jemal, Saraiya, et al., 2011). The clinical significance of melanoma is underscored by the fact that it has one of the highest life-years lost per fatality due to the fact that many of the patients who die from this disease are young and otherwise healthy (Burnet et al., 2005; Ekwueme et al., 2011).

Most melanomas arise on the skin, and are referred to as cutaneous melanomas. Extensive evidence supports an important causative role for ultraviolet radiation (UVR) exposure in these tumors. However, the role of UVR is less certain in other melanoma subtypes. Acral lentiginous melanomas arise from the skin on the palms of the hands, the soles of the feet, or under nailbeds, and thus are relatively protected from UVR. Mucosal melanomas arise from melanocytes on mucosal surfaces throughout the body, including the digestive and reproductive tracts, where there is no UVR exposure. Melanomas may also arise from melanocytes in the eye, and are referred to as uveal melanomas. Interestingly, molecular analyses have demonstrated that these anatomically defined subtypes are characterized by distinct patterns of DNA mutations, amplifications, and deletions (Curtin et al., 2005; Davies & Gershenwald, 2010).

The mainstay of treatment for primary melanomas remains surgery (Coit et al., 2009). Factors that predict a higher risk of recurrence of disease include increased tumor thickness, tumor ulceration, and increased mitotic rate of the primary tumor (Balch et al., 2009). For patients with regional lymph node metastases, the risk of relapse increases with increasing numbers of lymph nodes involved, and the degree of tumor burden. In addition to surgery, adjuvant treatment with high-dose interferon is approved for use in these patients. However, adjuvant interferon, which is given for 12 months and produces flu-like symptoms among other side effects, appears to have no significant impact on overall survival, and appears to prevent or delay relapse in a relatively small percentage of patients (Kirkwood et al., 2004). Patients with distant metastases from melanoma have a median overall survival of approximately 8 months. Clinical trials have demonstrated that cytotoxic chemotherapies are largely ineffective in this disease, with the only FDA-approved agent, dacarbazine (DTIC), achieving clinical responses in less than 10% of patients (Boyle, 2011; Tsao et al., 2004). As a result, other therapeutic approaches have been tested extensively in this disease, particularly immunotherapy. High dose bolus interleukin-2 (HD IL-2) was the first immunotherapy approved by the FDA for use in patients with stage IV melanoma. HD IL-2 has a modest response rate of ~15%, but it was approved because the majority of patients who achieve complete responses (~6%) remain free of disease durably (Atkins et al., 2000; Atkins et al., 1999). However, HD IL-2 is

an extremely toxic therapy that requires intensive care unit–level monitoring during administration, and it resulted in treatment-related deaths in approximately 2% of patients in early trials (Atkins et al., 1999; Phan et al., 2001). More recently, ipilimumab, a monoclonal antibody that stimulates the antitumor immune response by blocking the inhibitory CTLA-4 molecule on the surface of T-cells, was approved for use in advanced melanoma on the basis of two phase III trials that showed improvements in overall survival vs. standard therapies (Hodi et al., 2010; Wolchok et al., 2010). Ipilimumab achieves clinical responses in only ~10% of patients, and the responses sometime occur after initial progression, but clinical trials demonstrate 3- and 4- year disease control in 25–30% of patients.

4.1. BRAF

4.1.1. BRAF Mutations

BRAF is a serine-threonine kinase in the RAS-RAF-MEK-ERK signaling pathway. In 2001, a focused analysis of sequencing abnormalities in the genes encoding the RAF kinases identified frequent point mutations in the *BRAF* gene in melanoma, and at lower frequency in colon, thyroid, and ovarian cancers (Davies et al., 2002). Subsequent analyses have demonstrated that point mutations in *BRAF* are the most common somatic mutations in melanoma. The prevalence of *BRAF* mutations in cutaneous melanomas without chronic sun damage, which are the most common type, is ~45% (Hocker & Tsao, 2007; Jakob et al., in press). The prevalence of *BRAF* mutations is lower in cutaneous melanomas with chronic sun damage (~30%), acral lentiginous melanomas (10–15%), and mucosal melanomas (~5%), and they are not detected in uveal melanomas (Curtin et al., 2005; Hocker & Tsao, 2007; Woodman et al., 2012). Over 90% of the mutations in the *BRAF* gene detected in cancer result in substitutions of the valine at the 600 position, most frequently by glutamic acid (V600E, ~70% of *BRAF* mutations) or lysine (V600K, ~20%) (Hocker & Tsao, 2007; Jakob et al., 2011; Long et al., 2011). The V600 mutations of BRAF increase the catalytic activity of the protein 50- to > 200-fold, and result in constitutive activation of the pathway effectors MEK and ERK (Karasarides et al., 2004; Wan et al., 2004).

4.1.2. BRAF-Targeted Therapies

The high prevalence of activating *BRAF* mutations in melanoma suggested that inhibition of the RAS-RAF-MEK-ERK signaling pathway could be

an effective therapeutic strategy for the chemotherapy-resistant disease. Initial preclinical studies demonstrated that genetic inhibition of BRAF in melanoma cells with activating *BRAF* mutations inhibited tumor growth and survival (Hingorani et al., 2003). Similar inhibition was demonstrated in preclinical models with sorafenib, a multikinase small molecular inhibitor (Karasarides et al., 2004). Sorafenib was able to able to inhibit both wild-type and V600-mutant BRAF proteins, although it actually had higher affinity for several other kinases, including CRAF, VEGFR2, FLT-2, and c-KIT (Strumberg, 2005). Clinical testing of sorafenib in melanoma yielded disappointing results, with clinical responses observed in less than 5% of patients with single-agent therapy (Eisen et al., 2006). More promising results were seen in a phase I trial of the combination of sorafenib, paclitaxel, and carboplatin; however, a phase III trial demonstrated that sorafenib did not add any significant clinical benefit to the chemotherapy combination (Flaherty et al., 2008; Hauschild et al., 2009). Combined with the finding that most benign nevi, which have almost no malignant potential, have the same activating *BRAF* mutation that was found in the tumors (Pollock et al., 2003), the therapeutic value of BRAF became questionable.

The importance and clinical potential of BRAF in melanoma has now been demonstrated definitively by the development of second-generation, selective BRAF inhibitors. The most well-characterized of these agents is vemurafenib. Vemurafenib is a highly potent and selective inhibitor of V600-mutant BRAF proteins; its molecular IC_{50} for these proteins is ~10-fold lower than it is for wild-type BRAF protein, and >1000 lower than its affinity for most other kinases (Tsai et al., 2008). Vemurafenib inhibits the growth and survival of melanoma cells with activating *BRAF* mutations *in vitro*, and caused the regression of xenografts in animal models (Yang et al., 2010). Interestingly, treatment of melanoma cell lines with a wild-type *BRAF* gene results in hyperactivation of MEK and ERK signaling, and increased growth of tumor cells *in vitro* and *in vivo* (Halaban et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). In the phase I trial of vemurafenib in patients with metastatic melanoma, 81% of patients with a *BRAF V600E* mutation achieved unconfirmed clinical responses, while none of the five patients with a wild-type gene responded (Flaherty et al., 2010). Further clinical testing with vemurafenib has been limited to patients with activating *BRAF* mutations. The BRIM-3 phase III trial of patients with *BRAF V600E* mutations demonstrated that vemurafenib significantly improved clinical response rates (48% vs. 5%), progression-free survival, and

overall survival compared to dacarbazine, leading to FDA approval (Chapman et al., 2011). Comparable response rates of 50–70% have been observed with dabrafenib, a structurally unrelated selective inhibitor of V600-mutant BRAF proteins, in metastatic melanoma patients with activating *BRAF* mutations (Kefford et al., 2010; Trefzer et al., 2011).

While the clinical responses with vemurafenib and dabrafenib have been dramatic and impressive, unfortunately they are generally short-lived. In the phase I trial of vemurafenib, the median duration of clinical responses was approximately 7 months, which is very similar to early observations with dabrafinib (Flaherty, et al., 2010; Trefzer, et al., 2011). A number of mechanisms of secondary resistance to the selective BRAF inhibitors have been uncovered. Several of the alterations that have been detected result in reactivation of MEK and ERK signaling, including alternative splicing of the *BRAF* gene, concurrent mutations in *NRAS* or *MEK*, or increased expression of the kinase COT (Johannessen et al., 2010; Nazarian et al., 2010; Poulikakos et al., 2011; Solit & Rosen, 2011; Wagle et al., 2011). Alternatively, resistance can occur despite continued inhibition of MEK and ERK, but with molecular changes that activate other signaling pathways, particularly the PI3K-AKT network (Deng et al., 2012; Nazarian, et al., 2010; Villanueva et al., 2010; Xing et al., 2012). Interestingly, there is also evidence to support that activation of the RAS-RAF-MEK-ERK signaling pathway in melanoma cells regulates the ability of the immune system to recognize and respond to the tumor cells (Boni et al., 2010; Wilmott et al., 2011). Thus, combinatorial approaches of BRAF inhibitors with both targeted therapies and immunotherapies are currently being investigated as strategies to build upon the single-agent activity of these agents.

4.1.3. BRAF and Brain Metastasis

Brain metastasis is one of the most frequent complications of advanced melanoma, and one of the leading causes of death from this disease (Budman et al., 1978). Up to 60% of patients with metastatic melanoma will develop CNS involvement at some time during the course of their disease, and even higher rates of involvement have been reported in autopsy series (Sawaya et al., 2001; Sloan et al., 2009). The median survival from the diagnosis of melanoma brain metastasis is approximately 4 months (Davies et al., 2011; Raizer et al., 2008; Sloan, et al., 2009). A small proportion of patients who are diagnosed with resectable (i.e., <4) brain metastases, without concurrent extracranial metastases, may be long-term survivors with surgical removal of

the tumors (Sampson et al., 1998). However, most patients present with more extensive involvement. Whole-brain XRT may provide palliative benefit, but has minimal impact on survival. Temozolomide, a systemic chemotherapy which penetrates the blood–brain barrier and has the same active metabolite as dacarbazine, is frequently used in patients with brain metastases, but it achieves clinical responses in only ~5% of these patients (Agarwala et al., 2004). HD IL-2 is generally not used in patients with brain metastases, both due to concerns about intracerebral edema, and because early studies failed to demonstrate responses with HD IL-2 in patients with CNS involvement (Phan et al., 2001). Two of twelve patients with stable brain metastases treated with ipilimumab in a phase II trial achieved clinical responses in the brain, and both patients were still alive after 4 years (Weber et al., 2011) (Table 5.1).

A retrospective analysis of patients with stage IV melanoma who had been tested for activating mutations in *BRAF* and *NRAS*, which is the second most common (~20%) somatic mutation in this disease, found that patients with either mutation were approximately twice as likely to have evidence of CNS involvement at the time of diagnosis of distant metastases as patients who have normal copies of both of these genes (Jakob et al., in press). However, no prospective studies have evaluated this correlation to date. Little data is available at this time regarding the efficacy of vemurafenib in melanoma patients with brain metastases, as evidence of brain involvement was an exclusion criteria in the initial trials with that agent, although an impressive case report has been published (Fig. 5.1) (Rochet et al., 2011). An analysis of patients treated on the phase I trial of vemurafenib found that 25% of the patients had CNS involvement at the time of initial progression; 18% had progression in the brain only (Kim, Flaherty et al., 2011; Kim, Kim, et al., 2011; Kim, Lee, et al., 2011). A total of 10 patients with untreated, asymptomatic brain metastases were included in the phase I clinical trial of dabrafenib. Three of the patients had complete responses, and five had partial responses, for an impressive overall response rate of 80% (Long et al., 2010). Based on these promising results, a phase II trial of ~150 melanoma patients with brain metastases and documented *BRAF* mutation has recently completed accrual, and results are expected to be presented in the near future. A clinical trial testing the efficacy of vemurafenib in a cohort of BRAF-mutant melanoma patients with brain metastases is also ongoing. Similar to the experience with extracranial metastases, most of the patients with intracranial responses to BRAF inhibitors have gone on to develop resistance within a year. However, at this time there is no published

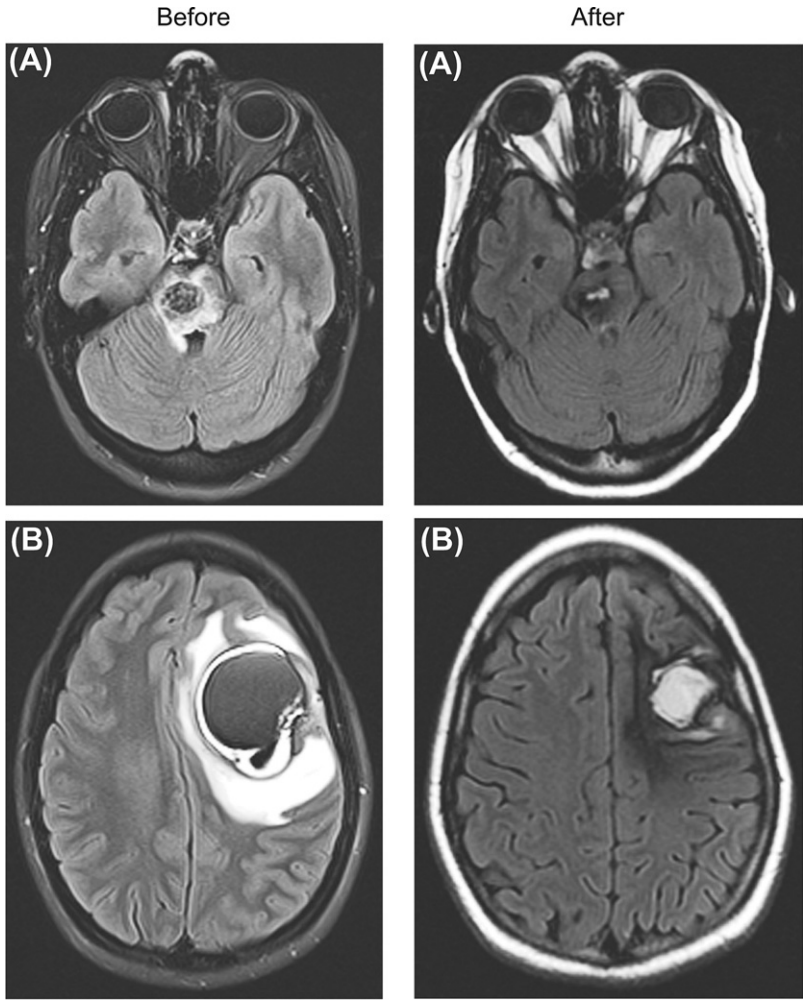

Figure 5.1 Clinical response of *BRAF*-mutant melanoma brain metastases with vemurafenib therapy. *Left:* baseline MRI of the brain showing symptomatic, progressive brain metastases following previous stereotactic radiosurgery. *Right*: MRI of the brain following treatment with vemurafenib for 6 months. (*Reprinted with permission from* The New England Journal of Medicine *(Rochet et al., 2011)).*

information about the potential mechanisms underlying the progression of CNS metastases.

4.1.4. c-KIT

The low prevalence of *BRAF* mutations in acral and mucosal melanoma led to investigations to identify other oncogenes in these tumors. Initial studies

focused on chromosomal regions which showed selective amplifications in these subtypes but not in cutaneous melanomas without chronic sun damage. One such region was 4q12, which contained several genes that encoded targetable proteins. Detailed analysis of this region demonstrated frequent focal amplifications of the *c-KIT* gene in acral (25%) and mucosal (30%) melanomas (Curtin et al., 2006). Further analyses have identified point mutations in the *c-KIT* gene in ~10% of acral melanomas and 20% of mucosal melanomas, some of which are also amplified (Woodman & Davies, 2010).

The *c-KIT* gene encodes the KIT protein, which is another receptor tyrosine kinase. Point mutations in *c-KIT* are detected in ~80% of gastrointestinal stromal tumors (GIST) (Hirota et al., 1998). Similar to melanomas, GISTs are highly aggressive tumors that are resistant to chemotherapy. However, small molecule inhibitors of KIT, such as imatinib, achieve clinical responses in more than 50% of GIST patients and are now the standard of care in this disease (Demetri et al., 2002). Previously, three phase II clinical trials of imatinib in unselected melanoma patients reported a total of one clinical response. However, multiple case reports have now described impressive clinical responses and benefit in metastatic melanoma patients with activating *c-KIT* mutations (Antonescu et al., 2007; Hodi et al., 2008; Quintas-Cardama et al., 2008; Woodman et al., 2009). Two phase II trials of imatinib in metastatic melanoma patients with *c-KIT* gene alterations have recently been reported. The reported clinical response rates of 15–25% compare favorably to the results of previous trials with imatinib in unselected melanoma patients, but interestingly are much lower than the rates observed in GIST patients (Carvajal et al., 2011; Guo et al., 2011).

There are no trials at this time to systematically address the efficacy of KIT inhibitors in *c-KIT*–mutant melanoma brain metastases. However, one report of a series of four melanoma patients with *c-KIT* mutations noted that although all of the patients responded to treatment with KIT inhibitors, three of the patients developed their initial relapse in the brain (Handolias et al., 2010).

5. CONCLUSION

The treatment of brain metastases remains challenging. The development of highly active, personalized targeted therapy approaches presents a clear opportunity to determine the clinical benefits of this approach in

patients with CNS involvement. However, as described above, this approach has had mixed results in these patients.

One of the clear challenges for the use of targeted therapies for brain metastases is the penetration of the blood–brain barrier. While there has been a tremendous emphasis on identifying molecular characteristics that correlate with sensitivity to specific therapies, even in the ideal patient the benefit of targeted therapies is highly dependent upon the achievement of significant target inhibition. This was demonstrated elegantly in the clinical development of vemurafenib for *BRAF*-mutant melanoma. Early phase testing of vemurafenib in patients with activating *BRAF* mutations demonstrated that the agent was well tolerated. However, increasing doses of the drug failed to significantly improve serum exposure, serum levels failed to reach concentrations that correlated with tumor regression in preclinical models, and no clinical responses were seen. The drug was subsequently reformulated, resulting in improved bioavailability, dose-proportional increases in serum levels, and clinical responses (Flaherty et al., 2010). Further supporting the importance of drug delivery to its target, analysis of a cohort of patients that underwent pretreatment and on-treatment biopsies demonstrated an almost linear relationship between the degree of MAPK pathway inhibition in the patients' tumors and the degree of tumor shrinkage achieved (Bollag et al., 2010). For CNS metastases, pharmacokinetic studies for several agents, including the studies described above with trastuzumab, gefitinib, and erlotinib, have consistently demonstrated that significantly lower levels of these agents are detected in the CSF than in the serum. These studies, and isolated patient vignettes, support that the doses of these agents may need to be increased in patients with brain metastases in order to achieve similar activity to that achieved with standard doses in extracranial metastases. An alternative approach is to develop drug delivery strategies that are able to overcome the blood–brain barrier, as exemplified by the activity of trastuzumab when administered intrathecally in patients who developed leptomeningeal involvement while receiving the agent systemically (Stemmler et al., 2006). Extensive research is also currently ongoing to identify modifications that may improve intracranial delivery of these agents (Soni et al., 2010).

While improved penetration of agents into the CNS may increase the efficacy of targeted therapies for brain metastases, there is also growing evidence that the molecular biology of these tumors can be markedly different than primary tumors or extracranial metastases (Chen & Davies, 2012). In addition to potential roles for HER2, EGFR, and BRAF, studies

have implicated a variety of molecules and pathways that increase the brain metastatic potential of primary tumors (Bos et al., 2009; Nguyen et al., 2009; Xie et al., 2006). However, there is also recent data to support that interactions between tumor cells and the microenvironment of the CNS may induce marked molecular changes in the tumor cells (Park et al., 2011). In that study, direct interactions between cancer cells and astrocytes *in vitro* largely recapitulated many of the changes observed in brain metastases in mice, suggesting that these supporting cells contribute significantly to this effect. Many of the genes that were upregulated in the tumor cells promote cellular survival, and co-culture of tumor cells with astrocytes induced marked resistance to chemotherapy (Kim, Flaherty et al., 2011; Kim, Kim, et al., 2011; Kim, Lee, et al., 2011). However, at this time it is not known if or how these changes impact the efficacy of targeted therapies.

The critical role of the microenvironment in the growth of brain metastases is also supported by studies examining the role and therapeutic potential of angiogenesis in these tumors. Single-agent treatment with various TKIs (i.e., sorafenib, sunitinib) that inhibit receptors that are critical to angiogenesis are approved therapies for patients with metastatic renal cell carcinoma (RCC). Analyses of clinical trials of TKIs in large cohorts of RCC patients support that these agents reduce the risk of CNS metastases, and they have some activity in patients with brain metastases (Gore et al., 2011; Massard et al., 2010; Stadler et al., 2010; Verma et al., 2011). In most cancers, however, antiangiogenic agents are used in combination with other therapies. Testing is ongoing to determine the safety and efficacy of these agents in patients with brain metastases, particularly in combination with effective targeted therapies (Lin & Winer, 2007; Metro & Fabi, 2012; Schettino et al., 2012).

Perhaps the greatest impediment to the development of improved treatments for patients with brain metastases has been the common practice of excluding these patients from clinical trials (Gounder & Spriggs, 2011). The recent impressive results in patients with brain metastases in the phase I trial of dabrafanib, and reports that demonstrate similar outcomes to patients without CNS involvement in early-stage clinical trials, have brought attention to and challenged this practice (Gounder & Spriggs, 2011; Long, et al., 2010; Tsimberidou et al., 2011). It must be acknowledged, however, that clinical evaluation of new agents in patients with CNS involvement has specific challenges, including the radiographic evaluation of response and progression, distinguishing neurological symptoms from side effects, and the need for invasive procedures to perform pharmacokinetic analysis.

However, each of these aspects is addressable with focused efforts and resources. In parallel, there is a need for improved understanding of the molecular pathogenesis and targeting of brain metastases through the development of additional preclinical models. Finally, the identification of brain metastases as a priority for drug development and testing will greatly facilitate advances in this critical era. Investment in these areas is likely to become increasingly important to improving the outcomes overall in patients with advanced cancer.

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ABBREVIATIONS

CNS central nervous system
CSF cerebrospinal fluid
XRT radiation therapy
ORR overall response rate
PFS progression-free survival
OS overall survival
nm nanomolar
kDa kilodalton
NSCLC non–small cell lung cancer
SCLC small cell lung cancer
PCI prophylactic cranial radiation
FISH fluorescence *in situ* hybridization
IHC immunohistochemistry
TKI tyrosine kinase inhibitor
UVR ultraviolet radiation
HD IL-2 high dose bolus interleukin-2
GIST gastrointestinal stromal tumor
HR hazard ratio

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CHAPTER SIX

Emerging Strategies for Targeting Cell Adhesion in Multiple Myeloma

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Abstract

Multiple myeloma (MM) is an incurable hematological cancer involving proliferation of abnormal plasma cells that infiltrate the bone marrow (BM) and secrete monoclonal antibodies. The disease is clinically characterized by bone lesions, anemia, hypercalcemia, and renal failure. MM is presently treated with conventional therapies like melphalan, doxorubicin, and prednisone; or novel therapies like thalidomide, lenalidomide, and bortezomib; or with procedures like autologous stem cell transplantation. Unfortunately, these therapies fail to eliminate the minimal residual disease that remains persistent within the confines of the BM of MM patients. Mounting evidence indicates that components of the BM—including extracellular matrix, cytokines, chemokines, and growth factors—provide a sanctuary for subpopulations of MM. This co-dependent development of the disease in the context of the BM not only ensures the survival and growth of the plasma cells but contributes to *de novo* drug resistance. In addition, by fostering homing, angiogenesis, and osteolysis, this crosstalk plays a critical role in the progression of the disease. Not surprisingly then, over the past decade, several strategies have been developed to disrupt this communication between the plasma cells and the BM components including antibodies, peptides, and inhibitors of signaling pathways. Ultimately, the goal is to use these therapies in combination with the existing antimyeloma agents in order to further reduce or abolish minimal residual disease and improve patient outcomes.

1. INTRODUCTION

Multiple myeloma (MM) was first described as a case of "mollities ossium" or abnormal softening of bone by Samuel Solly nearly 168 years ago in a women named Sarah Newbury (Solly, 1844). Solly reported Sarah having excruciating pain in the bone along with development of fatigue for

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which she was treated with rhubarb pill, infusion of orange peel, and opiate, if required. Solly's report was followed by the famous case of "mollities and fragilitas ossium" in Mr. M (later identified as Thomas Alexander McBean) who suffered from frequent bone fractures and was treated with a maintenance therapy of phlebotomy, cupping, and application of leeches followed by a prescription of steel and quinine along with opiates for relief (Macintyre, 1850). Sarah and Mr. McBean, both had "heavy deposits" in their urine and on autopsy, both their bones were found to be brittle and the bone marrow (BM) was found to be filled with red and gelatinous substance. Solly and Macintyre were unable to advance an explanation for the disease; however, they both entertained the hypothesis put forward by Friedrich Miescher stating that the disease was an inflammatory process that led to absorption and secretion of earthy matter from the bones into the urine (Macintyre, 1850; Solly, 1844). Mr. McBean's histological examination of the bone marrow was performed by the surgeon John Dalrymple and his urine sample was studied by the pathologist Henry Bence Jones (Macintyre, 1850).

John Dalrymple after studying the interior of Mr. McBean's affected bone noted that there were large number of nucleated cells of various size and shape, and majority of them were larger than an average erythrocyte. He also noted that the larger irregular cells often contained two or three nuclei (Rosenfeld, 1987). However, it was not until 1900 when Wright suggested that these abnormal cells within the BM of MM patient consisted of plasma cells or immediate descendants of these cells (Wright, 1900). It would take another couple of decades to show that these abnormal plasma cells shed copious amount of serum gamma globulin with antibody activity into the blood stream (Heremans et al., 1961; Longsworthet al., 1939; Tiselius & Kabat, 1939).

Henry Bence Jones from his studies on Mr. McBean's urine sample concluded that the sample contained enormous quantities of oxides of albumin—specifically, hydrated deutoxide of albumin ("Classics in oncology. Henry Bence Jones (1813–1873), 1978"). Furthermore, he emphasized the use of the identification of this oxide of albumin as a diagnosis for "mollities ossium" and for his efforts proteinuria associated with MM is called Bence Jones protein (Kahn, 1991). Almost 100 years later, Korngold and Lipari (1956) identified two classes of Bence Jones proteins (named kappa and lambda as a tribute to them) and demonstrated that antisera to these proteins also reacted with myeloma proteins in the blood. Finally, Edelman and Gally showed that the Bence Jones protein from MM patient's urine was identical to the light chain IgG monoclonal protein found in the serum of the

same patient, thus finally decoding the identity of the "albuminous" protein in the urine of MM patients (Edelman & Gally, 1962).

Today MM is clinically characterized by the accumulation of plasma cells in the BM and quantification of Bence Jones protein in the urine and peripheral blood is considered a surrogate marker of tumor burden. The prevailing hypothesis is that multistep genetic changes in the differentiated B cells along with cues originating from the BM microenvironment (see Fig. 6.1) lead to the transformation of these cells to malignancy [for

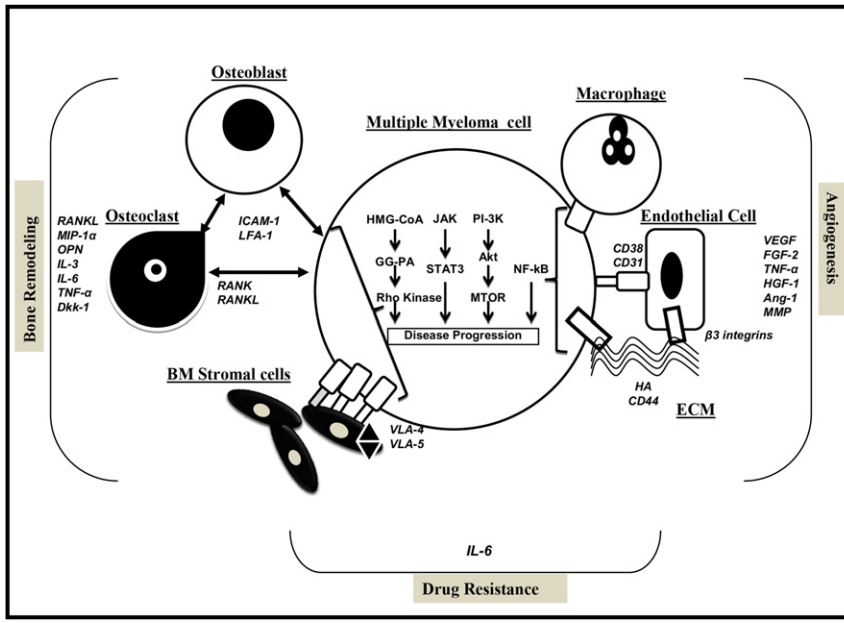

Figure 6.1 ***Disease progression in multiple myeloma.*** The figure depicts the three critical processes that aid in the progression of MM. All the three process, namely angiogenesis, bone remodeling, and drug resistance, requires participation of the adhesive component and the soluble factor component of the BM. So, for bone remodeling RANK/RANKL and ICAM-1/LFA-1 adhesion molecules along with the soluble factors RANKL, MIP-1α, OPN, IL-3, IL-6, TNF-α, and DKK-1 ensures that osteoclasts and osteoclastic acitivity is in an overdrive along with suppression of osteoblast. In angiogenesis, CD38/CD31 adhesion to the MM cells and β3 integrin adhesion to the ECM ensures new vascular formation by endotherlial cells and macrophages and the whole process is in turn aided by the soluble factors VEGF, FGF-2, TNF-α, HGF-1, Ang-1, and MMP. Finally, adhesion of the MM cells to the BMSCs and ECM ensure a CAM-DR phenotype. Taken together each of these process collectively benefits the MM cells by activating the PI3K/AKT, NF-κB, JAK/STAT3, and HMG-CoA/GG-PP/Rho Kinase pathway and ensures its survival and proliferation in the BM microenvironment. For color version of this figure, the reader is referred to the online version of this book.

review see Palumbo and Anderson (2011)]. Once transformed these malignant plasma cell produce monoclonal (M) proteins that can lead to renal failure caused by Bence Jones proteins (light chains) or to increased viscosity of the blood. In clinic, MM as a disease is diagnosed not only by the clinical manifestations like bone lesions and renal failure but also by the laboratory features like having clonal BM plasma cell population greater than or equal to 10%, presence of serum and/or urinary monoclonal protein, anemia, and hypercalcemia (Kyle & Rajkumar, 2009). MM accounts for 10% of all hematological malignancies and in United States, there will be approximately 21,700 estimated new cases of MM and approximately 10,710 patients will die of MM (Siegel et al., 2012). At present, the survival of myeloma patients has been vastly improved with the application of autologous stem cell transplantation and the introduction of novel therapeutic agents like the proteasome inhibitor bortezomib and immunomodulatory drugs like thalidomide and lenalidomide [for review see Kumar et al. (2008); Palumbo and Anderson (2011)]. Despite these advances in treatment regimens, MM remains incurable and therapeutic challenges still remain to be answered. This chapter reviews the role of adhesion in MM and enumerates the various strategies that have been explored to target adhesion for development of novel therapies in MM. Specifically, we have divided the review into two: the first part explains how adhesion plays a crucial role in the various aspects of MM disease progression like homing, angiogenesis, bone remodeling, and drug resistance and the second part enumerates the various therapeutic strategies that have been tested to target the disruption of these adhesion-facilitated processes.

2. ROLE OF ADHESION IN MM DISEASE PROGRESSION

The majority of MM cases arises from precursors like monoclonal gammopathy of undetermined significance (MGUS) or smoldering multiple myeloma (SMM) (Fonseca et al., 2009; Landgren et al., 2009). One of the most significant diagnostic difference between MM and MGUS or SMM is the complete lack of end-organ damage such as hypercalcemia, renal failure, anemia, and osteolysis in the later two compared to MM ("Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group," 2003). However, MGUS and SMM vary substantially in their risk of progressing to MM with 1% per year of MGUS compared to 10% per year of SMM

progressing to MM (Kyle et al., 2002, 2007). At present, even though the exact events that determine the progression of MGUS or SMM to MM remain elusive, it is becoming more and more apparent that BM microenvironment plays a key role in facilitating the progression of MM into a deadly disease (Hideshima et al., 2004; Kuehl & Bergsagel, 2002). In the following sections, we will discuss how this crosstalk between the myeloma cell and the BM microenvironment helps in the progression of the disease (see Fig. 6.1).

2.1. Homing to the BM

Adhesion molecules and chemokines play a crucial role in the homing of the myeloma cells to the BM. The homing pattern of mature plasma cells are faithfully copied by the myeloma cells and the key participants in this process for both the cell types is stromal cell-derived factor-1 (SDF-1) (also called CXCL12) and its receptor CXCR4 (Dar et al., 2005). In MM patients the SDF-1 level is elevated in the BM partly because of the hypoxia-driven SDF-1 upregulation by hypoxia-inducible factor-2 (HIF-2) and partly due to high osteoclast activity (Martin et al., 2010; Zannettino et al., 2005). At the same time, myeloma cells ubiquitously express the SDF-1 receptor CXCR4, which is further upregulated by cytokines and hypoxia (Kim et al., 2009; Trentin et al., 2007). Infact, myeloma cells incubated in hypoxic conditions (1% O_2) increased both the messenger and protein expression of CXCR4 (Kim et al., 2009). At the same time, Martin et al. (2010) have shown that under hypoxic conditions, HIF-2 binds to the SDF-1 promoter and increases its expression in MM cells which in turn secretes the over-expressed SDF-1 into its microenvironment. The coordinated upregulation of SDF-1 in the microenvironment and CXCR4 on the malignant plasma cell is critical for homing of the malignant clone to the BM compartment (Alsayed et al., 2007). Paradoxically, hypoxia, in addition to increasing the homing of circulating MM cells to the BM *via* the CXCR4/SDF-1 axis, also activated endothelial to mesenchymal transition-related machinery in the MM cells and decreased the expression of E-cadherin resulting in a decreased adhesion of MM cells to the BM, thus facilitating the mobilization of these cells into circulation (Azab et al., 2012).

Another important player in the homing mechanism is the family of heterodimer adhesive receptors called integrins (Desgrosellier & Cheresh, 2010). MM cells bind to the extracellular matrix (ECM) *via* the β1 integrin-mediated adhesion (Kibler et al., 1998). Of interest is the α5β1 receptor on the myeloma cells that bind to fibronectin (FN), which is shown to be

upregulated in the initial stages of MM disease; however the receptor is downregulated in circulating myeloma cells (Pellat-Deceunynck et al., 1995). In contrast to α5β1 receptor, integrin α4 can form a complex with either β1 subunit [and bind FN (through CS1) or vascular cell adhesion molecule-1 (VCAM-1)] or β7 subunit [and bind mucosal addressin cell adhesion molecule-1 (MAdCAM-1)] (Katz, 2010). Growth factors like hepatocyte growth factor (HGF) and insulin growth factor-1 (IGF-1) can stimulate the activity of α4β1 by stimulating the attachment of myeloma cells to FN and by affecting the adhesion and homing of myeloma cells, respectively (Holt et al., 2005; Tai et al., 2003). Apart from modulating integrin activity, cytokines like tumor necrosis factor α (TNFα) can actually upregulate the expression of α4β1 and thus enable adhesion and migration of MM cells (Hideshima et al., 2001).

In a MM mouse model, using the 5T33MMvv (cell lines originated in spontaneously developed MM mice and maintained by propagation in syngeneic mice) showed that these cells utilize IGF-1-dependent chemotaxis and CD44v6-dependent adhesion to bone marrow stromal cells (BMSCs) for specific homing into the BM (Asosingh et al., 2000). Moreover, direct contact of the MM cells to the BM endothelial cells were necessary for the upregulation of IGF-1 and CD44v6, which in turn facilitated homing of MM cells to the BM. Finally, MM cell surface markers like syndecan-1 (CD138) can directly bind FN and at the same time can also affect the activities of integrins (Morgan et al., 2007). Syndecan-1 can also help MM cells to adhere to type-I collagen and cause the release of matrix metalloproteinase (MMP) which can promote myeloma cell invasion into the BM (Barille et al., 1997).

2.2. Angiogenesis

Angiogenesis is a tightly regulated process involving the making of a new microvessel from existing vasculature (Stasi & Amadori, 2002). Increase in microvessel number within MM BM is correlative of poor prognosis and higher severity of the disease (Munshi & Wilson, 2001; Rajkumar et al., 2002; Swelam & Al Tamimi, 2010). One of the reasons for this increased vascularization of the BM is the increased need for oxygen and nutrients for the increased population of plasma cells residing in the BM which is typically a hypoxic environment. Indeed, analysis of control and 5T2MM-diseased mice showed that even though both normal and myeloma-infiltrated BM were hypoxic, the myeloma-containing BM showed significantly decreased

levels of hypoxia as measured by a decrease in the pimonidazole hypoxyprobe and a decrease in the expression of hypoxia-inducible factor-1α (HIF-1α), the surrogate marker of hypoxia (Asosingh et al., 2005). Furthermore, the initial hypoxia favors the growth of tumor initiating CD45 positive 5T2MM cells; however, with increased angiogenesis as the disease progresses, there is a switch to CD45 negative cells, which prefer the lower hypoxic conditions for their expansion and functionality (Asosingh et al., 2004; Asosingh et al., 2005).

2.2.1. Endothelial Cells

In MM, the vasculature formed within the BM, from endothelial cells, are very abnormal in that they are thin, unorganized, and heavily branched vessels (Vacca et al., 2003). They express CD133 cell surface marker which is a characteristic marker of the progenitor endothelial cells that are involved in new vessel formation (Ria et al., 2008). The myeloma cells along with the inflammatory cells (monocytes or macrophages) of the BM produce very high levels of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), which promotes differentiation of hematopoietic stem cells into endothelial cells and the formation of the new vessels (Ribatti et al., 2006). These endothelial cells express very high levels of β3 integrins which initiates their adherence to the ECM which provides both survival and proliferative cues to the endothelial cells (Hynes, 2002; Hynes et al., 2002). In addition, endothelial cells overexpress endoglin which upregulates the cell surface expression of CD31 on the endothelial cells giving it more tools to adhere to the myeloma cells *via* CD38/CD31 ligation (Vacca et al., 2003). Additionally, overexpression of E-selectin on the endothelial cell provides additional opportunities to interact with myeloma cells and enhance angiogenesis (Ria et al., 2010). Interestingly, very large proportions of circulating endothelial cells in MM patients carry the same chromosomal aberration as the myeloma cells of the patient, raising the speculation that both cell types may be derived from a common progenitor (Rigolin et al., 2006).

2.2.2. Macrophages

Macrophages are inflammatory white blood cells and have been shown to be significantly increased in numbers in BM aspirates of MM patients (Bingle et al., 2002; Zheng et al., 2009). In addition, direct cell contact between the macrophages and the MM cells protects the myeloma cells from caspase-dependent cell death induced by chemotherapeutic agents (Zheng et al.,

2009). One of the interesting characteristics of MM BM macrophages is its ability to mimic an endothelial phenotype by acquiring endothelial cell markers and forming capillary-like structures in the BM (Scavelli et al., 2008). This kind of mimicry requires that the MM macrophages be exposed to large amount of VEGF and basic FGF (bFGF) found in the BM and it is common to see BM aspirates of MM patients containing "mosaic" microvessels formed by a mixture of endothelial cells, macrophages mimicking endothelial cells, and normal macrophages (Scavelli et al., 2008).

2.2.3. Soluble Factors

Asosingh et al. (2004), in a MM mouse model, have shown that with MM disease progression, there is a shift in myeloma cells from CD45 positive cells to VEGF producing CD45 negative cells which supports angiogenesis. VEGF acts in an autocrine manner to induce growth and chemotaxis *via* the VEGFR1 receptor and at the same time it activates VEGFR2 on the BMSCs to produce interleukin-6 (IL-6) in a paracrine fashion (Dankbar et al., 2000; Podar et al., 2001). Increased IL-6, in turn, increase the adhesion between the MM cells and BMSCs which upregulates VEGF resulting in induction of angiogenesis (Chauhan et al., 1996; Hideshima et al., 2001). Additionally, activation of VEGF signaling pathway also inhibits antiangiogenic signaling chemicals like semaphorin 3A, thus ensuring that the BM is in a proangiogeneic state (Vacca et al., 2006).

Another important player in catalyzing angiogenesis in MM is the growth factor FGF-2 (Ribatti et al., 2007). FGF-2 concentration in the MM patient's BM aspirates and serum correlates with MM disease progression (Di Raimondo et al., 2000; Sato et al., 2002; Sezer et al., 2001). FGF-2 and IL-6 form a paracrine cross talk such that FGF-2 increases IL-6 expression in BMSCs, and IL-6 in turn upregulates FGF-2 expression and secretion from MM cells. The result of such interaction is increased angiogenesis helping MM growth and survival (Mitsiades et al., 2006).

Other soluble factors that also play a role in the angiogenesis induction in BM of MM patients include TNF-α, HGF-1, syndecan-1, Angiopoietin-1 (Ang-1), and MMP [for review see Ribatti et al. (2006)].

2.3. Bone Remodeling

In a healthy individual, there is a balanced process of bone remodeling ongoing between bone building, orchestrated by osteoblasts (osteogenesis), and bone resorption, orchestrated by osteoclasts (osteolysis). However, in

MM this homeostasis is tilted toward osteolysis leading to frail bones in more than 90% of the patients (Esteve & Roodman, 2007). This uncoupling of bone remodeling in MM is due to increased formation of osteoclasts with its activity stimulated by MM cells along with decreased differentiation of mesenchymal stromal cells (MSCs) and pre-osteoblast progenitors to osteoblasts (Giuliani et al., 2006).

2.3.1. Osteoclasts

One of the critical pathway toward osteoclast formation is the TNF receptor superfamily called receptor activator of nuclear factor-kappaB (RANK) and its activation through its ligand, RANK ligand (RANKL) (Edwards et al., 2008). RANK is expressed on the osteoclast progenitors, while RANKL is expressed on the cell surface of BMSCs, osteoblasts, activated lymphocytes, and MM cells (Farrugia et al., 2003; Heider et al., 2004). In normal physiology, BMSCs express osteoprotegerin (OPG) which act as a decoy receptor for RANKL and thus block any unheeded effects of osteoclasts activation. However, in MM there is an overexpression RANKL and total serum levels of RANKL correlates with lytic bone destruction and poor prognosis (Jakob et al., 2009). At the same time, Myeloma cells by adhering to BMSCs and osteoblast (by VLA-4/VCAM-1) can induce these cells to suppress their secretion of OPG by downregulating their expression (Giuliani et al., 2001). Another mechanism of decreasing the circulating levels of OPG is through binding of OPG to syndecan-1 on the MM cell surface leading to it internalization and degradation within the myeloma cells (Standal et al., 2002).

The activity of RANKL ligand can also be enhanced by a chemokine called macrophage inflammatory protein-1α (MIP-1α) acting through its receptor CCR1 and CCR5 (Choi et al., 2000; Oba et al., 2005). The amount of MIP-1α in the MM patient serum correlates with the intensity of bone lesions and moreover blocking MIP-1α in a mouse model of MM shows significant decrease in bone lesions and suppression of disease progression (Choi et al., 2001; Uneda et al., 2003). Furthermore, it has been shown that IL-3 secreted by the MM cells acts in the early stages of osteoclast formation followed by completion of osteoclast differentiation brought about by the effects of RANKL and MIP-1α. Indeed IL-3 can stimulate bone resorption and significantly potentiate the effects of RANKL and MIP-1α in MM (Lee et al., 2004). In addition, MM cells secrete osteopontin (OPN), HGF, and VEGF all of which directly or indirectly aid osteoclastogenesis (Alexandrakis et al., 2003; Nakagawa et al., 2000; Saeki et al., 2003).

One of the direct effects of MM cell-mediated osteoclast formation in the BM is that direct adherence of MM to osteoclasts supports myeloma cell proliferation and inhibits myeloma cell death (Abe et al., 2004; Hecht et al., 2008; Yaccoby et al., 2004). In addition to growth and survival, the interaction between MM cells and osteoclast can also protect the MM cells against antimyeloma agents possibly through the osteoclast-mediated secretions of OPN and IL-6 (Abe et al., 2004). Another interesting finding is that MM cells frequently form hybrids with osteoclasts such that such hybrid osteoclasts contain an additional MM nucleus (Andersen et al., 2007). At present, however, the function and activity of these hybrids are unknown.

2.3.2. Osteoblasts

MM cells inhibits proliferation and differentiation of osteoblast progenitors by secreting high levels of TNF-α, IL-7, and IL-3 (Ehrlich et al., 2005; Evans et al., 1992; Giuliani et al., 2005; Li et al., 2007). It has been suggested that this high levels of cytokines along with adhesion-dependent signaling involving ICAM-1/LFA-1 leads to apoptosis. Indeed, osteoblasts had a significantly increased rate of apoptosis when cultured with MM cells *in vitro* (Silvestris et al., 2004). Also, osteoblast differentiation from its progenitors requires bone morphogenetic protein type 2 (BMP-2) and co-stimulation of the Wnt/β-catenin signaling (Lin & Hankenson, 2011; Rawadi et al., 2003). However, MM cells produce copious amount of the Wnt signaling pathway inhibitor, Dickkopf-1 (DKK-1), which blocks Wnt co-receptor LRP5 and secreted frizzled-related protein-2 (sFRP2) which binds and sequesters soluble Wnt ligands (Oshima et al., 2005; Qiang et al., 2008; Tian et al., 2003). Therefore, at any given time within the MM BM milieu, there are sufficient inhibitors to inhibit BMP-2-mediated osteoblast differentiation. In addition, blocking of Wnt pathway also assures the suppression of OPG leading to uninterrupted binding of RANKL to RANK (Qiang, Chen, et al., 2008). Another way of suppression of OPG is by the cellular interaction between the myeloma cell and the osteoblast progenitor cells resulting in inhibition of the Runx2/Cbfa1 activity which is required for OPG induction (Giuliani et al., 2005). Interestingly, osteoblasts have been shown to possess strong antimyeloma activity, especially osteoblast that have been isolated from patients with advanced disease (Li et al., 2008). Specifically, decorin, a small leucine-rich proteoglycan produced and secreted by osteoblast inhibits angiogenesis and osteoclast formation thereby not only inhibiting MM cell growth but also inducing apoptosis (Li et al., 2008). These findings suggest that disease progression favors selection of MM cells

that can inhibit the formation of osteoblasts. Taken altogether, the MM BM microenvironment is conducive to suppression of osteoblast differentiation, a finding that likely contributes to lytic lesions found in MM patients.

2.4. Cell Adhesion-Mediated Drug Resistance

MM cells can bind to various components of the BM microenvironment, which includes the cellular component (BMSCs, MSCs, osteoclasts, osteoblasts, macrophages, endothelial cells, adipocytes, and fibroblasts) and the noncellular ECM (FN, collagen types I and IV, laminin, glycosaminoglycans, heparan sulfate, chondroitin sulfate, and hyaluronan (HA)). Studies have shown that myeloma cell interaction with the BM microenvironment bring about activation of signaling pathway and modulation of cytokine, chemokine, and growth factor production, all of which ultimately leads to the emergence of *de novo* drug resistance in MM cells *via* the process called cell adhesion-mediated drug resistance (CAM-DR) (Damiano et al., 1999).

MM patients show very high levels of plasma FN compared to healthy individuals and at the same time MM cells predominantly express $\alpha 4\beta 1$ (VLA-4) and $\alpha 5\beta 1$ (VLA-5), which can interact with FN and modulate MM cell survival (Jensen et al., 1993; Paizi et al., 1991). As precedence for this claim, multiple laboratories, using different cell types, have shown that adherence to FN *via* the integrins lead to cell survival (Higashimoto et al., 1996; Rozzo et al., 1997; Scott et al., 1997; Zhang et al., 1995). However, it was Damiano et al. (1999) who in 1999 demonstrated that adhesion of MM cells to FN through VLA-4 was sufficient to confer, not only cell survival, but more importantly, a drug-resistant phenotype against doxorubicin and melphalan. Since that finding, the mechanism associated with the integrin-mediated CAM-DR phenotype has been resolved and involves suppression of cell death (through downregulation of apoptotic protein Bim and regulating the cellular localization of c-FLIP$_L$), modulation of cell cycle signaling (through decreasing levels of $p27^{kip1}$ and inhibiting cyclin A and E-dependant CDK2 kinase activity), and inhibition of drug-induced DNA damage associated with topoisomerase II inhibitors (Hazlehurst et al., 2000; Hazlehurst et al., 2001, 2003; Shain et al., 2002; Yarde et al., 2009).

Another ECM component that is upregulated in MM is HA. Specifically, it has been shown that MSCs from the BM of MM patients upregulated and secrete high levels of HA (Calabro et al., 2002). Vincent et al., (2001) demonstrated that this increased HA induces survival and proliferation in MM cells through an IL-6 mediated pathway. Moreover, HA

anatagonized dexamethasone-induced apoptosis through a IL-6-dependent mechanism (involving downregulation of $p27^{kip1}$) and through a IL-6-independent mechanism (involving upregulation of Bcl-2 protein and NF-κB activation) (Vincent et al., 2001; Vincent et al., 2003). Furthermore, Ohwada et al. (2008) demonstrated that adhesion of MM to HA *via* its CD44 receptor lead to resistance against dexamethasone.

In addition to cell adhesion, the CAM-DR phenotype also benefits from the ensuing production of soluble factors as a direct result of adhesion of MM cells to the BM microenvironmental components. For example, Nefedova et al., (2003) showed that coculturing of MM cells, RPMI-8226 and NCI-H929, with BMSCs caused inhibition of cell death in the MM cell death induced by mitoxantrone. They concluded from their study that the resistance offered by the coculture was dependent on two separate mechanism: one arising from the cell-cell adhesion and the other arising from the soluble factor induced by this cell-cell adhesion (Nefedova et al., 2003). The mechanism was delineated further by Shain et al. (2009), who showed that adhesion of MM cell to FN *via* the β1 integrin allowed the activation of STAT3 *via* its association with gp130 which lead to upregulation of anti-apoptotic genes and resulted in cell survival. At the same time, the adhesion caused increased secretion of IL-6 which in turn helped overcome the G_1-S cell cycle arrest associated with FN adhesion, thereby resulting in cell proliferation (Shain et al., 2009). Finally, Hu et al. (2009) have shown that BM-derived growth factors like IGF-1 can increase the expression of the hypoxic surrogate marker HIF-1α. Increased HIF-1α correlated with activation of Akt and MAPK pathway resulting in protection against melphalan-induced cell death. Interestingly, inhibition of HIF-1α drastically reduced the IGF-1-induced expression of the anti-apoptotic protein survivin and reversed the protective effect of IGF-1 on melphalan-induced apoptosis (Hu et al., 2009).

Taken altogether, the existing data suggest that MM–BM interaction has a vital function in the progression of MM and targeting the interaction would provide a novel therapeutic strategy to cure MM.

3. THERAPIES TARGETING CELL ADHESION

Since the myeloma cells spend most of their time residing in the BM, it is imperative to understand the crosstalk occurring between the myeloma cell and its BM microenvironment, in order to identify the Achilles heel of

the disease and exploit the same. With that in mind we have tried to summarize below the various studies that have identified novel antimyeloma agents by examining and exploiting the interactions between the myeloma cells and its immediate environment. The present review does not cover agents like thalidomide, lenalidomide, and bortezomib, which have been extensively reviewed elsewhere [for review see Delforge (2011); Mohty et al. (2012); Palumbo & Anderson (2011)]. A summary of the list of agents discussed in the following section are provided in Table 6.1.

3.1. Agents That Directly Target Cell Adhesion

3.1.1. Antibodies

Antibodies can work either by blocking the action of adhesion molecules or by binding to the cell surface receptor and inducing cell death. Antibodies can induce cell death *via* two pathways. (i) Complement-dependent cytotoxicity involves the interaction between the Fc portion of the antibody with the classic complement-activating protein C1q leading to uptake of the myeloma cell and cellular fragmentation by antigen-presenting cells (Gancz & Fishelson, 2009). (ii) Antibody-dependent cell-mediated cytotoxicity, on the other hand, requires the activation of natural killer cells to mediate cell death (Ritchie et al., 2010).

3.1.1.1. Intercellular Adhesion Molecule-1 (ICAM-1) (CD54)

The expression of ICAM-1 has been shown to be elevated after chemotherapy in myeloma cells and unfortunately high ICAM-1 levels is a prognostic marker for poor response to chemotherapeutic agents (Schmidmaier et al., 2006). Huang et al., (1995) utilized UV3, a monoclonal antibody that recognizes human CD54 (ICAM-1) to demonstrate that it had therapeutic efficacy in SCID mice xenografted with the MM cell line ARH-77. Surprisingly, very low doses of UV3 were sufficient to prolong the survival of mice with early or advanced stages of the disease. It was further delineated that UV3 induced both antibody-dependent cell-mediated toxicity and complement-dependent cytotoxicity of ARH-77 cell line (Huang et al., 1995). The results of this experiment were further extended by Coleman et al., (2006), who demonstrated that only the Fc portion of the UV3 was critical for it antitumor activity in SCID mice xenografted with ARH-77. Presently, a trial utilizing a fully human immunoglobulin G1 antibody specific for ICAM-1 called BI-505 is recruiting volunteers for phase 1

Table 6.1 Summary of Antimyeloma Agents Targeting Cell Adhesion

	Molecular Target	References
A. Agents that directly target cell adhesion		
1. Antibodies	a. Intercellular adhesion molecule-1 (ICAM-1) (CD54) [UV3]	Huang et al., 1995 Coleman et al., 2006
	b. TNF receptor superfamily member 5 (CD40) [Lucatumumab; CHIR-12.12]	Tai et al., 2005
	c. CS1 [Elotuzumab; HuLuc63]	Tai et al., 2008 van Rhee et al., 2009
	d. Integrin α4 (CD49d) [Natalizumab]	Podar et al., 2011
	e. CD38 [Daratumumab]	de Weers et al., 2011
2. Antibodies conjugated to cytotoxic moieties	a. Neural cell adhesion molecule-1 (NCAM-1) (CD56) [Lorvotuzumab]	Tassone et al., 2004
	b. Syndecan-1 (CD138)	Ikeda et al., 2009
3. Virotherapy	a. ICAM-1 and decay accelerating factor (DAF)	Au et al., 2007
4. Peptides	a. Integrin α4 (CD49d) [HYD1]	Nair et al., 2009 Emmons et al., 2011)
5. Oligonucleotides	a. Defibrotide	Mitisades et al., 2009
B. Agents that indirectly target the cell adhesion apparatus		
1. Inhibitors of signal transduction pathways	a. HMG-CoA/GG-PP/ Rho-kinase pathway	Schmidmaier et al., 2004 Yanamandra et al., 2006
	b. RAS/cox-2 pathway	Nakamura et al., 2006
	c. NF-κB activation pathway	Walsby et al., 2010 Hideshima et al., 2006
	d. PI3-K/AKT activation pathway	Maiso et al., 2011 Ikeda et al., 2010
C. Agents targeting soluble factors		
1. Neutralizing Antibodies	a. Dickkopf-1 (DKK-1)	Fulciniti, et al., 2009
	b. Interleukin-6 (IL-6) [Siltuximab; CNTO 328]	Voorhees et al., 2007 van Zaanen et al., 1998

Table 6.1 Summary of Antimyeloma Agents Targeting Cell Adhesion—cont'd

	Molecular Target	References
	c. Vascular endothelial growth factor (VEGF) [Bevacizumab]	Attar-Schneider et al., 2012
2. Inhibition of actions of soluble factors	a. Hepatocyte growth factor (HGF)	Hov et al., 2004
	b. CCR1	Vallet et al., 2007
	c. Vascular endothelial growth factor (VEGF)	Podar et al., 2006
	d. Soluble intercellular adhesion molecule (sICAM-1)	Schmidmaier et al., 2007
	e. Transforming growth factor-β1 (TGF-β1)	Hayashi et al., 2004
	f. Fibroblast activation protein (FAP)	Pennisi et al., 2009
	g. Stromal cell-derived factor-1 (SDF-1)	Azab et al., 2009
D. Miscellaneous agents		
	a. PPARγ agonist	Wang et al., 2007
	b. Atiprimod	Neri et al., 2007
	c. KNK-437	Nimmanapalli et al., 2008
	d. Zoledronic acid	Corso et al., 2005

dose-escalation studies in relapsed/refractory MM patients to evaluate its efficacy and toxicity as a single agent (van de Donk et al., 2012).

3.1.1.2. CD40 (Lucatumumab; CHIR-12.12)

CD40 expression is very high in myeloma cells and stimulation by CD40L is important for myeloma cells to adhere to BM stromal cells, thereby resulting in increased production of IL-6 and VEGF in the BM milieu (Gupta et al., 2001; Pellat-Deceunynck et al., 1994; Urashima et al., 1995). Tai et al. (2005) evaluated the activity in MM of a human anti-CD40 antibody CHIR-12.12 that was generated in a strain of transgenic XenoMouse mice expressing human IgG1 antibodies and selected based on its inhibition of CD40L-induced biological signaling. The researchers first showed that CHIR-12.12 bound to CD138 expressing MM cell lines and CD138 expressing primary MM patient samples. The binding caused inhibition of CD40L-induced growth and survival of CD40-expressing

primary MM cells in the presence or absence of BMSCs. In addition, CHIR-12.12 not only decreased CD40L-induced MM cells adhesion to FN and BMSCs, but also blocks enhanced IL-6 and VEGF secretion in cocultures of MM cells with BMSCs (Tai et al., 2005). CHIR-12.12 was shown to inhibit CD40L-induced activation of PI3K/AKT, NFκB, and ERK. Finally, CHIR-12.12 was able to specifically induce antibody-induced cellular cytotoxicity in CD40-expressing MM cells, providing a rationale for its use in clinical trials.

Dacetuzumab (SGN-40) is another humanized partially agonistic anti-CD40 antibody that induces antibody-dependent cell-mediated cytotoxicity in CD40 positive MM cells (Hayashi et al., 2003). However, in a phase I dose-finding study in relapsed/refractory myeloma patients no objective clinical response was reported with use of Dacetuzumab as a single agent (Hussein et al., 2010).

3.1.1.3. CS1 (Elotuzumab; HuLuc63)

Tai et al. (2008), in an effort to search for ubiquitously expressed protein on MM cell that can be targeted by humanized antibodies, identified CS1 as a candidate antigen. They cataloged that 97% of the CD138 positive primary tumor cells from MM patients had high expressing levels of CS1 mRNA and its corresponding protein. Using a humanized anti-CS1 antibody, HuLuc63, they were able to show that blocking CS1 inhibited MM cell adhesion to BMSCs and induced antibody-dependent cellular cytotoxicity in these cells (Tai et al., 2008). Also, HuLuc63 demonstrated cytotoxic activity against primary MM cells that were resistant to bortezomib and heat shock protein (HSP)90 inhibitor. Finally, HuLuc63 showed significant tumor regression activity in three different xenograft models of human MM suggesting a need to test this antibody in clinical trials either alone or in combination with conventional therapies (Tai et al., 2008). In a later study, combination of bortezomib with HuLuc63 was very effective in enhancing the antimyeloma activity in a mouse model compared to each therapy alone (van Rhee et al., 2009).

3.1.1.4. Integrin α4 (CD49d) (Natalizumab)

As mentioned previously, adhesion of MM cells to FN *via* VLA-4 integrin is known to cause drug resistance in MM cell lines (Damiano et al., 1999). These preclinical findings indicate that α4 integrin may be an important target for increasing the efficacy of standard therapy in MM. Infact, anti-integrin-α4 antibody has been tested and found to have antimyeloma activity

as a single agent and in combination with melphalan, using a murine model of myeloma (Mori et al., 2004; Olson et al., 2005). More recently, Podar et al. (2011) evaluated the therapeutic potential of a recombinant humanized IgG4 monoclonal antibody that binds integrin-α4 in MM called Natalizumab. The study shows that integrin α4 expression was present in all the MM cell lines tested (MM.1S, RPMI-8226, INA-6, OPM2, and NCI-H929), but not in endothelial cells (HUVECs), BMSC cell lines (KM104, KM105), and primary BMSCs. Also, when tested in primary MM patient samples the *ITGA4* gene was upregulated in plasma cells from MM patients as compared to plasma cells from healthy donors (Podar et al., 2011). Natalizumab inhibited adhesion of MM cells to FN and BMSCs as well as disrupted the binding of the already adherent MM cells to BMSCs. Natalizumab not only abrogated the myeloma cell's proliferative effect on MM–BMSCs interaction but it also stopped VEGF-induced angiogenesis and VEGF and IGF-1-induced MM cell migration (Podar et al., 2011). Importantly, Natalizumab blocked MM cell adhesion and sensitized the cells to toxic effects of bortezomib in a MM-stroma coculture model. And finally, Natalizumab inhibited tumor growth, VEGF secretion, and angiogenesis in a SCID-Hu model utilizing INA-6 MM cells. In light of these findings, the researchers in the study think that Natalizumab should be studied further in a clinical setting preferably in combination with agents like bortezomib (Podar et al., 2011).

3.1.1.5. CD38 (Daratumumab)

Another cell surface protein that is differentially regulated in MM cells and normal myeloid cells is CD38, which is found to be highly expressed in MM patient cells (Lin et al., 2004). This observation led de Weers et al. (2011) to evaluate the role of CD38 as a potential therapeutic antibody target for the treatment of MM. The study utilized daratumumab, a high-affinity humanized antibody specific against a unique human CD38 epitope and found that daratumumab caused antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity in MM cells and in primary MM cells. Importantly, daratumumab maintained its activity in the presence of BMSCs indicating that it has antitumor activity in the BM microenvironment. Finally in a SCID mice xenograft model involving intravenous injection of CD38 expressing Daudi-luc MM cells, daratumumab reduced tumor burden (de Weers et al., 2011). The antibody is currently in a phase I/II safety and dose-finding trial for the treatment of MM.

3.1.2. Antibodies Conjugated to Cytotoxic Moieties

Because of the specificity of antibodies to selectively target tumor cells and due to the available technology to engineer humanized antibody, one strategy is to conjugate cytotoxics to a targeting antibody with a linker.

3.1.2.1. Neural Cell Adhesion Molecule-1 (NCAM-1) (CD56) (Lorvotuzumab)

Overexpression of NCAM-1 has been associated with MM (Kraj et al., 2008). Tassone et al. (2004) used HuN901, a humanized monoclonal antibody that binds with high affinity to CD56. HuN901 was conjugated to a potent antimicrotubular cytotoxic moiety called maytansinoid $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1) with the intension of delivering DM1 to CD56 expressing cells (Tassone et al., 2004). To evaluate the potential utility of CD56 as a target for antibody-based therapy, the researchers first observed the cell surface expression of CD56 in normal plasma cells and 15 patient MM cells and found 10 out of the 15 patients to express 3.2-fold higher expression of CD56 than normal plasma cells. Next, they increased their sample size to 28 patients and looked at the $CD38^{hi}CD45^{lo}$ MM cells and found 22 out of 28 patients expressed very high levels of CD56 (Tassone et al., 2004). Further, they found that HuN901-DM1 treatment selectively decreased the survival of CD56 positive MM cells and depleted CD56 positive cells from mixed cultures with CD56 negative cell line or adherent BMSCs. *In vivo* xenograft model using CD56 positive OPM2 MM cell line when subjected to treatment with HuN901-DM1 showed an inhibition of serum paraprotein secretion, inhibition of tumor growth, and increase in survival of the mice thus providing proof of principle for further drug development (Tassone et al., 2004).

3.1.2.2. Syndecan-1 (CD138)

The success of the previous study lead to the development of the murine/human chimeric CD138-specific monoclonal antibody nBT062 conjugated with cytotoxic maytansinoid derivatives to target MM (Ikeda et al., 2009). Specifically, three novel anti-CD138 antibody-maytansinoid conjugates, nBT062-SMCC-DM1, nBT062-SPDB-DM4, and nBT062-SPP-DM1 were developed varying in their linkage and maytansinoid moiety. All the three immunoconjugates inhibited cell growth in MM cell lines and primary MM patient cells while sparing the PBMCs from healthy volunteers (Ikeda et al., 2009). The inhibition of cell growth was observed to be due to cell cycle arrest followed by induction of apoptosis brought about by cleavage of

caspase 8, 9, and 3 and PARP. The unconjugated antibody, nBT062, completely blocked the cytotoxicity of the immunoconjugates. Further, all the three immunoconjugates blocked the adhesion of MM cells to BMSCs. Finally, nBT062-SPDB-DM4 and nBT062-SPP-DM1 significantly inhibited MM tumor growth and prolonged mice survival in both the SCID-hu mice model and in a xenograft model of MM cells being injected subcutaneously in SCID mice (Ikeda et al., 2009). The above two studies provide a framework supporting the clinical evaluation of immunoconjugates in MM patients.

3.1.3. Virotherapy

High expression of ICAM-1 levels on MM cell surface makes it an attractive target to design antibody therapies against MM (Huang et al., 1995). However, Au et al., (2007) utilized the specificity of oncolytic human enterovirus, Coxsackievirus A21 (CVA21), to target MM cells and mediate cell death through induction of apoptosis. Since CVA21 requires the coexpression of ICAM-1 and decay accelerating factor (DAF) to infect cells, the study first demonstrated by cell surface analysis that only MM cell lines (U266, RPMI-8266, and NCI-H929) and primary CD138 positive cells from 15 patient BM biopsies, but not PBMCs from normal donors, showed very high expression levels of ICAM-1 and DAF. Not surprisingly, MM cell lines and patient BM samples showed remarkable susceptibility to CVA21 lytic infection. In contrast, normal PBMCs and progenitor cells from patient samples were resistant to CVA21 infection showing the potential application of virotherapy as an antitumor agent in MM (Au et al., 2007).

3.1.4. Peptides

Peptides offer a promising future in targeting cell surface receptors with high specificity. Nair et al. (2009) have utilized a d-amino acid containing peptide (kikmviswkg), referred to as HYD1, to block $\alpha 4\beta 1$-mediated adhesion of MM cell line to FN and also to reverse the resistance associated with the BMSC coculture model. Further, HYD1 induced cell death in MM cell line (H929, 8226, and U266) without causing any cytotoxicity in PBMCs of healthy individuals. HYD1 induced cell death was necrotic in nature and was accompanied by loss of mitochondrial potential, loss of ATP, and increase in reactive oxygen species generation (Nair et al., 2009). More importantly, in the SCID-hu mice model with H929 cells, intraperitoneal injections of HYD1 lead to significant decrease in tumor

burden. In a follow-up study, Emmons et al. (2011) demonstrated that HYD1 was more potent in relapsed MM patient primary cells having high α4 integrin cell surface expression than in newly diagnosed MM patient primary cells having low α4 integrin cell surface expression. The authors conclude that HYD1 may represent a good candidate for pursuing trials in patients that are unresponsive to conventional therapy and have high levels of α4 integrin.

3.1.5. Oligonucleotides

MM cell interaction with the BMSCs leads to increases in proliferative cytokines, angiogenic cytokines, and adhesion molecule activation that all leads to decreased responsiveness of MM cells to cell death by antimyeloma agents. To address this, Mitsiades et al. (2009) tested the use of defibrotide, an orally bioavailable polydisperse oligonucleotide in MM. Defibrotide is polydisperse polydeoxyribonucleotide, derived from porcine mucosa by controlled depolymerization and has been shown to have antithrombotic, thrombolytic, and anti-adhesive effects (Eissner et al., 2002; Pescador et al., 1996). Mitsiades et al. (2009), specifically, wanted to evaluate whether defibrotide had activity as a single agent and if used in combination with other agents will it attenuate their activity. Secondly, they wanted to see whether defibrotide can interfere with the MM-stromal interaction and sensitize MM cells to chemotherapeutic agents. They found that defibrotide had no direct antimyeloma activity, at the same time it did not attenuate the antitumor activity of different class of antineoplastic drugs. However, in a coculture model of MM cells with BMSCs, defibrotide enhanced the activity of melphalan and dexamethasone. The sensitization of the MM cells was brought about by suppression of adhesive interactions between the two cell types leading to decreased NF-κB activity, which in turn resulted in decreased expression of cytokines, chemokines, and adhesion molecules (Mitsiades et al., 2009).

Defibrotide also had activity *in vivo* which lead to evaluation of defibrotide in a phase I/II study conducted to study the most appropriate dose of defibrotide in combination with melphalan, prednisone, and thalidomide in patients with relapsed and relapsed/refractory MM (Mitsiades et al., 2009; Palumbo et al., 2010). The results of this study showed that combination of melphalan, prednisone, and thalidomide with defibrotide showed antimyeloma activity and was favorably tolerated by patients confirming the role of this regimen in treating MM patients (Palumbo et al., 2010).

3.2. Agents That Indirectly Target Cell Adhesion

3.2.1. Inhibitors of Signal Transduction Pathways

3.2.1.1. HMG-CoA/GG-PP/Rho-Kinase-Pathway

Schmidmaier et al. (2004) showed that their four multiple myeloma cell lines NCI-H929, U266, RPMI-8226, and OPM2 when cocultured with the BMSCs resulted in strong reduction in chemosensitivity toward melphalan, treosulfan, doxorubicin, dexamethasone, and bortezomib. To assess the role of integrins in CAM-DR, they utilized a integrin inhibitor LFA703, a statin derivative that lacks HMG-CoA reductase activity, that has a strong inhibitory activity against $\alpha_L\beta_2$ integrins and found that LFA703 only reversed the CAM-DR by 50% in their coculture model (Schmidmaier et al., 2004; Weitz-Schmidt et al., 2001). However, inhibition of HMG-CoA reductase or its downstream mediators like geranylgeranyl transferase or Rho kinase by simvastatin, GGTI-298 or Y-27632, respectively, completely reversed CAM-DR associated with melphalan treatment. Of particular interest was their finding that the inhibition of HMG-CoA/GG-PP/Rho-protien/Rho-kinase pathway did not reduce the levels of IL-6 in the media and did not significantly downregulate the cell surface expression of VLA-4 and LFA-1 (Schmidmaier et al., 2004).

While the earlier study utilized geranylgeranyl transferase inhibitor to reverse CAM-DR, Yanamandra et al. (2006) utilized a farnesyl transferase inhibitor, tipifarnib, in combination with bortezomib to show cell death activity in BM microenvironment model. Interestingly, in this study the reversal of CAM-DR seen with the above-mentioned combination of drugs was not related to decreased MM cell adherence to BMSCs, but rather dependent on the activation of endoplasmic reticulum stress pathway.

3.2.1.2. RAS/cox-2 Pathway

Oncogenic *RAS* mutations is a common occurrence in MM patients and is associated with induction of the expression cox-2 (Neri et al., 1989; Sheng et al., 2001). Moreover, increased cox-2 expression correlated with aggressive disease and poor outcome in MM partly because of its ability to enhance binding of MM cells to FN (Hoang et al., 2006; Ladetto et al., 2005). In light of this, Nakamura et al. (2006) compared two cox-2 inhibitor (etodolac and meloxicam) and an immunomodulator (thalidomide) in their ability to inhibit proliferation and induce apoptosis in MM cell lines (RPMI-8226 and MC/CAR cells). Of the three drugs tested, they found that etodolac was far superior compared to meloxicam and thalidomide in

suppressing cell proliferation and inducing cell death in MM cells. Moreover, etodolac caused loss of mitochondrial membrane potential concurrent with activation of Caspase-9, -7, and -3 *via* a cox-2 independent pathway. More pertinent for this review, etodolac caused decreased adhesion of MM cells to BMSCs which correlated with etodolac-mediated downregulation of adhesion molecules VLA-4, LFA-1, CXCX4, and CD44 (Nakamura et al., 2006). Unfortunately, the reversal of CAM-DR in the presence of etodolac was not reported in this study.

3.2.1.3. NF-κB Activation Pathway

Since MM cell lines show constitutive activity of NF-κB which correlates with increased cell-cell contact and cytokine stimulation in BM microenvironment. Due to persistant and adhesion-mediated enhancement of NF-κB activation, this pathway is an attractive target for the treatment of MM (Annunziata et al., 2007; Gilmore, 2007). Part of the reason for this constitutive activity in patient MM cells was attributed to an as-yet-unknown proteinaceous secreted factor from the patient BMSCs acting in concert with IL-8 (Markovina et al., 2010). To further support NF-κB as a target for inhibition of MM cell growth, Walsby et al. (2010) evaluated the effect of the NF-κB inhibitor, LC-1, on MM cell lines H929, U266, and JJN3 and in plasma cells derived from MM patients. Their study showed that LC-1 showed toxicity in all the three cell lines tested by apoptosis through activation of caspase-3. The apoptosis was thought to be brought about by LC-1's ability to reduce the p65 subunit from accumulating and binding to its response element in the nucleus, thus causing a downregulation of NF-κB regulated antiapoptotic genes survivin and Mcl1. LC-1 also only preferentially killed CD38/CD138 positive patient MM cells while sparing the normal BM cells. Finally, LC-1 not only synergizes with melphalan, bortezomib, and doxorubicin but also is more potent in killing MM cells that are adhered to FN when compared to other conventional therapies like melphalan (Walsby et al., 2010).

Hideshima et al. (2006) studied the significance of IκB kinase (IKK) inhibition in MM cells in context of the BMSCs by using a inhibitor called MLN120B. They found that MLN120B induces growth inhibition in MM cell lines and augments TNF-α-induced cytotoxicity in MM.1S cells *via* inhibition of NFκB activity in a IKKβ-dependent manner. Addition of IL-6 or IGF-1 does not overcome the growth-inhibitory effect of MLN120B. Importantly, in a coculture model, MLN120B not only blocks both, the stimulation of cell growth and induction of IL-6 from BMSCs, but also

overcomes the protective effect of BMSCs against dexamethasone-induced cell death (Hideshima et al., 2006).

3.2.1.4. PI3-K/AKT Activation Pathway

The PI3-K/AKT/mTOR pathway is very essential in integrating the signals originating in the tumor microenvironment within the MM cells. Inspite of this, rapamycin, an inhibitor of this pathway is inefficient in treating MM. Maiso et al. (2011) hypothesized that the reason for the inefficient activity of rapamycin in MM was the inability of the drug to inhibit TORC2 from the mTOR complexes (TORC1/2). They decided to evaluate the effects of a dual TORC1/2 inhibitor, INK128, in MM cells. They found that in the 8 MM cell lines and 16 primary MM samples tested, the PI3-K/AKT/mTOR pathway was constitutively active. INK128 showed antimyeloma activity in all cell lines and primary MM cells without affecting the lymphocytes and the granulocytes populations derived from patient samples (Maiso et al., 2011). Furthermore the antimyeloma activity was attributed to cell cycle arrest leading to apoptosis. Importantly, this activity was not reversed in the presence of IL-6 or IGF-1 or when the cells were cocultured with BMSCs. Additionally, cells pretreated with INK168 and then injected in mice through tail vein injection did not home into the BM as compared to untreated cells which got cleared from circulation and into BM within 30 min (Maiso et al., 2011). Finally, the inhibitor INK128 showed good efficacy *in vivo* in reducing the MM cell burden in the mice BM, thus making a case for further clinical testing of TORC1/2 inhibitors in MM.

Ikeda et al. (2010) utilized a different approach in inhibiting the PI-3K/AKT pathway by using specific inhibitors of PI-3K interacting isoform p110δ. Ikeda et al. (2010) utilized two inhibitors of p110δ: CAL-101, which was used for all the *in vitro* studies; and IC48843, which was used in the *in vivo* studies. The study first showed that although only 2 MM cell lines (INA-6 and LB) out of the 11 tested showed expression of p110δ, 24 out of 24 primary patient derived MM cells expressed p110δ (Ikeda et al., 2010). Consequently, CAL-101 showed cytotoxicity in INA-6, LB, and MM cells derived from five patient specimens but not in the PBMCs from four healthy volunteers. Cell death was caused by apoptosis (both intrinsic and extrinsic) resulting from cleavage of caspase-8, 9, and 3, and PARP. This apoptosis was preceded by complete inhibition of AKT and ERK phosphorylation and also induction of autophagy in CAL-101 treated cells. CAL-101 also overcame MM cell survival and growth conferred by externally provided IL-6, IGF-1, and in BMSCs coculture (Ikeda et al.,

2010). Finally, *in vivo* efficacy of IC48843 was evaluated in a model where the MM cells are injected subcutaneously in SCID mice and in a SCID-Hu model. In both models, IC48843 significantly inhibited tumor growth and prolonged survival indicating the need to clinically evaluate these inhibitors.

3.3. Agents Targeting Soluble Factors

The BM microenvironment is filled with cellular entities capable of secreting growth factors, cytokines, and chemokines. Cross talk between the cellular components of the BM and between the EC and the cellular components leads to induction of soluble factors in an autocrine or a paracrine manner in majority of such interactions. These soluble factors act on their respective receptors resulting in activation of signaling pathways that favor myeloma cell growth, survival, and confer the ability to resist the actions of antimyeloma agents. Several strategies are available to inhibit the actions of these soluble factors in the BM and some of these are enumerated below.

3.3.1. Neutralizing Antibodies

3.3.1.1. Dickkopf-1 (DKK-1)

MM cell growth and progression is significantly affected by the cellular bone compartment. For example, osteoclasts support the survival and proliferation of myeloma cells, whereas osteoblasts inhibit myeloma cell growth (Abe et al., 2004; Yaccoby et al., 2006). Since MM cells produce Wnt inhibitor DKK-1, which in turn inhibits osteoblast production and moreover since DKK-1 serum levels correlate with bone lesions, Fulciniti et al. (2009) wanted to evaluate the activity of DKK-1 neutralizing antibody, BHQ880 in MM (Tian et al., 2003). *In vitro*, BHQ880 increased OB differentiation while neutralizing the negative effect of MM of osteoblastogenesis. BHQ880 was also effective in reducing the secretion of IL-6 in the MM-pre-osteoblast culture. BHQ880 significantly inhibits the growth of MM in the presence of BMSCs which was attributed the cumulative effects of (i) inhibition of MM cell adhesion on BMSCs and resultant decrease in production of IL-6, (ii) upregulation of β-catenin levels and downregulating NF-κB activity (Tian et al., 2003). Finally, in the SCID-hu mice model utilizing INA-6 cells, BHQ880 treatment lead to increased osteoblasts and osteocalcin levels providing a rationale that targeting DKK-1 could reduce bone disease associated with the progression of myeloma and directly inhibit growth of MM.

3.3.1.2. Interleukin-6 (IL-6) (Siltuximab; CNTO 328)

IL-6 is predominantly secreted by BMSCs and through activation of its receptor, IL-6R activates the JAK/STAT3, PI-3K/AKT, and MAPK pathways within the MM cell resulting in its growth and survival (Nilsson et al., 1990). Elevated levels of IL-6 and soluble IL-6R are found in the blood of MM patients and is considered as a marker for poor prognosis (Suematsu et al., 1990). However, murine antiIL-6 antibodies are ineffective as a single agent partly due to the development of host antibodies to the mouse IgG resulting in rapid clearance of the antibody from the patient's system (Bataille et al., 1995; Klein et al., 1991; Moreau et al., 2006). To circumvent this problem, Voorhees et al. (2007) evaluated the combination therapy of a chimeric human–mouse antibody against IL-6, CNTO 328, with the proteasome inhibitor, bortezomib. They found that CNTO 328 synergistically enhanced the cytoxicity of bortezomib in MM cell lines and in primary CD138 positive MM cells both in the presence and in the absence of BMSCs. This cytotoxicity was associated with activation of caspase 8, 9, and 3 in the MM cells. Further, the synergism was shown to be due to the inhibitory activity of CNTO 328 on bortezomib-induced induction and accumulation of HSP-70 and Mcl-1, respectively (Voorhees et al., 2007). Based on the safety profile of CNTO 328 and the preclinical data from this study, the combination is now being evaluated in clinical trials (van de Donk et al., 2012; van Zaanen et al., 1998).

3.3.1.3. Vascular Endothelial Growth Factor [Bevacizumab]

Attar-Schneider et al. (2012) set out to explore the efficacy of bevacizumab, an anti-VEGF antibody, on MM cell lines and BM samples. Bevacizumab caused cytostasis in both MM cell lines and primary BM samples and correlated with attenuation of downstream signaling proteins including mTOR, c-Myc, Akt, STAT3, (MM cell lines), and eIF4E translation initiation factor (MM cell lines and primary BM samples). Utilizing a constitutively Akt-expressing MM model, they showed that the effect of bevacizumab on viability is Akt-dependent (Attar-Schneider et al., 2012). This preclinical study highlights the utility of bevacizumab in combination with conventional antimyeloma therapies.

3.3.2. Inhibition of Actions of Soluble Factors

3.3.2.1. Hepatocyte Growth Factor

MM cells express hepatocyte growth factor (HGF) and its receptor c-Met and high levels of HGF in MM patients serum correlates with poor

prognosis for the patients (Iwasaki et al., 2002; Seidel et al., 1998; Seidel et al., 1998). Hov et al. (2004) examined the role of HGF receptor c-Met in MM by application of a novel selective inhibitor, PHA-665752, directed against the receptor. Specifically, they wanted to look at effects of PHA-665752 on four aspects of the actions of HGF in MM, namely proliferation of MM cells (ABNL-6 cells), migration of MM cells, and adhesion of MM cells to FN, and finally secretion of IL-11 from a sarcoma osteogenic cell line (Saos-2). They reported that PHA-665752 inhibited cell proliferation in MM cell lines and in primary patient CD138 positive cells. Additionally, the inhibitor completely abrogated the HGF-mediated adhesion of INA-6 MM cell line to FN. Also, PHA-665752 inhibited the IL-11 production induced by HGF in Saos-2 cells. Collectively, this study concluded that c-Met is an important target to inhibit proliferation, migration, and adhesion of MM cells (Hov et al., 2004).

3.3.2.2. CCR1

Chemokine CCL3 (MIP-1α) promotes osteoclast formation by acting through its receptors, CCR5 and CCR1 (Han et al., 2001). Since neutralizing antibodies to CCL3 reduces bone lesions and tumor burden, in MM mouse model, its receptor, CCR1, offers a promising therapeutic target for treatment (Oyajobi et al., 2003). Vallet et al. (2007) utilized MLN3897, a CCR1 inhibitor to demonstrate that in its presence, primary adherent PBMCs from normal donors failed to form osteoclasts in the presence of RANKL and M-CSF. This failure to form osteoclast was dependent on MLN3897's ability to interfere with the fusion of osteoclast precursors by inhibiting ERK activity and suppressing the expression of its downstream regulator c-Fos. Also, in the same study, MLN3897 inhibited the migration of MM cells induced by osteoclast culture supernatants and also abrogates the adhesion of MM cells to osteoclasts. Finally, MLN3897 completely reversed the MM cell survival and proliferative advantage conferred by osteoclasts in a coculture model providing a rationale for using MLN3897 in combination with traditional cytoxics used to treat MM (Vallet et al., 2007).

3.3.2.3. Vascular Endothelial Growth Factor

Studies have shown that VEGF is secreted by both MM and BMSCs and acts on the VEGF receptor on the MM cells, thereby inducing cell growth, survival, and migration (Podar & Anderson, 2005). Podar et al. (2006) demonstrated that pazopanib (GW786034B), an orally available small molecule tyrosine kinase inhibitor of VEGF-1, 2, and 3 could inhibit cell

growth, survival, and migration of MM cell lines (dexamethasone sensitive MM.1S, dexamethasone resistant MM.1R, doxorubicin sensitive RPMI, doxorubicin resistant RPMI, IL-6 dependent INA-6, OPM2, and U266). More importantly, Pazopanib blocks VEGF-mediated upregulation of ICAM-1 and VCAM-1 which concurrently downregulates the expression of VLA-4 and LFA-1 on MM cells. Consequently, pazopanib decreases the adhesion of MM cells to the endothelial cells and inhibits MM cell growth. Finally, pazopanib acts sysnergistically in inducing cytotoxicity with drugs like lenalidomide, bortezomib, and melphalan in a MM cell-endothelial cell coculture (Podar et al., 2006).

3.3.2.4. Soluble Intercellular Adhesion Molecule (sICAM-1)

Schmidmaier et al. (2007) showed that four MM cell lines (U266, RPMI-8226, OPM2, and NCI-H929) and eight primary MM patient cells had high levels of LFA-1 which correlated with significantly high amounts of sICAM-1 present in the serum of MM patients. To evaluate the role of sICAM/LFA-1 survival pathway, they utilized a LFA-1 inhibitor LFA878 and demonstrated that the inhibitor induced caspase-3 cleavage and apoptosis in MM cell lines. Further, treatment with the inhibitor led to decreased activation of the LFA-1/FAK/PI3-K/AKT survival pathway as seen by Western blotting. Finally, combination of LFA878 with src inhibitor significantly increased cell death as compared to LFA878 alone indicating a novel therapeutic option in mediating cell death in MM (Schmidmaier et al., 2007).

3.3.2.5. Transforming Growth Factor-β1

Adhesion of MM cells to patient BMSCs results in a huge induction of transforming growth factor-β1 (TGF-β1), which in turn results in the secretion of IL-6 (Urashima et al., 1996). Hayashi et al. (2004) demonstrated that addition of TGF-β1 or adhesion of MM cell to BMSCs increased the secretion of IL-6 and VEGF and triggered cell proliferation in MM cells. They then used a TGF-β receptor I kinase inhibitor SD-208 and showed that it not only significantly inhibited the secretion of IL-6 and VEGF from BMSCs (triggered by either TGF-β1 or adhesion of MM cells to BMSCs), but also decreased tumor cell growth triggered by MM cell adhesion to BMSCs. Part of the activity of SD-208 was attributed to its ability to block TGF-β1-triggered nuclear accumulation of Smad2/3 and HIF-1α (Hayashi et al., 2004).

3.3.2.6. Fibroblast Activation Protein

Ge et al. (2006) have shown that fibroblast activation protein (FAP) is a BM microenvironment factor that is upregulated in osteoclast when cocultured with primary MM cells. Moreover, knocking down of FAP in the osteoclasts resulted in reduced survival of MM cells when they were cocultured with the osteoclasts. In light of this study, Pennisi et al. (2009) wanted to evaluate if FAP is a target that can give a viable therapeutic option in MM pathogensis. The study utilized a dipeptide boronic-acid DASH (dipeptidyl peptidase (DPP) IV activity and/or structure homologs) inhibitor PT-100. PT-100 is a cell permeable inhibitor that can specifically inhibit the activity of FAP at low nanomolar concentrations (Pennisi et al., 2009). PT-100 significantly reduced the survival of MM cells cocultured with osteoclasts without having any direct cytotoxic effect on the myeloma or mature osteoclast population. In the same way, PT-100 inhibited osteoclast differentiation and subsequent pit formation without affecting the resorption activity of mature osteoclast or the differentiating abilities of osteoblasts. Part of the explanation for its action is that it reduces p38 activity in osteoclast along with significant downregulation of CD44. Finally, in a SCID-hu model, PT-100 reduced osteoclast activity, bone resorption, and tumor burden, demonstrating its value in MM pathogenesis (Pennisi et al., 2009).

3.3.2.7. Stromal Cell-Derived Factor-1 (SDF-1)

The homing of MM cells to the BM depends upon chemokines, especially the chemokine SDF-1 and its receptor CXCR4 (Alsayed et al., 2007; Kucia et al., 2005). In addition to homing SDF-1 also induces modest proliferation of MM cells by induction of ERK, MAPK, and AKT (Hideshima et al., 2002). AMD3100, an inhibitor of CXCR4, has shown to be very efficient in mobilizing HSCs and MM cells from the BM into the peripheral blood (Alsayed et al., 2007; Grignani et al., 2005). In light of this, Azab, Runnels, et al. (2009) wanted to test whether MM cells can be mobilized into circulation by AMD3100 and rendered sensitive to antimyeloma treatments. Their study demonstrated that AMD3100 enhanced the sensitivity to bortezomib by disrupting the adhesion of MM cells to BMSCs. The reversal of drug resistance was mechanistically attributed to AMD3100-induced inhibition of AKT and accumulation of cleaved PARP in MM cells in coculture with BMSCs and treated with bortezomib (Azab, Runnels, et al., 2009).

The same research group utilized ROCK inhibitor, Y27632, and rac1 inhibitor, NSC23766, to inhibit SDF-1-induced polymerization of actin and activation of LIMK, src, FAK, and cofilin in MM cell lines and patient

samples (Azab, Azab, et al., 2009). *In vivo* treatment with both inhibitors resulted in reduced homing of MM.1S cells to murine BM niches. Both the above-reported studies confirm the role of the SDF-1/CXCR4 axis in homing and as a viable therapy option in MM for increasing the efficacy of standard therapy.

3.4. Miscellaneous Agents

In this section, we have listed agents that have multiple mechanism of action all of which culminate in decreased expression of adhesion molecules on the cell surface of the MM cells or the BMSCs. It is evident that more work will need to be done to understand mechanisms that drive expression of cell adhesion receptors. Additionally, it must be noted that although reports are linked to change in expression, inhibitors denoted in the following sections have multiple mechanisms of action and thus difficult to tease out the direct role of reducing expression of adhesion on the overall phenotype.

3.4.1. PPARγ Agonist

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear superfamily that functions as ligand-dependent transcription factor and has been shown to be expressed in IL-6 responsive MM cells (Wang et al., 2004). Moreover, PPARγ ligands have shown to induce apoptosis in MM cells (Eucker et al., 2004; Ray et al., 2004). Wang et al., (2007) utilized a natural and synthetic agonist of PPARγ 15-d-PGJ2 and troglitazone, respectively, and demonstrated that these agonists reduced the binding of KAS6/1 myeloma cells to HS-5 BMSCs by downregulating the expression of VCAM-1 and ICAM-1 on the stromal cells while their respective receptor expression, VLA-4 and LFA-1, on the myeloma cells remain unchanged. The agonists were equally effective in inhibiting cell growth in MM.1R, a drug-resistant MM cell line when compared to its parent sensitive cell line, MM.1S (this cell line is resistant to dex due to truncated GR receptor), and thus not anticipated to be cross resistant to other clases of agents. The agonists also suppressed the adhesion-mediated secretion of IL-6 from the BMSCs by inhibiting the transcriptional activity of 5'CCAAT/enhancer-binding protein β (C/EBPβ) and NF-κB on the IL-6 promoter. The inhibition of transcriptional activity was brought about by forming of complexes between C/EBPβ and PPARγ and between PGC-1 and PPARγ, thus inhibiting their respective abilities to upregulate the expression of IL-6 gene (Wang et al., 2007).

3.4.2. Atiprimod

Atiprimod, an orally active anti-inflammatory drug, inhibits MM cell growth, induces apoptosis, inhibits JAK2/STAT3 and NF-κB activation pathway, and downregulates the antiapoptotic proteins BCL-2, Bcl-XL, and Mcl-1 (Amit-Vazina et al., 2005; Hamasaki et al., 2005). Neri et al. (2007) utilized gene expression and microarray data analysis to show that Atiprimod-treated MM cells have downregulation of genes involved in adhesion (ITGB4, ITGB8, CDH3, and CDHF9), cell cycle progression (PTPNR21, PTPRN2, and TGF-β2), and upregulation of pro-apoptotic genes (TNF15, TRAILR3, p21, and v-Fos). Further *in vivo* evaluation in SCID-Hu models either using MM cell lines or primary MM cells showed reduced tumor burden in Atiprimod-treated mice confirming the ability of this inhibitor to overcome the protective effects of BM milieu (Neri et al., 2007).

3.4.3. KNK-437

High levels of HSP70 is associated with drug resistance in many forms of malignancies (Chant et al., 1995; Sliutz et al., 1996). Nimmanapalli et al., (2008) have demonstrated that adhesion of the MM cells to BMSCs or FN induced the transcription of *HSPA4* mRNA and its translated protein HSP70. Furthermore, addition of IL-6 significantly increased the expression of HSP70 in MM culture alone or adherent to FN. The researchers were then able to demonstrate that the use of the HSP inhibitor, KNK-437, interferes with the induction of *HSPA4* mRNA successfully inhibited the adhesion of MM cells to FN and patient-derived primary stromal cells (Nimmanapalli et al., 2008; Yokota et al., 2000). Furthermore, KNK-437 was not only able to induce cell death in 8226 cells, melphalan resistant 8226-LR5 cells, primary CD138 cells but could also significantly reverse CAM-DR to melphalan in these cell lines.

3.4.4. Zoledronic Acid

All the studies enumerated above show the effects of inhibitors on the expression of adhesion molecules in MM cells. However, Corso et al. (2005) evaluated the effect of the biphosphonate zoledronic acid on BMSCs. In the study, BMSCs were isolated from eight patients with MM and then treated with increasing concentration of zoledronic acid. They reported that zoledronic acid caused decreased proliferation and increased apoptosis in BMSCs (Corso et al., 2005). Further, zoledronic acid treated BMSCs secreted less amount of IL-6 and had reduced expression of adhesion molecules like CD106, CD54, CD49d, and CD40. Even though the study does not

delineate the mechanism for its action, they conclude that the cumulative effect on the BMSCs might explain the antitumor activity of zoledronic acid (Corso et al., 2005).

4. CONCLUSION

Experimental evidence continues to support the critical role of the BM microenvironment in mediating *de novo* drug resistance and disease progression. Thus it is essential that target identification and drug discovery consider the complexity of multicellular model systems. As reviewed in this chapter, multiple targets and new inhibitors have been identified for interrupting survival signals which are coopted by the MM cell from the microenvironment. It is likely that many of these agents may have minimal activity as a signal agent and thus it is critical to design rationale combination strategies, and appropriate design of these trials will be important for ensuring clinical success. The other challenge will be to determine the redundancy and whether targeting upstream or downstream will be the most efficacious strategy. Finally, currently it is unclear of the heterogeneity of patient variability for coopting survival mechanisms. For example, it is feasible that during initial drug selection in some patient specimens VLA-4 will be the dominant pathway for conferring CAM-DR, while in others VLA-5, CD44, or perhaps chemokine driven inside–out activation of VLA-4 may be the dominant pathway. Thus it will be critical to move toward a personalized approach for targeting the CAM-DR phenotype associated with cell adhesion as well as for inhibitors of soluble factors that confer drug resistance or contribute to bone disease.

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ABBREVIATIONS

Ang-1 angiopoitin-1
BM bone marrow
BMP-2 bone morphogenetic protein type 2

BMSCs bone marrow stromal cells
CAM-DR cell adhesion-mediated drug resistance
CVA21 coxsackievirus A21
DAF decay accelerating factor
DKK-1 dickkopf-1
ECM extracellular matrix
FAP fibroblast activation protein
FGF-2 fibroblast growth factor-2
FN fibronectin
HA hyaluronan
HGF hepatocyte growth factor
HSP heat shock protein
ICAM-1 intercellular adhesion molecule-1
IGF-1 insulin growth factor-1
IKK IκB kinase
IL-6 interleukin-6
MAdCAM-1 mucosal addressin cell adhesion molecule-1
MGUS monoclonal gammopathy of undetermined significance
MIP-1α macrophage inflammatory protein-1α
MM multiple myeloma
MMP matrix metalloproteinase
MSCs mesenchymal stromal cells
NCAM-1 neural cell adhesion molecule-1
OPG osteoprotegrin
OPN osteopontin
PPARγ peroxisome proliferator-activated receptor γ
RANK receptor activator of nuclear factor-kappaB
RANKL RANK ligand
SDF-1 stromal cell-derived factor 1
sFRP2 secreted frizzled-related protein-2
SMM smoldering multiple myeloma
TGF-β1 transforming growth factor-β1
TNF-α tumor necrosis factor-α
VCAM-1 vascular cell adhesion molecule-1
VEGF vascular endothelial growth factor

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CHAPTER SEVEN

Targeting Notch Signaling for Cancer Therapeutic Intervention

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Abstract

The Notch signaling pathway is an evolutionarily conserved, intercellular signaling cascade. The Notch proteins are single-pass receptors that are activated upon interaction with the Delta (or Delta-like) and Jagged/Serrate families of membrane-bound ligands. Association of ligand-receptor leads to proteolytic cleavages that liberate the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates to the nucleus, where it forms a complex with the DNA-binding protein CSL, displacing a histone deacetylase (HDAc)–corepressor (CoR) complex from CSL. Components of a transcriptional complex, such as MAML1 and histone acetyltransferases (HATs), are recruited to the NICD–CSL complex, leading to the transcriptional activation of Notch target genes. The Notch signaling pathway plays a critical role in cell fate decision, tissue patterning, morphogenesis, and is hence regarded as a developmental pathway. However, if this pathway goes awry, it contributes to cellular transformation and tumorigenesis. There is mounting evidence that this pathway is dysregulated in a variety of malignancies, and can behave as either an oncogene or a tumor suppressor depending upon cell context. This chapter highlights the current evidence for aberration of the Notch signaling pathway in a wide range of tumors from hematological cancers, such as leukemia and lymphoma, through to lung, skin, breast, pancreas, colon, prostate, ovarian, brain, and liver tumors. It proposes that the Notch signaling pathway may represent novel target for cancer therapeutic intervention.

1. INTRODUCTION

1.1. Overview of the Notch Signaling Cascade

Notch families are single-pass transmembrane proteins that have dual functions as both cell surface receptors and nuclear transcriptional regulators. The Notch was initially noticed to be responsible for the specific phenotype displayed as "notches" at the wing blades of *Drosophila melanogaster* (Fig. 7.1) by John S. Dexter in 1914 and the alleles of the Notch gene were identified in 1917 by Thomas Hunt Morgan (Blaumueller et al.,

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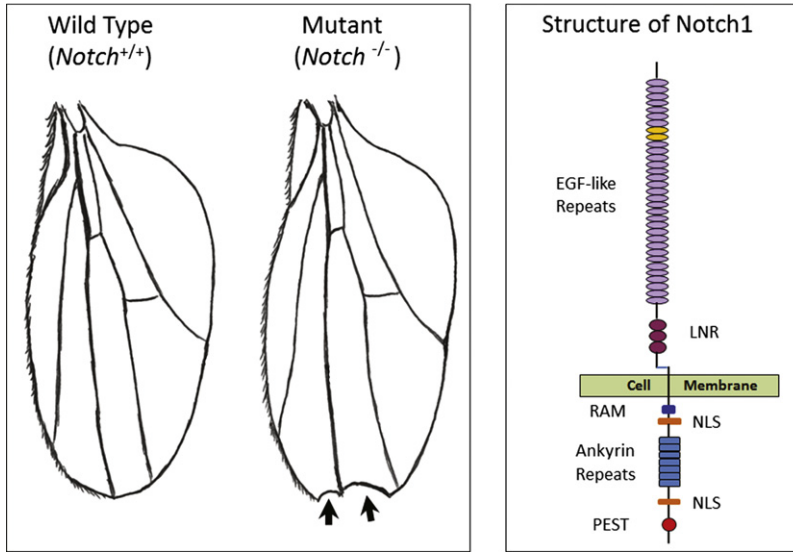

Figure 7.1 Illustration of phenotype displayed as "notches" at the wing blades of *Drosophila melanogaster* in which *Notch* gene is mutated/deleted (*left*). Schematic illustration of molecular structure of transmembrane Notch 1 protein (*right*). For color version of this figure, the reader is referred to the online version of this book.

1997). The molecular analysis and gene sequencing was independently undertaken by Spyros Artavanis-Tsakonas and Michael W. Young in the1980s (Schroeter et al., 1998; Wharton et al., 1985). In mammals, the Notch families have four receptors (Notch1–4). Each Notch receptor is synthesized as a full-length precursor protein (300–350 kDa) consisting of extracellular, transmembrane, and intracellular domains that correlate with different cellular functions. Extracellular domain of Notch has 29–36 small cysteine knotmotifs called EGF-like repeats which is responsible for ligand binding (Deftos et al., 2000; Martinez Arias et al., 2002), a heterodimer domain, and three LNR (Lin-12, Notch repeats) domains, followed by transmembrane domain, ankyrin repeats, and a PEST motif (Kopan et al., 1994). The unprocessed Notch precursors are cleaved at the S1 site by furin-like convertase within the Golgi apparatus and reassembled as a heterodimer on the cell surface (Blaumueller et al., 1997). There are five Notch ligands (Jagged 1–2, Delta-like (Dll) 1, 3, and 4) in mammals. Notch ligands are also transmembrane proteins. It means that Notch ligand-expressing cells typically must contact with the Notch-expressing cell for signaling to occur. Both the Jagged and Dll proteins are members of the DSL (Delta/Serrate/LAG-2) family and they have multiple EGF

repeats on extracellular domain for interaction with Notch receptors. In addition, Jagged proteins have a cysteine-rich domain (Wilkin et al., 2004). Notch signaling activation is initiated by ligand-receptor binding between two adjacent cells. This interaction of the ligand–receptor induces a conformational change in Notch receptors that leads to two successive proteolytic cleavages in Notch receptors. The first cleavage is mediated by metalloprotease (ADAM17 (A Disintegrin and Metalloprotease 17)/TACE (TNF-α Converting Enzyme)) at the extracellular domain (S2) (Brou et al., 2000; Mumm et al., 2000). This makes Notch susceptible to the second cleavage at the transmembrane domain (S3), which is carried by γ-secretase, a five-subunit complex. The γ-secretase complex is composed of presenilin1 and 2, nicastrin, Pen-2, and Aph1 (De Strooper et al., 1999; Schroeter et al., 1998). Following these two cleavage steps, the Notch intracellular domain (NICD) is released to the cytoplasm, and enters into the nucleus to activate the transcription of Notch target genes. Following NICD translocation into the nucleus, NICD binds to a transcriptional repressor CSL (also known as CBF1, or RBP-Jκ) to displace the corepressor complex. Binding with NICD switches CSL into an activated state. Additionally, the NICD/CSL complex recruits co-activators, such as Mastermind-like (MAML) (Wu & Griffin, 2004) and p300, whichfacilitate the transcriptional activation of Notch target genes (Wallberg et al., 2002) (Fig. 7.2). Primary Notch target genes include two families of transcriptional factors, Hes (Hairy and E (spl)) and Herp (Hes-related repressor protein) (also known as Hey/Hesr/HRT/CHF/gridlock). The helix-loop-helix domain in both Hes and Herp families determines the dimerization of Hes and Herp proteins. Homo- or hetero-dimers of Hes and/or Herp bring about repression of transcription by interacting with other corepressors or sequestering transcriptional activators (Iso et al., 2003). Other Notch target genes include cyclins D1 (Ronchini & Capobianco, 2001), p21 (Rangarajan et al., 2001), NF-κB (Cheng et al., 2001), pre-Tα (pre-T-cell receptor alpha chain) (Reizis & Leder, 2002), GATA3 (Amsen et al., 2007), NRARP (Lamar et al., 2001), c-Myc (Weng et al., 2006), and Deltex1 (Izon et al., 2002).

In addition to the canonical activation of the Notch pathway, there is increasing evidence that Notch can signal in CSL-independent modes (Martinez Arias et al., 2002). For instance, activation of CSL-dependent Notch signaling can prevent the differentiation of C2C12 cells upon serum withdrawal, and this is likely to occur by inhibiting the function of the muscle-specific transcription factor MyoD (Kopan et al., 1994).

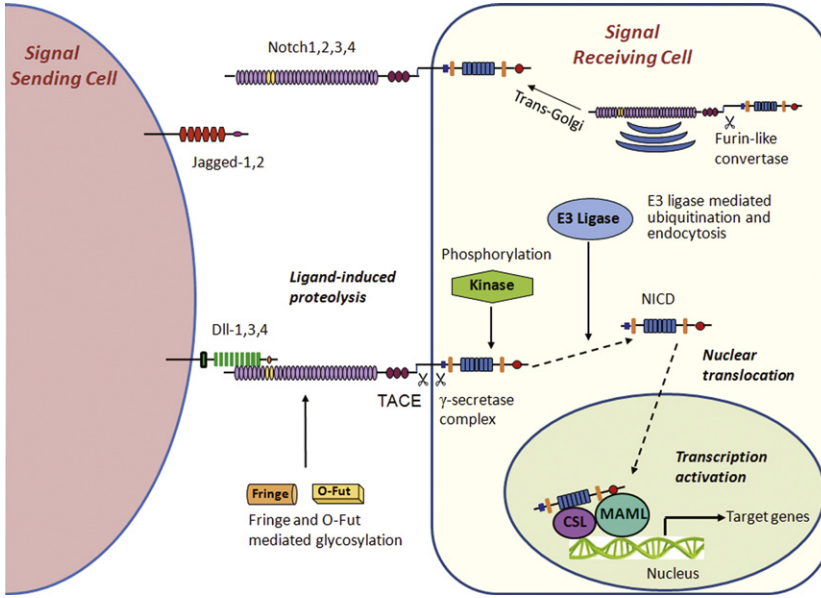

Figure 7.2 The Notch signaling cascade. The Notch receptors (Notch1–4) are single-pass transmembrane proteins that are activated by the Delta-like and Jagged families of membrane-bound ligands expressed on adjacent cells. Upon furin-mediated trans-Golgi digestion, Notch proteins are transported to the plasma membrane and form matured heterodimer on the cell surface. Interaction with ligands leads to two additional proteolytic cleavages (TACE and γ-secretase complex) that liberate the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates to the nucleus, where it forms a complex with the DNA binding protein CSL. Co-activators, such as MAML, are recruited to the NICD-CSL complex, leading to the transcriptional activation of Notch target genes. Notch receptors can be posttranslationally modulated by glycosylation, which are mediated by the enzymes of the glycosyltransferase Fringe and *O*-fucosyl transferase 1 (*O*-Fut), and phosphorylation. In addition, Notch can be regulated by different E3 ligases to undergo ubiquitination and subsequent proteolysis or endocytosis. For color version of this figure, the reader is referred to the online version of this book.

1.2. Modulation of Notch Pathway

Notch signaling is an unusual signaling pathway because of its activity independent of secondary messengers for amplification. Notch pathway can be modulated at various levels. Pathway-intrinsic, including feedback regulation of receptor and ligand transcription, glycosylation, differential intracellular trafficking, and receptor and ligand ubiquitination and endocytosis, as well as pathway-extrinsic mechanisms, including cross talk between Notch and other major signaling mechanisms, modulate Notch signaling,

contributing to the versatile output (Bruckner et al., 2000; Callahan & Egan, 2004; Haines & Irvine, 2003; Kidd et al., 1998; Nie et al., 2002).

One characteristic of Notch signaling is the involvement of multiple enzymatic modulations, which serve to regulate Notch signal transduction. Besides ligand-triggered, metalloprotease and γ-secretase-mediated proteolytic cleavages, and furin-mediated Notch maturation, Notch signaling can be regulated by four E3 ligases (Su(dx)/Itch, Sel-10, Neutralized, and LNX (ligand of Numb-protein X)) to undergo ubiquitination and subsequent proteolysis. Notch endocytosis by a different class of E3 (Nedd4) promotes the degradation of Notch whereby activation of the Notch signaling is attenuated/terminated (Lai, 2002; Le Borgne et al., 2005; Sakata et al., 2004; Wilkin et al., 2004). LNX also can ubiquitinate the Numb, a Notch antagonist for degradation, which enhances/stabilizes the Notch pathway activation (Callahan & Egan, 2004; Nie et al., 2002).

Moreover, Notch receptors are posttranslationally modified by glycosylation (Bruckner et al., 2000) and phosphorylation (Kidd et al., 1998), adding further complexity to the regulation of Notch signaling. The Notch receptors can be glycosylated extracellularly at the EGF-like repeats. Enzymes that process the extracellular posttranslational modification include the glycosyltransferase Fringe and *O*-fucosyl transferase 1 (*O*-Fut). Fringe enzymes add *N*-acetyl-glucosamine to the O-linked fucose to inhibit the binding of Notch receptors to Jagged. In contrast, Fringe potentiates Delta-initiated Notch activation (Haines & Irvine, 2003). The mechanism underlying such a ligand-dependent regulatory effect remains unclear. The Notch protein is phosphorylated variably on serines of the cytoplasmic domain (Kidd et al., 1989). The phosphorylated NICD can preferentially associate with Su(H). Formation of NICD/Su(H) complex may determine the subcellular location of NICD (Kidd et al., 1998) . The studies of Notch posttranslational modification by enzymes provide both a direction for further elucidation of the mechanisms that regulate Notch activation and a new paradigm for the role of enzymatic modifications in Notch-related diseases, especially cancers.

2. NOTCH SIGNALING IN CANCER

The Notch pathway is an evolutionally conserved signaling pathway that has been implicated in a wide variety of processes, including cell fate determination, tissue patterning and morphogenesis, cell differentiation,

proliferation, and death. Notch signaling is, therefore, one of the critical pathways in embryonic development and patterning. Given that tumorigenesis and organ development are believed to share similar mechanisms, it is not surprising that developmental pathways, such as Notch, Wnt, and Hedgehog, are employed by tumor cells for their development and progression. Highly aggressive tumor cells have been shown to carry many characteristics of embryonic progenitor cells and use the Notch signaling pathway to promote their survival. Dysregulation of the Notch pathway has been associated with a wide range of cancers (Balint et al., 2005; Santagata et al., 2004; Wang, Zhang, et al., 2006). The Notch pathway could be either oncogenic or tumor suppressive depending on the tissue and organ site in which it is expressed (Table-7.1). However, how does activation of a single pathway give rise to two opposite outcomes in different cell types and contexts remains to be a mystery. One explanation for this seemingly paradoxical response is that canonical Notch pathway turns on/off different tissue/cell-specific target gene(s) or downstream pathway(s) that determine the ultimate effect of Notch signaling. For example, in keratinocytes, perhaps only CSL binds the *p21* promoter, thereby Notch functions as a tumor suppressor in this type of cells. Another potential explanation is that it depends upon other cooperative signaling(s). For instance, Notch1-deficient mice develop spontaneous, highly vascularized basal cell carcinoma (BCC)-like tumors. In both mouse and human, BCC is frequently associated with deregulated Hedgehog (Shh) signaling, and Notch1-deficiency in the mouse skin leads to increased Gli2 expression, which is a downstream component of the Shh pathway (Nicolas et al., 2003). Another pathway that seems to be deregulated as a consequence of loss of Notch1 is Wnt pathway, which results in increased β-catenin-mediated signaling in hyperproliferative skin and primary tumor lesions, suggesting that Notch might suppress Wnt signaling in the skin (Nicolas et al., 2003). The cross talk between these pathways comprehensively determines the identity and threshold of downstream pathway(s) which controls cell fate. With respect to the different roles of Notch in cancers, further studies are needed to specifically identify the underlying mechanisms.

The general mechanisms of deregulation of Notch signaling characterized in cancers include chromosomal translocation (t (7, 9))-resulted constitutive expression of NICD (Ellisen et al., 1991), gain-of-function mutations in Notch1 in human T-cell acute lymphoblastic leukemia (T-ALL) (Weng et al., 2004), gene amplification of Notch3 in ovarian serous carcinoma (Nakayama et al., 2007), and the low levels of the Notch

Table 7.1 Involvement of Aberrant Notch Signaling in a Wide Variety of Cancers. Notch Signaling may Act as a Tumor Suppressor or a Promoter Depending on the Type of Tumor

Tumor Type	Notch/Ligand	Function	References
T-ALL	Notch1	oncogenic	Weng et al., (2004)
AML	Jagged1	oncogenic	Tohda et al., (2005)
B-CLL	Notch1, Notch2/Jagged1, Jagged2	oncogenic	Rosati et al. (2009)
Diffuse large B-cell lymphoma	Notch2	oncogenic	Lee et al. (2009)
Marginal zone lymphoma	Notch2	oncogenic	Troen et al. (2008)
Multiple myeloma	Notch1, Notch2/Jagged1	oncogenic	Jundt et al. (2004)
pre-B-ALL	Notch1−4	Tumor suppressive	Nefedova et al. (2004); Zweidler-McKay et al. (2005)
Breast cancer	Notch1, Notch4	oncogenic	Dievart et al. (1999); Raafat et al. (2004)
Human Breast cancer	Notch1/Jagged1	oncogenic	Reedijk et al. (2005)
Human Breast cancer	Notch2	Tumor suppressive	Parr et al. (2004)
SCC	Notch1	Tumor suppressive	Proweller et al. (2006)
Melanoma	Notch1	Oncogenic	Balint et al. (2005); Bedogni et al. (2008); Liu et al. (2006)
NSCLC	Notch3	Oncogenic	Haruki et al. (2005); Konishi et al. (2010)
ACL	Notch1/Jagged1, Dll1, Dll4	Tumor suppressive	Zheng et al. (2007)
SCLC	Notch1	Tumor suppressive	Sriuranpong et al. (2001, 2002)

(*Continued*)

Table 7.1 Involvement of Aberrant Notch Signaling in a Wide Variety of Cancers. Notch Signaling may Act as a Tumor Suppressor or a Promoter Depending on the Type of Tumor—cont'd

Tumor Type	Notch/Ligand	Function	References
CRC	Notch1/Jagged1, Jagged2, Dll4	Oncogenic	Jubb et al. (2009); Meng et al. (2009); Reedijk et al. (2008)
Pancreatic cancer	Notch1, Notch3, Jagged2, Dll4	Oncogenic	Miyamoto et al. (2003); Mullendore et al. (2009); Sawey et al. (2007)
Glioblastoma	Notch2	Oncogenic	Fan et al. (2010)
Ovarian cancer	Notch1, Notch3?	Oncogenic	Hopfer et al. (2005)
Prostate cancer	Notch1	Oncogenic	Shou et al. (2001)
Prostate cancer	Notch1	Tumor suppressive	Gupta et al. (2008)
Liver cancer	Notch1	Tumor suppressive	Qi et al. (2003); Viatour et al. (2011)
Kaposi's sarcoma	Notch1, Notch2, Notch4	Oncogenic	Curry et al., (2005); Lan et al. (2006)

antagonist Numb in human breast cancers (Pece et al., 2004). One main difficulty in the Notch study is to address how this simple, direct pathway gives rise to two opposite effects in different cell types and contexts. This review recapitulates the recent studies about the multifunctions of Notch and the potential therapeutic implications in cancers.

2.1. Notch in Hematological Tumors

Notch activation has been implicated in tumorigenesis of various hematological diseases, including leukemias, lymphomas, and multiple myeloma. In 1991, it was discovered that the chromosomal translocation (t (7; 9)) leads to constitutive activation of Notch1 in human T-ALL (Ellisen et al., 1991). Afterwards, the gain-of-function mutations in Notch1 receptor located at heterodimerization (HD) domain–encoding locus (exon 26 and 27), transcriptional activation domain, and PEST domain (exon 34) (Weng et al., 2004) were identified as a novel mechanism for the constitutive activation of Notch1 in human T-ALL. Most Notch-dependent T-ALL cell lines and about 20% of primary T-ALL cell lines have mutations both in HD domains and PEST domains. When mutations occur at both sites in human T-ALL, they can produce synergistic effects in Notch activation (Weng et al., 2004). c-Myc has been characterized to be a direct target of Notch1 in Notch-dependent T-ALL cell lines. Notch1 stimulates the transcription of *c-Myc* by binding to its promoter through a region containing a conserved CSL binding site (Weng et al., 2006). In addition, stimulation of the mTOR pathway by mitogens requires concurrent Notch signals in T-ALL cell lines (Chan et al., 2007). Interestingly, the effect of Notch1 withdrawal on the mTOR pathway can be rescued by enforced expression of c-Myc. This data indicates that c-Myc acts as an intermediary protein in between Notch and mTOR (Chan et al., 2007).

Although Notch activation represents a common feature in T-ALL pathogenesis, the role of Notch signaling in acute myeloid leukemia (AML) is not remarkable. Gain-of-function mutations of Notch have been seldomly established for AML (Fu et al., 2006; Palomero et al., 2006). Previous studies showed that even though Notch1 activation remains low in primary AML cells, the Notch ligand Jagged1 is widely expressed (Chiaramonte et al., 2005; Tohda & Nara, 2001). A recent study indicates that the ligand stimulation of Jagged1 in primary AML cells from 12 patients has no effects on the self-renewal of AML cells, but instead promotes the differentiation of

AML cells (Tohda et al., 2005). However, the underlying mechanism of how Notch signaling relates to the abnormal growth of AML remains unclear.

Notch receptors (Notch1 and Notch2) and their ligands (Jagged1 and Jagged2) are also constitutively expressed in B-chronic lymphocytic leukemia (B-CLL), but not normal B-cells. Moreover, Notch activation in B-CLL is accompanied with cellular inhibitor of apoptosis protein 2 (c-IAP2) and X-linked inhibitor of apoptosis protein (XIAP) expression. These represent additional novel potential therapeutic targets for the treatment of this disease (Rosati et al., 2009).

Notch1 has been implicated in the determination of T-cell fate and the maturation of early T-cells in the thymus (Radtke et al., 1999). In contrast, Notch2 is widely expressed in mature B-cells and is indispensable for the development of marginal zone B cell lineage. In a study by Lee et al. (2009), five diffuse large B-cell lymphoma samples were found to harbor Notch2 mutations. These mutations are located on the PEST domain of Notch2, and confer increased activity to Notch2 receptors. This suggests that gain-of-function of Notch2 mutations plays a role in the oncogenesis of diffuse large B-cell lymphoma (Lee et al., 2009). In addition, activating mutations in Notch2 are also involved in marginal zone lymphomas, another type of B-cell malignancy (Troen et al., 2008). Although the mutations of Notch are not widely identified in B-cell tumors, high levels of active Notch receptors and ligands (Jagged1) have been reported in B-cell malignancy (Jundt et al., 2004; Lee et al., 2009; Zweidler-McKay et al., 2005). Collectively, these findings suggest a ligand-dependent Notch activation in B-cell tumors. Some studies demonstrate that activation of Notch signaling induces growth arrest and apoptosis in B-cell tumors, including human B-cell leukemia, Hodgkin's disease, and multiple myeloma (Nefedova et al., 2004; Zweidler-McKay et al., 2005). However, a number of studies provide opposite evidence concerning the role of Notch in B-cell malignancy, showing that active Notch actually promotes the proliferation of B-cell tumors (Jundt et al., 2002, 2004; Lee et al., 2009). To explain the discrepancy, further investigations and more meticulous examinations are necessary. Notch may exert different roles at different stages of B-cell development. It has been noted that Notch has an inhibitory effect during B progenitor commitment (Souabni et al., 2002). On the contrary, Notch may have positive effects on B-cell lineage during the later stage (Tanigaki et al., 2003).

2.2. Notch in Solid Tumors

Deregulation of Notch pathway has been connected with the tumorigenesis in a variety of solid cancers. Depending upon the type of tumor, Notch signaling can function as either a tumor promoter or a suppressor.

2.2.1. Breast Cancer

Breast cancer is the most common malignancy in women, accounting for one quarter of all female cancer (Jemal et al., 2010). The tumorigenic activity of Notch in breast cancer has been established in mouse models. In 1987, the insertion of mouse mammary tumor virus (MMTV) into the *Notch4* locus, referred to as *int3* in the Czech II mouse strain was discovered (Gallahan & Callahan, 1987), providing the first link between Notch and breast cancer. This group further reported that the MMTV-mediated insertion led to the truncated form of Notch4 protein, which is constitutively active. Besides Notch4, involvement of Notch1 in the formation of murine mammary tumors has also been identified. Notch1 is mutated by MMTV insertion and the truncated form of Notch1 functions as an oncogene in the development of mammary carcinomas (Dievart et al., 1999). Although the correlation of aberrant Notch signaling with mammary tumors is well established in murine models, such a correlation to human breast cancer is less robust. Callahan and his coworkers observed that expression of human-int3 (Notch4/In3) in transgenic mice blocked normal mammary development and induced the formation of breast tumors with an increased latency (average 18 months) (Raafat et al., 2004). However, in most studies regarding human breast cancers, activated Notch is only detectable at the protein level, rather than the mRNA level (Clarke et al., 2005; Parr et al., 2004; Reedijk et al., 2005). Parr's data further shows that Notch1 is increased in poorly differentiated breast tumors, while high level of Notch2 is associated with a higher chance of survival, suggesting Notch1 exerts a tumor-promoting function and Notch2 functions as a tumor suppressor in human breast cancers (Parr et al., 2004). Very recently, Robinson et al. (2011) have identified a novel genetic mechanism employed by Notch gene families in breast cancer. Using paired-end transcriptome sequencing to explore the landscape of gene fusions in a panel of breast cancer cell lines and tissues, they observed that individual breast cancers have a variety of expressed gene fusions, and identified recurrent gene rearrangements in Notch gene family. They also demonstrated that Notch-family gene fusion has substantial phenotypic effect in breast epithelial cells. Breast cancer cell

lines harboring Notch gene rearrangements are uniquely sensitive to inhibition of Notch signaling both *in vitro* and *in vivo*.

So far, many studies have indicated that the Notch signaling plays an oncogenic role in breast cancers mainly through its interaction with other signaling pathways in mammary tumorigenesis. The well-characterized pathways which have the interactions with the Notch signaling during the oncogenesis of breast cancer include Ras, Erb2, TGF-β, and Wnt signaling pathways. Four of seven cases of Notch1-positive human breast ductal carcinomas are H-Ras positive. This data suggests that Notch1 is the downstream effector of Ras signaling (Weijzen et al., 2002). Furthermore, 80% of the mice with transgenic human Ras developed mammary tumors. Conversely, in mice with transgenic Ras and Notch inhibitor Deltex, only 20% developed mammary tumors. This highlights the cooperative functions of Ras and Notch in the development of breast cancers (Weijzen et al., 2002). Of interest, tumors co-expressing high levels of Notch1 and Jagged1 correlate with poor survival of human breast cancers (Reedijk et al., 2005). The human ErbB2 protein is a receptor tyrosine kinase that belongs to the human epidermal growth factor receptors (hEGFRs) family (Coussens et al., 1985). The amplification and overexpression of the *ErbB2* gene occurs in 20–30% of human breast cancers. ErbB2 behaves as an oncogene in collaboration with Notch1 in the development of mouse mammary tumors (Dievart et al., 1999). The overexpression of ErbB2 suppresses Notch activity and leads to decreased expression of canonical Notch target genes, including Hey1, Hes1, and Hes5 (Osipo et al., 2008). Furthermore, inhibition of ErbB2 by trastuzumab, a tyrosine kinase inhibitor (TKI), increases Notch1 activity and sensitizes the breast cancer to a GSI (γ-secretase inhibitor) (Osipo et al., 2008). This data suggests that combination of GSI with chemotherapy including trastuzumab may increase the efficacy of trastuzumab and reverse the resistance to ErbB2-targeted therapies.

2.2.2. Skin Cancer

Basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma, originated from keratinocytes and melanocytes, respectively, are three different types of skin cancers. Notch signaling has been observed to have dual functions in skin cancers, depending on the cell type and context. As a consequence of loss of Notch1 activation in murine skin, basal cell carcinoma–like tumors are developed, suggesting that the Notch pathway exerts tumor suppressive effects in the skin (Nicolas et al., 2003). Inhibition of Notch signaling by dominant negative–MAML1 (DN-MAML1) in

transgenic mice promotes the formation of cutaneous squamous cell carcinoma and dysplastic precursor lesions, suggesting that the canonical Notch pathway confers epidermal skin cells a protection against cutaneous SCC (Proweller et al., 2006). A study by Demehri et al., (2009) shows that Notch1 promotes tumorigenesis of skin cancer by disrupting the skin barrier integrity and producing a wound-like stromal microenvironment. In contrast, evidence suggesting the tumorigenic activities of Notch1 signaling in melanoma has emerged. It has been demonstrated that Notch1 is activated in melanoma, and active Notch1 promotes progression of primary melanoma towards an advanced stage (Balint et al., 2005; Liu et al., 2006; Pinnix et al., 2009). In addition, active Notch1 confers a transformed phenotype to primary melanocytes *in vitro* (Pinnix et al., 2009). Findings further indicate that Notch1 signaling is indispensable for Akt and hypoxia to transform melanocytes, suggesting Notch1 is the downstream effector of Akt and hypoxia during melanomagenesis (Bedogni et al., 2008). The molecular mechanism whereby the Notch signaling promotes melanoma progression has not been fully determined while previous studies revealed several potential downstream pathways, such as β-catenin pathway, Mel-CAM, N-Cadherin, and MAPK pathway, which might mediate the oncogenic effect of the Notch signaling (Balint et al., 2005; Liu et al., 2006; Pinnix et al., 2009). Further understanding of the precise role of Notch in specific skin cancers may help us develop a rationale for novel Notch-based therapeutics.

2.2.3. Lung Cancer

Like its dual functions in skin cancer, Notch signaling may also behave as either an oncogene or a tumor suppressor in lung carcinomas, depending on the tumor cell type. In non–small cell lung cancers (NSCLC), one study showed that Notch3 mediated signaling is active and promotes the growth of lung tumors (Haruki et al., 2005). In fact, inhibition of Notch3 by MRK-003, a GSI, reduces tumor cell proliferation and induces apoptosis in human NSCLC (Konishi et al., 2010). However, Chen et al. reported that Notch1 protein is downregulated in NSCLC cell lines and expression of constitutively active Notch1 in adenocarcinoma of the lung (ACL) cells causes cell death. These data suggest that the opposite functions of Notch signaling are highly context dependent. Interestingly, under hypoxic conditions, Notch1 is dramatically upregulated, which seems to be essential for cell survival in ACL, a type of NSCLC (Chen et al., 2007). These results indicate that oxygen concentration determines the biological effects of Notch1 signaling in ACL. A similar observation of the expression of Notch1–3 in the cell line

A549 and SPC-A-1 of the human lung adenocarcinoma has also been obtained (Zheng et al., 2007). Overexpression of NICD inhibits the growth of the lung adenocarcinoma A549 cells *in vitro* by induction of cell cycle arrest and suppresses tumor growth of A549 in nude mice (Zheng et al., 2007). These findings suggest that the Notch signaling may function as a tumor suppressor in human lung adenocarcinoma cells. As a comparison, Notch1 and Notch2 have low-level expression in small cell lung cancers (SCLC), and overexpression of Notch causes growth inhibition in SCLC cells (Sriuranpong et al., 2001, 2002).

In addition, alterations of the Notch pathway in lung cancer have been reported. Westhoff et al. (2009) have observed that Notch signaling is altered in approximately one-third of NSCLCs. In ~30% of NSCLCs, loss of Numb expression leads to increased Notch activity, while in a smaller fraction of cases (around 10%), gain-of-function mutations of the Notch1 gene are present. They also found that activation of Notch pathway correlates with poor clinical outcomes in NSCLC patients without TP53 mutations (Westhoff et al., 2009). On the other hand, Wang et al. (2011) have observed loss-of-function mutations in Notch1 and Notch2 in lung (and cutaneous) squamous cell carcinoma. Notch aberrations in lung squamous cell carcinoma include frameshift and nonsense mutations, leading to receptor truncations as well as point substitutions in key functional domains that abrogate Notch signaling.

2.2.4. Colorectal Cancer

Colorectal cancer is the second most common cause of malignancy deaths worldwide (Parkin, 1994). The early growth of colorectal tumors requires angiogenesis (Goodlad et al., 2006; Korsisaari et al., 2007), which is dependent on the increased expression of proangiogenic factors (e.g., vascular endothelial cell growth factor-A (VEGF-A)) (Ferrara et al., 1991; Korsisaari et al., 2007). The Notch ligand, Dll4, is expressed by endothelial cells (Indraccolo et al., 2009; Thurston et al., 2007) and can be induced by VEGF (Liu et al., 2003) and hypoxia through hypoxia-inducible-factor (HIF)-1α (Patel et al., 2005). A recent study has found that Dll4 is highly expressed in the endothelium of a large cohort of colon cancers and this expression is dramatically correlated with VEGF and hypoxia (Jubb et al., 2009). It implicates that Dll4-Notch pathway may be a potential therapeutic target of colon cancer. The Notch1 receptor has been discovered to be active in response to chemotherapy in colon cancer cells (Meng et al., 2009). Downregulation of Notch1 signaling with GSI sensitizes colon cancer cells

to chemotherapy, whereas overexpression of NICD increases resistance to chemotherapy. Therefore, suppression of Notch1 signaling may be a novel therapeutic target to increase the sensitization of colon cancer cells to chemotherapy (Meng et al., 2009). Reedijk et al. have suggested that expression of Jagged ligands and Notch1 as well as Notch receptor activation are constant features of human colon cancers, thus application of GSIs and other anti-Notch therapeutics may benefit patients with this disease (Reedijk et al., 2008).

2.2.5. Pancreatic Cancer

Pancreatic cancer is one of the most aggressive human malignancies. Aberrant activation of the Notch pathway is commonly observed in pancreatic cancer (Miyamoto et al., 2003; Mullendore et al., 2009; Sawey et al., 2007; Wang, Zhang, et al., 2006). High-level expression of Notch ligands, including Jagged2 and Dll4, are detectable in the majority of pancreatic cancer cell lines. Inhibition of Notch pathway either by siRNA targeting Notch1 or by means of GSI (GSI18) alleviates anchorage-independent growth in PANC-1 cells, indicating that sustained Notch activation is required for pancreatic cancer maintenance (Mullendore et al., 2009; Wang, Zhang, et al., 2006). Similarly, a study by Wang et al. showed that inhibition of γ-secretase activity by GSI reduced the growth of premalignant pancreatic duct–derived cells in a Notch-dependent manner and the tumor development in a murine model of pancreatic ductal adenocarcinoma (PDAC) (K-Ras, p53 L/+ mice). These data suggest that Notch pathway is essential for PDAC progression. Interestingly, TW-37, a small molecule of Bcl2 family proteins, is able to inhibit cell growth and induce apoptosis in pancreatic cancer through a downregulation of the Notch1 activity (Wang, Azmi, et al., 2009). This finding suggests that the antitumor agent TW-37 plays an inhibitory role in pancreatic tumor growth, at least, partially through the inactivation of Notch signaling. Suppression of Notch3 by Notch3-specific siRNA can increase gemcitabine-induced caspase-mediated apoptosis in pancreatic cancer through inactivation of PI3K/Akt-dependent pathway, suggesting Notch3 is a potential therapeutic target for pancreatic cancer (Yao and Qian, 2009).

2.2.6. Glioblastoma

Glioblastoma (GBM) is the most common malignant brain tumor in adults. Despite recent advances in surgery, imaging, chemotherapy, and radiotherapy, outcome in GBM remains poor and recurrence remains high.

Therefore, novel efficient strategies are desperately needed to treat this disease. One study showed that inhibition of Notch by GSIs or shRNA sensitizes glioma stem cells to radiation at clinically relevant doses. Such results suggest that integrated Notch signaling is involved in radioresistance of glioma stem cells (Wang, Wakeman, et al., 2009). Another similar study demonstrates that Notch2 activation in GBM neurospheres increases their growth *in vitro* and Notch blockade with GSIs depletes the stem-like cells required for GBM *in vivo* and *in vitro* (Fan et al., 2010). This data suggests that GSIs might be applied as useful chemotherapeutic agents by targeting cancer stem cells in gliomas. Hence, a combination regimen of GSIs and radiotherapy may be a highly efficacious strategy in the treatment of malignant GBM. Interestingly, activation of Notch signaling in GBM can result from ligand stimulation from endothelial cells that nurture self-renewal of cancer stem cells (Zhu et al., 2011).

2.2.7. Ovarian Cancer

Ovarian cancer is such an aggressive disease that the overall mortality rate reaches to about 50% (Berg & Lampe, 1981). It has been suggested that Notch signaling functions as a tumor promoter in ovarian carcinoma. Several Notch pathway components are expressed in epithelial ovarian tumors. Ovarian carcinomas express higher Hes1 protein levels than adenomas, indicating a stronger Notch pathway activation. Constitutive activation of Notch1 pathway by overexpression of NICD in A2780 ovarian carcinoma cells promotes their proliferative and survival advantage (Hopfer et al., 2005). In addition, *Notch3* gene amplification is found to occur in more than half of the ovarian serous carcinomas (Park et al., 2006). Moreover, abundant NICD expression in three ovarian cancer cell lines as well as in 16 of 21 (76%) human ovarian cancer samples has been reported. Antagonizing NICD via siRNA results in a significant growth inhibition in all three ovarian cancer cell lines (Rose et al.,).

2.2.8. Prostate Cancer

Prostate cancer is one of the most frequently diagnosed tumors in men and the second leading cause of cancer-related death in the United States. It has become a significant health problem (Jemal et al., 2009). Notch signaling is required for embryonic and postnatal prostatic growth and development, for proper cell lineage specification within the prostate, as well as for adult prostate maintenance and regeneration following castration and hormone replacement. Evidence for Notch as a regulator of prostate cancer

development, progression, and metastasis has also emerged (Leong & Gao, 2008). Downregulation of Notch1 and Jagged1 has been shown to inhibit prostate cancer cell growth, migration/invasion, and to induce cell apoptosis *in vitro*. These effects are achieved through inactivation of Akt, mTOR, and NF-κB signaling pathways (Ma et al.; Wang et al., 2009). Consistently, activation of Notch signaling attenuates its inhibitory effect on prostate cancer cell migration (Kim et al.,). Interestingly, prostate cancer cell lines C4-2B and PC3 that are derived from bone metastases express Notch1 while LNcaP and DU145 which are not derived from bone metastases lack Notch1 receptor (Mamaeva et al., 2009). These findings are consistent with observations made by another group (Zayzafoon et al., 2004). Therefore, Notch1 appears to be critical for prostate cancer metastases. However, whether Notch is a promoter or a suppressor in prostate cancer metastasis remains unclear. A metastasis-promoting function of Notch in prostate cancer has been suggested *in vivo*. In transgenic prostates from TRAMP mice, prostate cancer cells that metastasize to the lymph nodes exhibit high levels of Notch1 mRNA (Shou et al., 2001). In contrast, Notch signaling may play a metastasis-inhibiting function in prostatic neuroendocrine cancer. In 12T-10 transgenic mice, prostate cancer cells frequently undergo neuroendocrine differentiation with subsequent metastasis to the lungs (Masumori et al., 2001). These lung metastases are shown to express MASH1 protein (Gupta et al., 2008), thus indicating a downregulation of Notch signaling. Similarly, liver metastases from an NE-10 transplantable tumor model derived from 12T-10 prostate tumors exhibit robust MASH1 protein expression (Gupta et al., 2008). Therefore, Notch signaling may negatively regulate the metastasis of neuroendocrine prostate cancer cells, and promote the metastasis of prostate cancer cells that lack a neuroendocrine phenotype.

2.2.9. Liver Cancer

Hepatocellular carcinoma (HCC) accounts for 80–90% of liver cancers and is one of the most prevalent carcinomas throughout the world. Notch signaling may function as a tumor suppressor in HCC. It has been shown that activation of Notch signaling in both mouse and human HCC cells is sufficient to block their expansion *in vitro* (Qi et al., 2003; Viatour et al., 2011). HCC cells with enforced expression of NICD undergo cell cycle arrest in G2 and display increased apoptotic activity. Similarly, *in vivo* modulation of Notch pathway activity with DAPT, a γ-secretase inhibitor and potent inhibitor of Notch signaling, results in accelerated cancer

development in retinoblastoma (RB) triple knockout (TKO) mice (Viatour et al., 2011). Consistently, liver-specific inactivation of *Notch1* expression, although not sufficient to promote HCC, leads to the proliferation of hepatocytes in mice (Croquelois et al., 2005). Therefore, liver-specific activation of the Notch pathway may provide novel treatment approaches in HCC.

2.2.10. Kaposi's Sarcoma

Kaposi's sarcoma (KS) is a common neoplasm in HIV-1-infected individuals causing significant morbidity and mortality. Infection by KS-associated herpesvirus (KSHV) is a key factor in the development of KS. KSHV causes a predominantly latent infection in the infected host. Interestingly, one of the mechanisms underlying the oncogenic effect of KSHV has been ascribed to Notch pathway activation. It has been revealed that the replication and transcription activator (RTA) encoded by ORF50 activates its downstream genes and initiates viral lytic reactivation through functional interaction with RBP-Jκ (CSL), which is a major downstream effector of the Notch signaling pathway. It suggests that RTA can takeover the function of Notch signaling pathway and mimic the activities of NICD to modulate gene expression. On the other hand, activation of Notch signaling may react with RTA promoter to reactivate KSHV from latency. Lan et al., (2006) have demonstrated that NICD is elevated in KSHV latently infected pleural effusion lymphoma (PEL) cells. NICD can activate the RTA promoter in a dose-dependent manner, and force expression of NICD in latently infected KSHV-positive cells and initiate full blown lytic replication with the production of infectious viral progeny (Lan et al., 2006). Curry et al. (2005) have also observed elevated levels of activated Notch1, 2, and 4 as well as downstream target gene Hey1 and Hes1 in KS tumor cells *in vivo* and *in vitro* compared to endothelial cells, the precursor of the KS cell. Blocking Notch signaling by gamma-secretase inhibitors (GSI) in primary and immortalized KS cells induces KS cell apoptosis *in vitro*. Furthermore, injection of GSI into xenografted KS tumor on mice causes tumor growth inhibition and tumor regression. These findings indicate that KS cells overexpress activated Notch and interruption of Notch signaling inhibits KS cell growth. Thus, targeting Notch signaling may be of therapeutic value in KS patients.

2.3. Notch in Cancer Stem Cells

Cancer stem cells (CSCs) or tumor-initiating cells (TICs) were initially identified in human acute myelogenous leukemia (AML) (Lapidot et al.,

1994) and similar cancer stem-like cells were subsequently identified in a variety of solid tumors. CSCs are characterized by tumorigenic properties and the ability to self-renew, form differentiated progeny, and develop resistance to therapy. Therefore, targeting CSCs becomes a promising approach in cancer therapeutics. CSCs are known to use many of the same signaling pathways that are found in normal stem cells, such as Wnt/β-catenin, Hedgehog, and Notch for their self-renewal and differentiation. Several studies have shown that the Notch pathway activation promotes stem cell self-renewal and survival while inhibits differentiation in brain tumors (Androutsellis-Theotokis et al., 2006; Pierfelice et al., 2008). Notch signaling is also implicated to be involved in self-renewal and survival of the $CD34^{+}/CD38^{+}$ CSCs in AML (Gal et al., 2006). Moreover, Phillips et al., (2007) have demonstrated that Notch signaling is required for mediating the promoting effect of recombinant human erythropoietin on self-renewal and survival of breast CSCs. In addition, IL-6 signaling may also rely upon Notch3 activity to maintain self-renewal of mammary CSCs (Sansone et al., 2007). Overall, although the study of the Notch signaling in CSCs is still in its infancy, accumulating evidence nonetheless suggests a central role of Notch signaling in the regulatory network of the "stemness" of CSCs, thus targeting Notch pathway is likely to provide sustained benefits for cancer treatment.

2.4. Notch in Tumor Angiogenesis

Neoplastic angiogenesis is one of the requirements for tumor growth and metastasis (Hanahan & Weinberg, 2000), as tumor greater than one cubic centimeter must develop its own blood supply to avoid necrosis. VEGF plays a key role in tumor angiogenesis, as does other pathways, including Notch (Zeng et al., 2005). Both Dll4 and VEGF are known as genes where loss of a single allele leads to embryonic lethality due to disrupted vascular hierarchy (Carmeliet et al., 1996; Gale et al., 2004; Krebs et al., 2004). In mammals, many studies have demonstrated that Dll4 is induced by VEGF in tumor vasculature and functions downstream of VEGF to inhibit the VEGF-induced vessel growth, forming a negative feedback loop to inactivate VEGF (Lobov et al., 2007; Suchting et al., 2007). It suggests that VEGF-induced Dll4 negatively inhibits tumor angiogenesis. However, studies have shown that blockade of the Dll4-Notch pathway in mice induces tumor angiogenesis. Inhibition of Dll4 delays tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007).

This paradoxical phenomenon could be explained by analyzing the functionality of blood vessels. The microvasculature formed from the enhanced tumor angiogenesis has poor integrity and perfuses the tumor poorly, thereby increasing hypoxia in tumors. In other words, Dll4 blockade causes the formation of nonfunctional vasculature and brings about a delay in tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007) (Fig. 7.3). Therefore, Dll4 has become a potential anti-angiogenic therapeutic target. Moreover, when combined with anti-VEGF treatment, Dll4 blockade is even more efficient in controlling tumor growth (Noguera-Troise et al., 2006). Concordantly, Li et al. (2007) have illustrated that Dll4 expressed in tumor cells activates Notch pathway in mice endothelial cells and improves tumor vascular function.

There are two advantages with respect to anti-Dll4 tumor therapy. First, the viability of treated animals is not compromised by administration of anti-Dll4 antibodies or soluble Dll4 ligand. Second, unlike the GSI, treatment

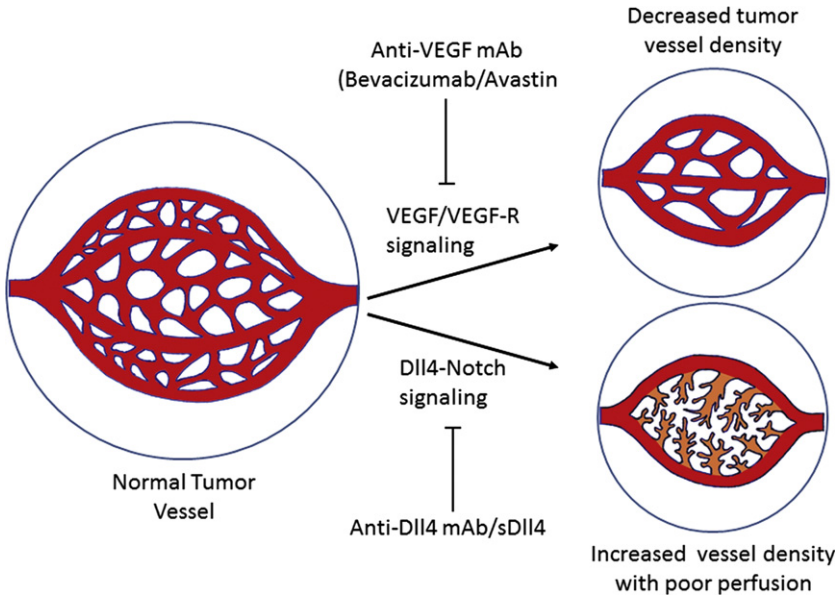

Figure 7.3 Two antitumor angiogenesis models. (a) Neutralizing VEGF-VEGFR signaling by anti-VEGF monoclonal antibodies (Bevacizumab/Avastin) inhibits tumor vessel formation and reduces tumor size. (b) Antagonizing Dll4-Notch signaling by either anti-Dll4 antibodies or soluble Dll4 paradoxically promotes blood vessel formation but inhibits tumor growth. Reduced tumor growth is resulted from poor perfusion of newly formed capillaries. For color version of this figure, the reader is referred to the online version of this book.

with anti-Dll4 antibodies has no observable side effects on homeostasis in mice small intestine (Ridgway et al., 2006). Due to the implication of Dll4-Notch pathway in immunity (Fung et al., 2007; Maillard et al., 2003; Mukherjee et al., 2009), further studies are needed to determine whether these anti-Dll4 therapies have nonangiogenic effects. Moreover, since hypoxia is induced in response to inhibition of Dll4 in tumors (Noguera-Troise et al., 2006), additional investigations about the combination of anti-Dll4 treatment and other therapies are necessary.

However, the high-promising anti-Dll4 therapy also brings about new challenges. It has been reported that chronic Dll4 blockade causes pathological activation of endothelial cells, disrupts normal organ homeostasis, and induces vascular tumors, raising important safety concerns (Yan et al., 2010). More careful studies are required in this aspect.

2.5. Notch in Tumor Stromal Cells

Tumors have come to be understood to function as complex tissues in which numerous infiltrated and recruited host cells also play critical roles (Anton & Glod, 2009; Lorusso & Ruegg, 2008). These nonneoplastic cells constitute the tumor-associated stroma. The tumor stroma is comprised of endothelial cells and pericytes that together form tumor vasculature, infiltrated inflammatory cells including macrophages and lymphocytes, fibroblasts/myofibroblasts which are derived from the local existing fibroblasts, recruited bone marrow–derived mesenchymal stem cells (MSCs) (Liu et al., 2009), as well as the extracellular matrix (ECM). All of these components communicate with each other and with the neoplastic cells to contribute to tumor initiation, progression, and development of life-threatening metastasis (Fig. 7.4). Notch in endothelial cell–dependent tumor angiogenesis has been reviewed separately in part II-C. Involvement of the Notch signaling in modulating other tumor stromal cells, specifically fibroblasts and inflammatory cells, has also been revealed.

Fibroblasts are major components of tumor stroma and critically involved in regulating tumor growth, metastasis, and angiogenesis through secretion of soluble factors, including CXCL12/SDF-1α, TGF-β, PDGF, IGF, FGF, VEGF, synthesis of ECM, such as fibronectin, collagen, and matrix metalloproteases (MMPs), and direct cell–cell interaction (Allinen et al., 2004; Bhowmick et al., 2004; Lynch & Matrisian, 2002; Midwood et al., 2004; Olumi et al., 1999). Infiltrated/recruited tumor stromal fibroblasts are activated in tumor tissue and are termed as cancer-associated-

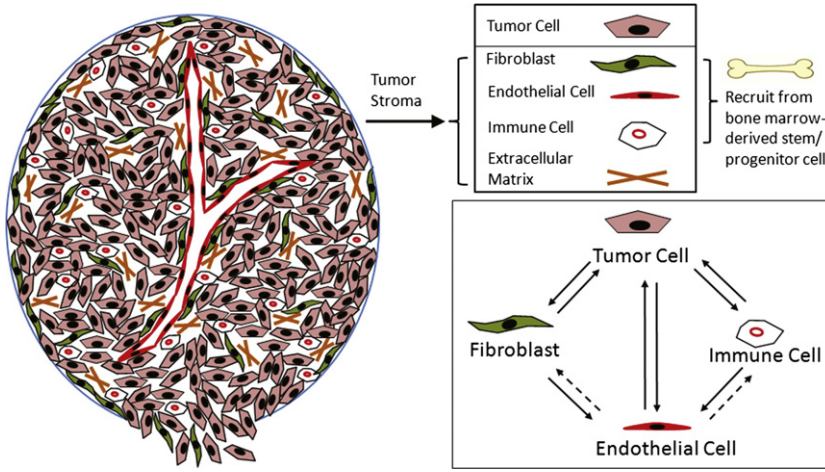

Figure 7.4 Tumor is a complex tissue containing numerous infiltrated and recruited stromal cells, including endothelial cells, fibroblasts, and immune cells, in addition to neoplastic cells. These stromal cells interact with neoplastic cells and play critical roles in tumor development and progression (solid arrow: relationship is defined; dash arrow: relationship is uncertain). For color version of this figure, the reader is referred to the online version of this book.

fibroblasts (CAFs) (Orimo & Weinberg, 2006). Recent work has demonstrated that CAFs extracted from invasive human breast carcinomas are more capable in the promotion of the growth of mammary carcinoma cells and tumor angiogenesis compared to cells derived from outside of tumor masses (Orimo et al., 2005). In addition, stromal fibroblasts can render non-tumorigenous cells to gain a permanently transformed phenotype (Hayward et al., 2001). Moreover, CAFs can even mediate resistance to antiangiogenic therapy (Crawford et al., 2009). Fibroblasts, thus, may represent promising therapeutic targets in the prevention and treatment of tumor growth and survival. The cellular activity of fibroblasts is regulated by a variety of signals, including the Notch signaling. The Notch signaling appears to serves as a "molecular switch" in controlling the biological function of stromal fibroblasts within tumor tissue. The work by Shao et al. (2011) demonstrates that activation of Notch pathway is able to convert fibroblasts from "tumor promoters" to "negative regulators". Fibroblasts engineered to constitutively activate Notch1 pathway significantly inhibited tumor growth and tumor angiogenesis in a mouse tumor xenograft model. It points to Notch pathway activation playing a negatively regulatory role in controlling cellular behavior of fibroblasts. This finding is consistent with other studies conducted in fibroblasts. For instance, Notch pathway activation via either

overexpression of NICD or stabilization of NICD by ablation of Sel-10 (Fbxw7), a negative regulator of Notch signaling, results in cell cycle arrest and apoptosis in mouse embryonic fibroblasts (Bhowmick et al., 2004). In addition, inhibition of Notch signaling by soluble forms of the Dll1 and Jagged1 ligands has been found to induce fibroblast growth factor receptor (FGFR)-dependent transformation of NIH 3T3 fibroblasts *in vitro* (Hayward et al., 2001). Therefore, manipulation of Notch signaling may serve as an innovative strategy to target tumor microenvironment by modulating tumor-regulatory function of stromal fibroblasts.

Infiltration and/or recruitment of inflammatory cells, including lymphocytes, macrophages, mast cells, and monocytes, into tumor tissue have long been thought of as a reaction of the host to fight against neoplasm. The role as "policeman/fighter" for inflammatory cells within tumor tissues to find, kill, and clean up neoplastic cells is the center of the theory of "immune surveillance." However, it is now understood that inflammatory cells within tumor tissues also play important roles in promoting tumor development and progression (Hanahan & Weinberg, 2011). These infiltrated/recruited inflammatory cells are often activated by tumor cells in the tumor microenvironment through either cell–cell interaction and/or by tumor-produced soluble proteins. Tumor-infiltrating inflammatory cells are named tumor-associated (or -activated) cells, for instance, tumor-associated macrophages (TAM). These tumor-associated/-activated inflammatory cells act as "accomplice/lackey" to aid in tumor malignancy. They are rich sources of cytokines, growth factors, and ECM that activate important signal transduction pathways in tumor cells, including NF-κB, JAK/STAT, and PI3K/Akt/mTOR, which regulate the expression of genes controlling tumor cell growth, survival, and chemosensitivity. Many of these soluble proteins also facilitate tumor angiogenesis, thus indirectly promote tumor growth and metastasis.

Given that the Notch signaling is known to play an important role in the regulation of development of hematopoietic and immune cells (Radtke et al., 2010), it is speculated that Notch signaling is involved in modulating the tumor-promoting effect of tumor-associated/-activated inflammatory cells. Studies have indeed approved a critical role for Notch signaling in determining the phenotype and function of TAM. TAM participate in immune responses to tumors in a polarized manner: classic M1 macrophages produce IL-12 to promote tumoricidal responses, whereas M2 macrophages produce IL-10 and promote tumor progression. Wang et al. demonstrated that Notch signaling plays critical roles in the determination of M1 versus M2 polarization of macrophages, and that compromised Notch pathway

activation can lead to the M2-like TAM. They observed that the M2-like TAM have a lower level of Notch pathway activation in tumor tissue. Forced activation of Notch signaling increases M1 response that produces IL-12. This process is independent of M1 or M2 inducers. When Notch signaling is blocked, the M1 inducers induce M2 response at the expense of M1. Macrophages deficient in Notch signaling show TAM phenotypes. Forced activation of Notch signaling in macrophages enhances their anti-tumor capacity (Wang et al., 2010). Therefore, regulation of TAM phenotype and function through manipulation of Notch signaling may serve as an alternative strategy to target tumor microenvironment.

3. NOTCH PATHWAY AS POTENTIAL THERAPEUTIC TARGETS IN CANCER

A growing body of research and clinical evidence are in support of Notch's oncogenic or tumor suppressive role in a wide variety of cancers. It, therefore, places Notch signaling as a potential target for cancer therapeutics. An extensive understanding of Notch signaling cascade and its interaction with other pathways has provided us with insightful information for the identification of molecular targets to design effective therapeutic strategies (Fig. 7.5).

3.1. GSI Therapy

Aberrant Notch signaling has been extensively linked to cancer and tumorigenesis. Ligand binding to the extracellular domain of the Notch receptor triggers intramembranous cleavage of the Notch receptor, carried out by the γ-secretase complex, resulting in cytoplasmic release of the NICD (De Strooper et al., 1999). Therefore, blocking transmembranous proteolytic cleavage of Notch by GSIs could be a promising strategy for Notch-targeted therapeutics. The strategy inhibits NICD production, thus suppressing the downstream transcriptional events.

Over the past decades, synthetic GSIs have been successful in treating Alzheimer's disease, where defective γ-secretase cleavage of the substrate molecule amyloid precursor protein (APP) generates an Aβ42 variant of Aβ40 peptides, consequently resulting in plaque formation (Lichtenthaler et al., 1997). Since the proteolytic processes in Notch signaling activation are comparable with the processes involved in APP cleavage, GSIs are also capable of inhibiting the activation of Notch receptor, which offers an attractive targeted therapy for tumors dependent on aberrant Notch activity.

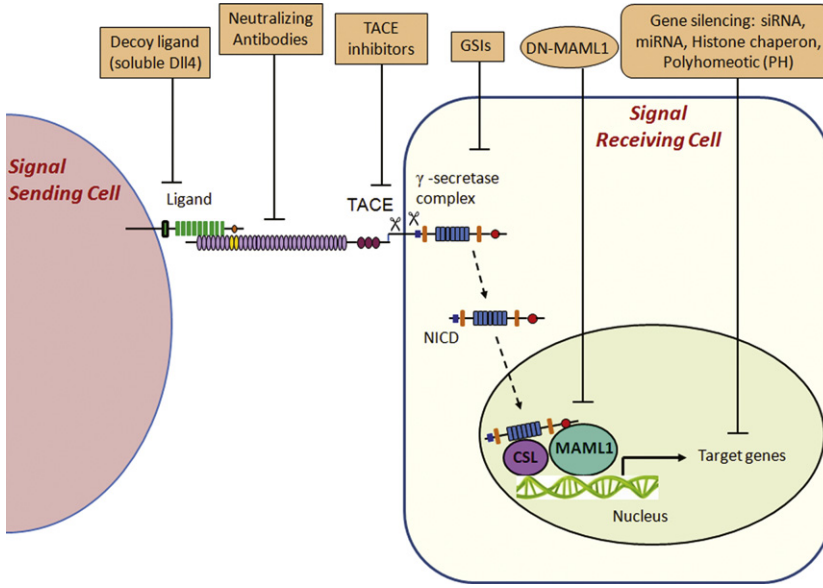

Figure 7.5 Potential cancer therapeutics by targeting Notch signaling. These include decoy Notch ligand (soluble Dll4), disruption of two proteolytic cleavages by TACE inhibitor and GSIs, gene silencing by siRNAs, miRNAs, Histone chaperon and Polyhomeotic (PH) techniques, and transcriptional regulation (DN-MAML1). For color version of this figure, the reader is referred to the online version of this book.

It has been reported that treatment of T-ALL with GSIs including compound E, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-Sphenylglycine t-butyl ester), MRK-003 and YO01027 induces cell cycle arrest and apoptosis (Lewis et al., 2007; Masuda et al., 2009; O'Neil et al., 2006; Weng et al., 2004). Treatment of medulloblastoma (MB) in a xenograft mice model with dipeptide GSI, DAPT, leads to decreased cell proliferation and increased apoptosis, suggesting that Notch activation contributes to human MB proliferation and survival (Hallahan et al., 2004). Studies using synthetic GSI, dibenzazepine (DBZ), led to the conversion of proliferative crypt cells into postmitotic goblet cells in $Apc^{-/-}$ mice, suggesting GSIs might be of therapeutic benefit in colorectal cancer (van Es et al., 2005). It has been noted that GSI1 suppresses breast cancer cell survival by promoting a cell cycle arrest at G2/M, which further triggers apoptosis (Rasul et al., 2009). Similarly, GSI-XII induces apoptosis of myeloma cells. Moreover, GSI-XII dramatically improves the sensitivity of myeloma cells to chemotherapeutic drugs such as doxorubicin and maphalan, representing a promising strategy for therapeutic intervention in multiple myeloma (Nefedova et al., 2008).

RO4929097 is a newly developed GSI with high selectivity and efficacy. This potent GSI has been proven to have an *in vitro* γ-secretase inhibitory activity. Of note, RO4929097 produces a less transformed, slow growing phenotype, rather than inhibiting tumor cell proliferation or inducing apoptosis. RO4929097 is active following oral administration and currently being tested in a phase I multidose escalation in patients with solid tumors (Luistro et al., 2009). The phase I study of another GSI, MK0752, for patients with advanced breast cancer is ongoing (http://clinicaltrials.gov/ct2/show/NCT00106145). Moreover, the exploratory study of MK0752 in combination with tamoxifen or letrozole to treat early stage breast cancer is currently under way. In addition, PF-03084014 has been tested in a phase I dose-escalating study to determine its safety in patients with advanced solid tumors and T-ALL (http://clinicaltrials.gov/ct2/show/NCT00878189).

While solid tumors have responded favorably to GSI, the majority of human T-ALL cell lines are not susceptible to these treatments. The molecular basis of GSI resistance in T-ALL remains to be clarified. One study suggests that FBW7 mutations produce dominant-negative FBW7 alleles and confer GSI resistance in T-ALL cells (O'Neil et al., 2007). Another study indicates that mutations on PTEN (a tumor suppressor) confer resistance to GSI therapy in human T-ALL cells. Loss of PTEN and constitutive activation of AKT in GSI-resistant T-ALL cells increase glucose metabolism and bypass the requirement of Notch1 signaling to sustain cell growth (Palomero et al., 2008; Palomero et al., 2007). In some human T-ALL cells, represented by CEM and Jurkat J6, when combined with chemotherapy drugs, GSI (compound E) antagonizes the effect of chemotherapy by decreasing apoptosis. Compound E also induces the expression of antiapoptotic gene Bcl-xl mRNA and protein in CEM and Jurkat J6 cells (Pinnix et al., 2009).

The studies summarized above demonstrate a potential clinical application of GSI in antitumor therapy. However, one of the challenges is the inhibitor-associated side effect, especially cytotoxicity in the gastrointestinal tract (GIT) (Barten et al., 2006). For example, inhibition of Notch by GSI reverses glucocorticoid resistance in T-ALL and glucocorticoid treatment antagonizes the effects of Notch inhibition in the intestinal epithelium and protects from GSI-induced gut toxicity. Thus, combination therapies of GSIs and glucocorticoid can enhance the therapeutic efficacy in human T-ALL (Real et al., 2009). Advantages of GSI treatments include ease of administration, low cost, and oral bioavailability. In addition, it can block the activation of all four Notch receptors. However, unselectively blocking

of all Notch homologues could also be disadvantageous since Notch proteins may have opposite effects in some tumors (O'Neil et al., 2007). Furthermore, such compounds cause significant toxicities following chronic oral administration (Barten et al., 2006; Wong et al., 2004) and acquire resistance (O'Neil et al., 2007; Palomero et al., 2007). Another disadvantage is that since γ-secretase has a wide variety of targets other than Notch receptors, GSIs indiscriminately inhibit many signaling pathways (Lleo, 2008). Shelton et al. (2009) have developed a di-coumarin family of inhibitors that selectively inhibit APP cleavage by γ-secretase. They have revealed that the di-coumarin compounds induce a conformational change of γ-secretase by binding to an allosteric site that causes selective inhibition of Aβ42. This class of allosteric inhibitors provides the basis for development of Alzheimer disease therapeutic agents (Shelton et al., 2009). It follows that a broad number of drugs with sufficient specificity and affinity for inhibition of Notch receptor cleavage could be discovered for cancer therapy.

3.2. Other Therapeutic Approaches to Notch Signaling Inhibition

In addition to interfering with the cleavage of Notch receptors using GSIs, Notch ligand can be targeted using the more specific monoclonal antibodies (mAbs). mAbs selectively targeting Dll4 have been demonstrated to inhibit Notch signaling in endothelial cells and cause defective endothelial cell differentiation (Ridgway et al., 2006). Furthermore, neutralizing Dll4 with a Dll4-selective antibody dysregulates tumor angiogenesis and inhibits tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006). Remarkably, the combination of antihuman Dll4 and antimouse Dll4 results in additive antitumor activity in colon tumors (Hoey et al., 2009). In a NOD/SCID mice model of human colon cancer, administration of anti-Dll4 inhibits tumor growth and reduces cancer stem cell (CSC) frequency, indicating CSC might be the target for this drug (Hoey et al., 2009). Conversely, some mAbs have been implicated to specifically induce proteolytic cleavages in Notch3 (Li et al., 2008). The activating antibody (256A-13) binds to overlapping epitopes on one face of Notch3 and mimics certain effects of ligand-induced Notch activation (Li et al., 2008). These observations suggest that it is possible to develop antibodies that selectively modulate the activities of individual Notch receptors. The Notch-specific structural domain is the key toward the design of specific mAbs for Notch receptors. Currently, these mAbs are being developed and characterized as

antiangiogenic therapeutic agents (Noguera-Troise et al., 2006; Thurston et al., 2007; Yan & Plowman, 2007). Using phage display technology, (Wu et al 2010). generated highly specialized antibodies that are able to discriminate Notch1 versus Notch2 function. They have found that selective blocking of Notch1 inhibits tumor growth in preclinical models through two mechanisms: inhibition of cancer cell growth and deregulation of angiogenesis. Whereas inhibition of Notch1 plus Notch2 causes severe intestinal toxicity, inhibition of either receptor alone reduces or avoids this effect, demonstrating a clear advantage over pan-Notch inhibitors.

Modulation of Notch signaling by other pathway components has also come to light. It has been shown that Notch1 is induced by PI3K/Akt pathway in human arterial endothelial cells (Liu et al., 2003) and in melanoma development (Bedogni et al., 2008). GSK3-α/β act as negative regulators of Notch1 (Jin et al., 2009), and Notch2 was downregulated by GSK3β (Espinosa et al., 2003). Phyllopod, a transcriptional target of the EGFR pathway, can block Notch signaling pathway (Nagaraj & Banerjee, 2009). Inhibition of these pathways may indirectly modulate Notch signaling under certain circumstances.

microRNAs (miRNAs) are small (19–22 nts) noncoding regulatory RNA molecules that regulate diverse cellular processes (Pillai et al., 2007). Various miRNAs regulate the Notch pathway by binding to the 3′-untranslated region (3′-UTR) of Notch target mRNA. miRNA-34a has been found to be deregulated in human gliomas and forced miRNA-34a expression inhibits *in vivo* brain tumor growth by targeting multiple oncogenes (c-Met, Notch1 and Notch2) (Li et al., 2009). miRNA-34a molecule can also inhibit human pancreatic cancer stem cell renewal potential via the direct modulation of the downstream effectors of Notch1/2 and Bcl2 (Ji et al., 2009). These studies suggest that restoration of tumor suppressor miRNA34 may provide a promising therapy for human gliomas and pancreatic cancers. In the screening of metastatic MB cell lines, miRNA 199b-5p has been observed to be a modulator of Notch signaling via its targeting of Hes1. Downregulation of Hes1 expression negatively regulates the proliferation rate and anchorage-independent growth of MB cells (Garzia et al., 2009). Small interfering RNA (siRNA) is another type of RNA interference that has been used to inhibit Notch pathway activation (Cohen et al., 2009; Ono et al., 2009; Yao and Qian, 2009). Theoretically, any Notch pathway components can be targeted by specific siRNA. Thus, siRNAs- and/or miRNAs-mediated gene–targeting approaches hold significant promise as potential anticancer therapeutic agents.

Evidence that histone chaperons play a diverse function during chromatin transactions is emerging (De Koning et al., 2007; Eitoku et al., 2008). ASF1, one of the H3/H4 chaperons, has been found to be required for repression of E(spl) Notch target genes through interactions with the Su(H)/H DNA binding complexes in *Drosophila*. These findings reveal that histone chaperons can act as gene regulators in silencing Notch-targeted genes (Goodfellow et al., 2007). However, the molecular mechanism by which ASF1 achieves gene silencing has yet to be delineated. A study by Moshkin et al. (2009) demonstrates that the histone chaperons ASF1 and NAP1 facilitate removal of histone marks by two silencing complexes, LAF and RLAF, respectively, in different manners during Notch silencing. Modulation of histone chaperons involved in the Notch pathway silencing might be a useful strategy in disease therapeutics.

PcG (polycomb Group) gene encodes another epigenetic factor that silences Notch target genes. PcG proteins are involved in many physiological processes, including repression of homeotic gene transcription and modulation of cell proliferation (Schuettengruber et al., 2007). A mutation in the gene locus (ph) encoding the PcG protein Polyhomeotic (PH) induces cell proliferation (Martinez et al., 2009). In conjugation with Ras protein, these cells promote metastasis. PcG proteins are found to bind to many genes in the Notch pathway and control their transcription. When Notch is inhibited by either RNA interference or a dominant-negative form of the Notch pathway components, the over-proliferative phenotype of ph mutant cells can be reversed. It suggests that PH protein acts as a tumor suppressor in controlling cell proliferation by silencing Notch pathway components.

It is also possible to deregulate Notch pathway at the posttranslational level by inhibiting the ubiquitination of Notch ligands for endocytosis (Fontana & Posakony, 2009; He et al., 2009) or blocking the fucosylation of Notch receptors (Okajima & Irvine, 2002; Stahl et al., 2008).

Interestingly, a study by van Tetering et al. shows that ADAM10/Kuz metalloprotease, but not ADAM17/TACE, is the main protease responsible for Notch1 cleavage at site 2 (S2) upon DSL ligand binding under physiological conditions in mouse fibroblast cells. However, ADAM10 may not be required for ligand-independent cleavage of Notch1 receptors harboring some types of gain-of-function T-ALL mutations (van Tetering et al., 2009). Consistently, some other studies report that the ADAM requirement for Notch receptor activation is cell-context dependent. Specifically, ADAM10/Kuz is absolutely required for ligand-induced Notch activation,

while Notch signaling independent of ligands requires ADAM17/TACE (Bozkulak & Weinmaster, 2009; Delwig & Rand, 2008). Identification of new drugs targeting the rate-limiting S2 cleavage may prove to be an interesting strategy to be exploited.

A DN-MAML1 at the length of 13–74 residues has been demonstrated to antagonize Notch signaling and cell proliferation in T-ALL cell lines (Maillard et al., 2004; Weng et al., 2003). This DN-MAML1 forms a structure of α-helix that binds to the extended groove formed by the assembly of NICD and CSL in human and *Caenorhabditis elegans* (Nam et al., 2006; Wilson & Kovall, 2006). These data suggest that Notch transactivation complex (NICD-CSL-MAML1) might be a useful target for Notch inhibition by such α-helix like peptides. Recently, Moellering and colleagues have prepared peptide segments of the MAML1 binding site, and constrained them into α-helical conformation by hydrocarbon "'staples". They reason that the stapled peptides bind to the CSL-NICD complex, preventing full length MAML1 from binding and thereby directly inhibiting the transcription of Notch-targeted genes (Arora & Ansari, 2009; Moellering et al., 2009).

Very excitingly, studies have shown that "natural agents", which are typically nontoxic to humans, including sulforaphane, quercetin, curcumin, genistein, and others, are able to inhibit Notch expression or increase the sensitivity of tumor cells to several chemotherapeutic agents (Kallifatidis et al., 2011; Kawahara et al., 2009; Wang, Banerjee, et al., 2006; Wang et al., 2006a, b), thereby suggesting their suitability in the treatment of particular types of tumors.

4. CONCLUSION

Aberrant Notch activation is linked to cancer since 1991 when mammalian Notch1 was first identified as part of the translocation t(7;9) in a subset of human T-ALL. Since then, aberrant Notch signaling has been found in many solid and hematopoietic tumors. Depending on tumor type, Notch signaling activation can function as either an oncogene or a tumor suppressor. Notch signaling interferes with differentiation, proliferation, survival/apoptosis, and possibly self-renewal of tumor cells. It is also involved in the modulation of tumor angiogenesis and activities of tumor stromal cells. Accumulating evidence has emerged over the past decade that strongly supports the hypothesis that Notch signaling is one of

the most promising novel therapeutic targets in cancer treatment. Improved strategies for the clinical application of Notch pathway targeted therapies will need to consider: (i) Specificity. Four Notch receptors may have distinct, even opposite, effects depending on cell context and tumor types. Notch2 is oncogenic in embryonal brain tumor growth while Notch1 inhibits the tumor growth (Fan et al., 2004). Notch1 and Notch3 have overlapping functions in inducing murine mammary tumor phenotypes (Hu et al., 2006). Two key Notch ligands, Jagged1 and Dll4, have been implicated in tumor angiogenesis. However, these two Notch signaling components regulate tumor angiogenesis by diverse mechanisms. Inhibition of Dll4 paradoxically induces increased tumor angiogenesis but reduced tumor growth, because newly growing tumor vessels are not functional with poor perfusion capacity. In contrast, Jagged1 expression in tumor cells promotes the growth of tumor vessels, suggesting a proangiogenic role of Jagged1 in tumors (Dufraine et al., 2008). Hence, new classes of specific Notch inhibitory molecules, such as novel GSI compounds that are capable of selectively inhibiting specific Notch receptors, or specific anti-Notch inhibitory antibodies which could be engineered to block the specific member of Notch receptors in the tumor cells, need to be developed. Complete understanding of the mechanism of individual Notch ligand/receptor's function in different tumors will greatly increase our ability to improve the anticancer regimen under specific circumstances. In addition, identification of biomarkers to guide the selection of specific anti-Notch medicine or predict the response of various tumor cells to anti-Notch treatment will be a significant plus. (ii) Combined therapies. At present, it is difficult to achieve satisfactory therapeutic accomplishments with Notch-targeted monotherapy, given the fact that Notch signaling interacts with many other pathways, including PI3K/Akt, NF-κB and STAT3. Appropriate combination of Notch inhibitors with other individual medicines may prove to be synergistically beneficial in the clinical setting. Moreover, combined therapy will not only increase the antitumor effects of these drugs, but also improve their therapeutic window. (iii) Efficacy versus toxicity (side effect). In considering GSI-associated acute toxicity, the balance between efficacy and toxicity of GSI should be taken into account in future clinical applications. A new parenteral drug formulation aiming to avoid the toxic effects of GSI in the gut should be developed. Finally, targeting the Notch signaling pathway by natural agents may represent an alternative for overcoming drug toxicity and resistance.

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ABBREVIATIONS

ACL adenocarcinoma of the lung
ADAM A disintegrin and metalloprotease
AML acute myeloid leukemia
APP amyloid precursor protein
BCC basal cell carcinoma
B-CLL B-chronic lymphocytic leukemia
CAF cancer-associated-fibroblasts
c-IAP2 cellular inhibitor of apoptosis protein 2
CSC cancer stem cell
DBZ dibenzazepine
DLL Delta-like
DN-MAML1 dominant negative–mastermind like 1
DSL Delta/Serrate/LAG-2
EMC extracellular matrix
FGFR fibroblast growth factor receptor
GBM glioblastoma
GIT gastrointestinal tract
GSI γ-secretase inhibitor
HCC hepatocellular carcinoma
HD heterodimerization
hEGFRs human epidermal growth factor receptors
Herp Hes-related repressor protein
Hes Hairy and E (spl)
HIF hypoxia-inducible factor
KS Kaposi's sarcoma
KSHV KS-associated herpesvirus
LNR Lin-12, Notch repeats
LNX ligand of Numb-protein X
MAML mastermind-like
MB medulloblastoma
MMPs matrix metalloproteases
MMTV mouse mammary tumor virus
MSC mesenchymal stem cell
NICD Notch intracellular domain
NSCLC non–small cell lung cancers
***O*-Fut** *O*-Fucosyl transferase
PcG polycomb Group

PDAC pancreatic ductal adenocarcinoma
PEL pleural effusion lymphoma
PH Polyhomeotic
RB retinoblastoma
RBP-Jκ CSL
RTA replication and transcription activator
SCC squamous cell carcinoma
SCLC small cell lung cancers
Shh Hedgehog
siRNA small interfering RNA
TACE TNF-α converting enzyme
T-ALL T-cell acute lymphoblastic leukemia
TAM tumor-associated macrophages
TKI tyrosine kinase inhibitor
TKO triple knockout
VEGF vascular endothelial growth factor
XIAP X-linked inhibitor of apoptosis protein.

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CHAPTER EIGHT

Stem-Like Cells and Therapy Resistance in Squamous Cell Carcinomas

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Abstract

Cancer stem cells (CSCs) within squamous cell carcinomas (SCCs) are hypothesized to contribute to chemotherapy and radiation resistance and represent potentially useful pharmacologic targets. Hallmarks of the stem cell phenotype that may contribute to therapy resistance of CSCs include quiescence, evasion of apoptosis, resistance to DNA damage, and expression of drug transporter pumps. A variety of CSC populations within SCCs of the head and neck and esophagus have been defined tentatively, based on diverse surface markers and functional assays. Stem-like self-renewal and differentiation capacities of these SCC subpopulations are supported by sphere formation and clonogenicity assays *in vitro* as well as limiting dilution studies in xenograft models. Early evidence supports a role for SCC CSCs in intrinsic therapy resistance, while detailed mechanisms by which these subpopulations evade treatment remain to be defined. Development of novel SCC therapies will be aided by pursuing such mechanisms as well as refining current definitions for CSCs and clarifying their relevance to hierarchical versus dynamic models of stemness.

1. INTRODUCTION

Squamous cell carcinomas (SCCs) of the digestive tract share a distinct biology and arise almost exclusively within the mucosa of the head and neck and proximal third of the esophagus. Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide (Argiris et al., 2008). In the United States, smoking is the major risk factor for SCC of the head and neck or esophagus, with heavy alcohol use serving as a potent cofactor. Oncogenic human papilloma viruses were also recently

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recognized as an important and growing etiology for HNSCCs of the tonsil and base of the tongue, with HPV-16 predominating among the multiple oncogenic subtypes.

Currently, advanced stage HNSCCs require multimodality therapies that may combine surgery, radiation, cytotoxic chemotherapy, and/or targeted therapy against the epidermal growth factor receptor (EGFR). Yet, these aggressive treatments continue to produce high rates of recurrence as well as severe treatment-related disabilities for long-term survivors. Over the past decade, the cancer stem cell (CSC) hypothesis has emerged as a new paradigm for many solid tumors, with CSCs proposed to play a broad role in intrinsic resistance to existing drugs and radiation therapy. Pursuing strategies that pharmacologically target HNSCC CSCs therefore holds potential for benefit in the form of improved survival and decreased treatment-related morbidity.

Conceptually akin to normal stem cells, CSCs were originally conceived as a minority subset of malignant cells with capacity for both unlimited self-renewal and hierarchical differentiation. They are predicted to show additional hallmarks of normal stem cells including resistance to DNA damage and apoptosis, allowing them to evade both drugs and radiation and subsequently drive tumor repopulation posttherapy (Fig. 8.1). In addition, CSCs have been attributed with enhanced migratory and invasive capacity, which may occur in association with an epithelial to mesenchymal transition (EMT)-related gene signature. Here, we briefly delineate the evolving conceptual framework of the CSC hypothesis. In this context, we review multiple working definitions of CSC subpopulations within SCCs. We subsequently appraise the early evidence regarding the significance of these subsets in intrinsic therapy resistance and the mechanisms underlying this resistance.

2. CSCs

2.1. Hierarchical CSC Model

A hierarchical CSC model posits that ongoing tumor propagation requires a minority subset of tumor cells with phenotypic traits shared with normal adult stem cells. These cells are deemed necessary to sustain the bulk of a tumor comprised of rapidly proliferating and terminally differentiated cells. By dividing asymmetrically, CSCs simultaneously renew themselves and generate a hierarchy of more differentiated lineages that lack independent tumor propagating ability.

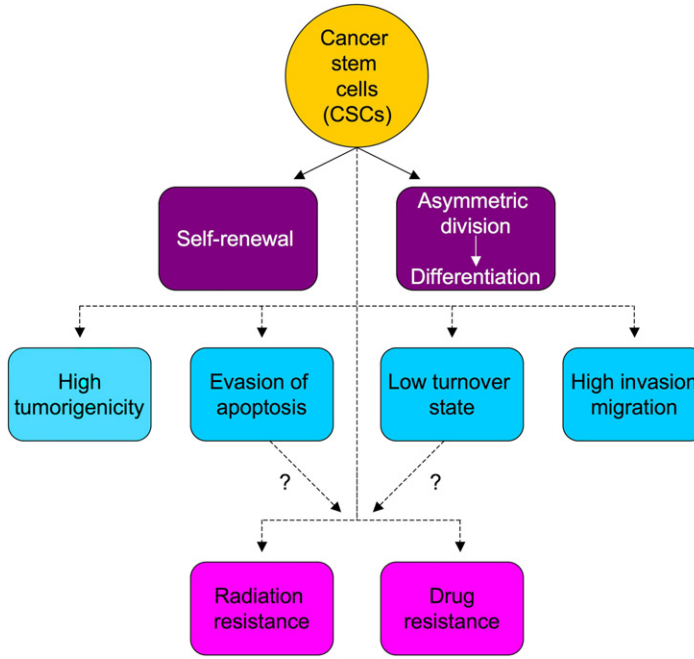

Figure 8.1 Proposed biological properties of HNSCC CSCs. HNSCC CSCs are defined primarily by their capacities for self-renewal and differentiation. They also can possess several additional CSC traits (high tumorigenicity, low-turnover, high invasion/migration, evasion of apoptosis), some of which may contribute to their resistance to chemo- and radiotherapies. For color version of this figure, the reader is referred to the online version of this book.

The hierarchical CSC model was first supported experimentally by Dick et al., who identified a subpopulation of acute myeloid leukemia (AML) cells that were $CD34^{high}$ $CD38^{low}$ and could generate xenograft tumors fully recapitulating the cell surface marker heterogeneity of the original tumor (Bonnet & Dick, 1997; Lapidot et al., 1994). In contrast, more differentiated $CD34^{low}$ and $CD34^{high}$ $CD38^{high}$ cells were not tumorigenic. Corroborating findings in AML and chronic myelogenous leukemia have since been described across multiple model systems, including genetically engineered mice, and in patients (Lane & Gilliland, 2010). Over the past decade, solid tumors have also been dissected to identify subpopulations showing enhanced tumorigenicity in xenograft models in conjunction with stem cell-like phenotypes in *in vitro* assays. The $CD44^{high}$ $CD24^{low}$ subset in breast cancer was the first such example and has become perhaps the most extensively characterized population in this regard (Al-Hajj et al., 2003;

Visvader & Lindeman, 2008). Since then, stem-like subpopulations have been similarly defined across numerous solid tumor types including brain, prostate, and colon (Collins et al., 2005; Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004).

2.2. Dynamic CSC Model

There is growing evidence that some cells in solid tumors meet the experimental criteria used for CSCs, but do not adhere to a strict hierarchical model of stemness. Specifically, putative non-CSC populations may revert to the CSC state when provided a permissive microenvironment and thus also contribute to tumor propagation. For instance, more differentiated, luminal breast cancer cell phenotypes transition to the $CD44^{high}$ $CD24^{low}$ CSC state and allow tumor propagation when coinoculated with irradiated carrier cells (Gupta et al., 2011). In malignant melanoma, multiple markers of subpopulations with CSC properties have been defined (Boiko et al., 2010; Schatton et al., 2008). However, engraftment of even single human melanoma cells has been shown feasible with simple xenograft assay modifications (Quintana et al., 2008). Furthermore, the lack of CSC marker enrichment among engrafted cells in such modified xenograft experiments supports an absence of hierarchical organization based on currently used melanoma markers (Quintana et al., 2010). Accordingly, expression of the H3K4 histone demethylase JARID1B induces a stem-like state in melanoma cells and is required for long-term tumor propagation, and yet $JARID1B^{low}$ and $JARID1B^{high}$ phenotypes are highly plastic and undergo rapid interconversion (Roesch et al., 2010). A comparable epigenetic transition regulated by another JARID1 family member, JARID1A, was shown to be rapidly and reversibly induced by exposure to cytotoxic and EGFR-targeted therapy (Sharma et al., 2010). Such dynamic reversibility between CSC and non-CSC populations has implications for any pharmacologic approach, which must then simultaneously target multiple epigenetic cell states to achieve tumor eradication. At present, the degree to which the current definitions of SCC CSCs conform to hierarchical or dynamic models of stemness remains largely untested.

3. CSCs IN SCCs

3.1. Defining SCC CSCs

CSCs in SCCs have been defined by diverse methodologies using cell lines, primary tumor specimens, and patient-derived xenografts (PDXs).

A number of assays (sphere formation, Hoechst dye exclusion, Aldefluor®) and markers (e.g., CD44, CD133) have been used to identify, isolate, and subsequently characterize CSC populations in SCCs (Table 8.1). Expression of these markers is associated with a variety of other proteins associated with stemness, differentiation, apoptosis regulation, and/or drug resistance (Table 8.2). In distinguishing SCC CSCs, investigators have relied upon two cardinal features of stem cells: self-renewal and differentiation. Though controversial in its interpretation, serial xenotransplantation in animal models remains a key functional assay for self-renewal and lineage capacity and thus for evaluating the stemness of a tumor subpopulation (Clarke et al., 2006). Such studies in SCCs have largely been performed using xenotransplantation of human cells to immune deficient mice rather than in syngeneic mouse models. High tumor formation ability at low cell numbers in limiting dilution assays is used as a correlate of stemness. Self-renewal and differentiation are confirmed based on the subpopulation forming tumors of comparable heterogeneity upon secondary passage. A central caveat of such studies is that cells with innate CSC properties in a human tumor may not necessarily coincide with those that engraft most efficiently in the mouse microenvironment. Also, modifications in assay conditions have been shown to dramatically affect the frequency of human cancer cells determined to be tumor forming (Quintana et al., 2008). In this regard, changes in tumor disaggregation methods, Matrigel use, and coinjection of nonmalignant carrier cells are all known to alter xenotransplantation assay results.

3.2. Sphere-Forming SCC Cells

First used to define neural stem cells (Reynolds & Weiss, 1992), sphere-formation assays select stem cells by growing a bulk population at low density on a nonadherent substrate, in the absence of serum and the presence of defined growth factors. Outgrowth of individual stem cell clones is represented by floating sphere formation. Spheres can be subsequently disaggregated and passaged under distinct culture conditions promoting self-renewal versus differentiation. Variations of this assay are now widely used both for defining stem-like subpopulations *in vitro* and assessing the self-renewal and differentiation potential of populations selected by other criteria. Accordingly, some studies designate SCC CSCs based solely on the sphere-formation assay, while others use it as a measure of self-renewal and differentiation. Pastrana et al. provide critical review of this assay,

Table 8.1 CSCs in Squamous Cell Carcinoma of the Esophagus, Head, and Neck

Assay/Marker(s)	Description	Origin of SCC	Stem-Like Properties
Sphere forming	Single cells that can form spheres in an *in vitro* assay are considered to possess both the ability to self-renew and differentiate	HNSCC[a] (Lim et al., 2011), laryngeal (Chen, Wei, et al., 2011), tongue (Chen, Wei, et al., 2011; Chiou et al., 2008), and gingival (Chiou et al., 2008)	Self-renewal (serial propagation), differentiation, expression of stemness markers, increased colony formation/invasion, tumor forming, propensity for quiescence (G_0/G_1, Ki-67)
Side population (SP)	Small populations of cells that do not accumulate appreciable levels of Hoechst 33342 dye, likely due to increased efflux	Tongue (Loebinger et al., 2008; Sun et al., 2010; Tabor et al., 2011), laryngeal (Yanamoto et al., 2011), buccal (Yajima et al., 2009), and esophageal (Li et al 2011)	Self-renewal (sphere formation), differentiation (reproduce heterogeneity), increased proliferation/colony formation, expression of stemness marker and drug resistance genes, tumor forming
$ALDH^{high}$	Cells that have high levels of aldehyde dehydrogenase activity measured by its conversion of a fluorescent substrate to a negative product which is retained intracellularly, marking the ALDH-expressing cells	HNSCC[a] (Chen et al., 2010), buccal, retromolar trigone (Chen et al., 2009), laryngeal (Chen, Wei, et al., 2011; Clay et al., 2010), tongue (Chen, Wei, et al., 2011; Clay et al., 2010) and oropharyngeal (Clay et al., 2010), gingival (Tang et al., 2011)	Self-renewal (sphere-formation), increased proliferation/colony formation/invasion, expression of stemness markers, tumor forming

$CD44^{+}$	Cell surface glycoprotein; most frequently expressed surface marker on CSLCs	HNSCC[a] (Prince et al., 2007), hypopharyngeal (Chikamatsu et al., 2012; Okamoto et al., 2009), gingival (Chikamatsu et al., 2012), esophageal (Zhao et al., 2011)	Self-renewal (sphere formation, serial propagation), differentiation (reproduce heterogeneity), increased proliferation/invasion/migration, expression of stemness markers and drug resistance genes, tumor forming
$CD133^{+}$	Cell surface glycoprotein; marker for CSLCs in some solid tumor types	Tongue (Chen, Wu, et al., 2011; Zhang et al., 2010), buccal (Zhang et al., 2010), gingival (Chen, Wu, et al., 2011)	Self-renewal (sphere formation), differentiation (reproduce heterogeneity), increased colony formation/invasion, expression of stemness markers, tumor forming
$GRP78^{mem+}$	Glucose regulated protein78; mediator of endoplasmic reticulum homeostasis, anchored at the plasma membrane	Tongue, gingival (Wu et al., 2010)	Self-renewal (sphere-formation), differentiation (reproduce heterogeneity), increased colony formation/invasion, expression of stemness markers, tumor forming
c-Met^{+}	Tyrosine kinase receptor for hepatocyte growth factor	Oropharyngeal, buccal, tongue (Sun & Wang, 2011)	Self-renewal (serial transplantation), differentiation (reproduce heterogeneity), increased colony formation, expression of stemness genes, tumor forming
$p75^{NTR+}$	Low-affinity neurotrophin receptor; mediates neuronal survival, differentiation, and apoptosis	Esophageal (Huang et al., 2009)	Self-renewal (sphere formation, serial passage), differentiation (reproduce heterogeneity), tumor forming

[a]Specimen(s) of unknown origin.

Table 8.2 Differentially expressed markers in squamous cell carcinoma CSCs

Positive markers		
Oct4, Sox2, Nanog, Nestin, Bmi1, Klf4, Notch, p63, hTERT, β-catenin	Stemness	Chen et al., 2009, 2010; Chiou et al., 2008; Chikamatsu et al., 2012; Krishnamurthy et al., 2010; Lim et al., 2011; Prince et al., 2007; Tabor et al., 2011; Tsai et al., 2011; Wu et al., 2010; Yanamoto et al., 2011; Zhang et al., 2010; Zhao et al., 2011)
ABCG2, ABCB1, ABCA3, ABCA5, ABCC1	Drug resistance	(Chen et al., 2009; Chiou et al., 2008; Li et al., 2011; Lim et al., 2011; Okamoto et al., 2009; Sun et al., 2010; Tabor et al., 2011; Tsai et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011; Zhao et al., 2011)
BCL2, BCL2A1, BCL2L1, BNIP1, NAIP, CFLAR	Apoptosis	(Chikamatsu et al., 2012; Yajima et al., 2009)
Snail, Twist, vimetin, N-Cadherin, EpCAM(ESA)[a]	EMT (mesenchymal)	(Biddle et al., 2011; Chen et al., 2009; Chen, Wu, et al., 2011; Chen et al., 2011; Lo et al., 2011, Tabor et al., 2011)
Negative markers		
CK5(14), CK4(13), CK18, involucrin	Differentiation	(Lim et al., 2011; Wu et al., 2010; Zhao et al., 2010)
E-cadherin, EpCAM(ESA)[a]	EMT (epithelial)	(Biddle et al., 2011; Chen et al., 2009; Chen, Wu, et al., 2011; Lo et al., 2011)

[a] $CD44^{high}ESA^{high}$, $CD44^{high}ESA^{low}ALDH^{high}$ denote distinct, dynamic epithelial-like and mesenchymal-like CSC populations, respectively (Biddle et al., 2011).

highlighting the strengths and limitations to its interpretation (Pastrana et al., 2011).

In contrast to some other solid tumor types, most SCC cell lines and primary tumor cells are relatively inefficient in sphere-forming capacity under currently used assay conditions. Sphere-forming cells were identified in only 3 of 47 primary HNSCC specimens dissociated to single cells and grown in tumor sphere medium (Lim et al., 2011). These spheres were passaged as secondary and tertiary spheres, demonstrating self-renewal. HNSCC sphere-derived cells possessed increased colony formation in soft agar relative to their counterparts passaged under differentiating conditions. Chiou et al. enriched for CSCs by culturing two HNSCC cell lines under sphere-forming conditions, producing populations with increased activity in invasion and colony-forming assays *in vitro* (Chiou et al., 2008). In addition, xenografting these sphere-forming cells in limiting dilution assays demonstrated increased tumorigenicity as well as enhanced invasion and neovascularization. Similarly, spheres generated from certain HNSCC cell lines can be serially passaged, and cells derived from the spheres have been shown to be highly invasive *in vitro* (Chen, Wei, et al., 2011). These sphere-derived HNSCC cells also showed increased aldehyde dehydrogenase activity, another common CSC marker (Section 3.4).

3.3. Side Populations in SCC

Goodell et al. originally identified a small subset of bone marrow cells with increased efflux of the vital DNA binding dye, Hoechst 33342. This subset of cells, termed side population (SP) cells, based on their location in 2D flow cytometry plots, were demonstrated to contain hematopoietic stem cells (Goodell et al., 1996). Since that time, SP cells have been associated with stemness in other tissue types and used to isolate CSC candidates in various cancers, including SCC.

The frequency of SP cells in primary SCCs and cell lines reportedly varies from 0.2 to 3%. HNSCC SP cells have shown increased sphere formation, self-renewal over serial passage, and differentiation to restore normal tumor heterogeneity (Loebinger et al., 2008; Sun et al., 2010; Tabor et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011). HNSCC SP cells can also have increased *in vitro* proliferative and colony-forming capacities (Loebinger et al., 2008; Tabor et al., 2011) as well as enhanced tumorigenicity *in vivo* (Loebinger et al., 2008; Yanamoto et al., 2011). SP cells have also been defined in primary HNSCCs, accounting for about 0.5% of the

tumor (Yanamoto et al., 2011). SP cells isolated from primary esophageal squamous cell carcinomas (ESCCs) show comparable behavior, with increased colony formation *in vitro* and xenograft tumor formation at a limiting dilution of only 100 cells (Li et al., 2011).

3.4. Aldehyde Dehydrogenase Activity and SCC CSCs

The aldehyde oxidative function of the aldehyde dehydrogenase family of enzymes participates in retinoic acid biosynthesis and is thus innately linked to the regulation of squamous epithelial differentiation (Douville et al., 2009). High aldehyde dehydrogenase isoform 1 (ALDH1) activity has been detected in some normal stem cell populations, particularly hematopoietic progenitor cells (Kastan et al., 1990), and subsequently used to isolate CSC candidates in different cancers, including SCCs. A widely used assay for ADLH1 activity is based on the fluorochrome Aldefluor® (BODIPY-conjugated aminoacetaldehyde, Storms et al., 1999), which passively diffuses into the cell and is converted by ALDH1 to BODIPY-aminoacetate. This product is retained within the cell, resulting in a green fluorescence of $ALDH^{high}$ cells.

$ALDH^{high}$ cells isolated from primary HNSCCs were shown to be more tumorigenic as xenografts than $ALDH^{low}$ cells in two studies, but with relatively modest differences in limiting dilution. Specifically, 3000 $ALDH^{high}$ cells formed tumors in all mice injected, whereas $ALDH^{low}$ cells were not tumorigenic until more than 10,000 cells were used (Chen et al., 2009, 2010). $ALDH^{high}$ cells from primary HNSCC specimens form tumors in mice from as few as 500 cells and recapitulate original tumor histology and heterogeneity with respect to ALDH1 activity (Clay et al., 2010). $ALDH^{high}$ HNSCC cells also appear to have more proliferative and invasive potential as well as higher sphere-forming capacity than $ALDH^{low}$ or parental populations (Chen et al., 2009; 2010; Chen, Wei, et al., 2011).

One study further fractionates $ALDH^{high}$ HNSCC cells based on high expression of the cell surface marker CD44 and low expression of CD24 (Chen et al., 2009). CD44 is a cell surface marker used to define CSCs in multiple tumor types including SCCs (Section 3.5.1) and CD24 is a negative CSC marker in breast cancer that has failed consistent validation in SCCs. $ALDH^{high}/CD44^{+}/CD24^{-}$ cells possessed higher tumorigenicity than the $ALDH^{high}$ or CD44+/$CD24^{-}$ cells subsets alone and showed the highest *in vitro* proliferation, colony formation, invasion, and sphere formation of all the subsets (Chen et al., 2009). Similarly, addition of CD49f, a normal stem

cell marker, to selection of $ALDH^{high}$ cells identified a subpopulation with enhanced stemness features in the HNSCC HEp3 cell line. Notably, these $CD49f^{+}/ALDH^{high}$ cells demonstrated a nonhierarchical plasticity with the non-CSC phenotypes defined based on these two markers (Bragado et al., 2012).

3.5. CSC Makers in SCC

3.5.1. CD44

The cell surface glycoprotein CD44 is a receptor for matrix hyaluronic acid. The functions of multiple splice variants of this molecule remain poorly understood but may hold significance in the progression of several malignancies (Naor et al., 2002, Naor et al., 2008; Zoller, 2011). CD44 has become the most commonly used cell surface marker for CSCs across multiple tumor types and is perhaps the most universally validated CSC marker in HNSCCs at present. Prince et al. identified a subpopulation (<10%) of CD44-expressing cells in primary HNSCC specimens with CSC properties (Prince et al., 2007). These $CD44^{+}$ HNSCC cells were highly tumorigenic compared with $CD44^{-}$ cells and successfully propagated in serial xenotransplantation assays. Tumors formed from sorted $CD44^{+}$ cells reproduced the original tumor morphology and heterogeneity with respect to CD44 expression. In a subsequent study, ALDH1 activity was combined with CD44 to select CSCs from primary HNSCCs (Krishnamurthy et al., 2010). $CD44^{+}/ALDH^{high}$ cells showed enhanced xenograft tumorigenicity and formed tumors that recapitulated the heterogeneity of the original. This study also described a "gradient of stemness" with respect to colony-forming efficiency: $CD44^{+}/ALDH^{high} > CD44^{+}/ALDH^{low} > CD44^{-}/ALDH^{low}$.

It is important to note that $CD44^{+}$ cells are not consistently a minority subset in HNSCCs, forming up to 80% of cells in many tumors (Joshua et al., 2012). Resembling these tumors, most HNSCC cell lines are nearly 100% $CD44^{+}$, and yet a few cell lines have been further fractionated by some investigators based on distinctions in CD44 cell surface level. Such a $CD44^{high}$ subpopulation (2.1%) isolated from a HNSCC cell line displayed increased sphere-formation, proliferation, migration, and invasion (Okamoto et al., 2009) as well as high CD133 and low CD24 expression, surface signatures of CSCs in other cancers. Furthermore, HNSCC cells grown in tumor sphere media are enriched for $CD44^{high}$ cells (Chikamatsu et al., 2012). In select ESCC cell lines, higher cell surface CD44 levels correlate with tumorigenicity and induced differentiation of these cells decreases CD44 expression (Zhao et al., 2011).

3.5.2. CD133

CD133 was initially described as a cell surface marker specific for hematopoietic stem cells (Miraglia et al., 1997, Yin et al., 1997) and subsequently has been pursued extensively as a CSC marker across multiple tumor types (Keysar & Jimeno, 2010). While not a broadly validated marker in SCCs, the existence of a subpopulation of $CD133^+$ cells has been reported in certain HNSCC cell lines (1–2% cells) as well as in primary tumor tissues (1–3%) (Zhang et al., 2010). These $CD133^+$ cells isolated from HNSCC lines showed increased sphere formation compared with $CD133^-$ cells, and HNSCC-derived spheres were enriched for $CD133^+$ cells (Zhang et al., 2010). This subpopulation also exhibited higher xenograft tumorigenicity than $CD133^-$ cells and gave rise to both $CD133^+$ and $CD133^-$ cells. Silencing CD133 expression abrogated sphere formation in two HNSCC cell lines and simultaneously decreased colony formation, migration, and invasion while promoting differentiation (Chen, Wu, et al., 2011).

3.5.3. Other Markers

3.5.3.1. c-Met

Signaling by the receptor tyrosine kinase c-Met has been implicated in the progression of a variety of cancers including HNSCC (De Herdt & Baatenburg de Jong, 2008; Di Renzo et al., 2000; Gentile et al., 2008). Sun and Wang report a subpopulation of c-Met^+ cells in three PDXs of HNSCCs that display CSC properties (Sun & Wang, 2011). The c-Met^+ subset was shown to have enhanced tumorigenicity, with as few as 100 cells forming xenograft tumors that were similarly heterogeneous and could be serially passaged. c-Met^+ HNSCC cells were metastatic by intracardiac injection whereas c-Met^- cells were not. As expected, cells positive for both c-Met and CD44 were more tumorigenic than single marker positive cells.

3.5.3.2. GRP78

Expression of membrane-bound 78 kDa glucose-regulated protein ($GRP78^{mem}$) is another potential regulator of stemness and tumorigenicity in HNSCC cells (Wu et al., 2010). GRP78 (also known as binding immunoglobulin protein BiP) is an endoplasmic reticulum chaperone protein relevant to embryonic stem cell (ESC) survival (Gonzalez-Gronow et al., 2009; Luo et al., 2006). GRP78 has also been shown to play a role in HNSCC growth and metastatic potential (Chiu et al., 2008). Wu et al. observed increased expression of $GRP78^{mem}$ in six HNSCC cell lines grown

in tumor sphere media. Characteristic of CSCs, isolated GRP78^{mem+} cells were sphere-forming, tumorigenic *in vivo*, and generated both GRP78^{mem+} and GRP78^{mem-} cells. SiRNA-mediated silencing of GRP78 diminished sphere formation and drove cells toward a differentiated phenotype (involucrin^{+}/CK18^{+}).

3.5.3.3. p75NTR

The low-affinity neurotrophin receptor p75^{NTR} regulates neuron survival, differentiation, and apoptosis and has been used as a marker of various stem and progenitor cell populations (Boiko et al., 2010; Campagnolo et al., 2001; Okumura et al., 2003; Qi et al., 2008; Yamamoto et al., 2007). Cells positive for p75^{NTR} from four ESCC cell lines showed increased capacity to be passaged as spheres as well as higher tumorigenicity than p75^{NTR-} cells *in vivo* (Huang et al., 2009).

3.5.4. Stemness Markers in SCC CSCs

3.5.4.1. Oct4, Sox2, and Nanog

The transcription factors Oct4, Sox2, and Nanog are required to maintain pluripotency and self-renewal in ESCs (Boyer et al., 2005; Loh et al., 2006). Enhanced expression of these factors is often observed in SCC CSCs, supporting the innate stemness of subpopulations currently defined based on other current markers. Sphere-forming SCC CSCs have been shown to express increased levels of Oct4, Nanog, and Sox2 mRNA and protein (Chiou et al., 2008; Chen, Wei, et al., 2011; Lim et al., 2011). Nestin, an intermediate filament protein widely employed as a marker of neural stem cells (Park et al., 2010), is also increased in HNSCC tumor spheres (Chiou et al., 2008; Lim et al., 2011). ALDH^{high} HNSCC subpopulations show expression patterns similar to ESCs including high expression of Oct4, Nanog, and Sox2, as well as nestin and the transcription factor Klf4 (Chen et al., 2009, 2010). Elevated Oct4 and Nanog mRNA levels are also present in SP cells (Sun et al., 2010; Tabor et al., 2011). Similarly, CSC subpopulations expressing the cell surface proteins CD44 and CD133 also display heightened levels of one or more of these factors (Chen, Wu, et al., 2011; Chikamatsu et al., 2012; Zhang et al., 2010).

3.5.4.2. Bmi1

Bmi1 is a member of the Polycomb family of transcription repressors, which have been implicated in processes that regulate stem cell fate (Park et al., 2004). Bmi1 is necessary for efficient self-renewal of adult hematopoietic

and neuronal stem cells (Molofsky et al., 2003; Park et al., 2003). SCC CSCs defined by various methods show high expression of Bmi1 (Chen et al., 2010; Chikamatsu et al., 2012; Huang et al., 2009; Prince et al., 2007; Yanamoto et al., 2011). Knockdown of Bmi1 in $ALDH^{high}$ CSCs significantly inhibited the colony-forming and invasion capacities of these cells, supporting a role for Bmi1 in regulating a CSC state in HNSCC (Chen et al., 2010). Furthermore, microarray analysis revealed a shift away from an ESC-like gene profile upon Bmi1 downregulation.

3.5.4.3. Other Stemness Markers

Various other factors known to play roles in stem cell regulation are also found to be differentially expressed in SCC CSCs. For example, p63, a marker of tissue-specific stem cells in squamous epithelia (Pellegrini et al., 2001), is upregulated in $p57^{NTR+}$ ESCC cells. The Wnt/β-catenin and Notch pathways play key regulatory roles in adult stem cells in various tissues (Blanpain et al., 2006; Brabletz et al., 2009; Conboy & Rando, 2001; Fre et al., 2005; Korkaya et al., 2009). Notch1 signaling normally drives keratinocyte differentiation in squamous epithelia but appears to have alternate, stemness-promoting functions upon malignant transformation (Ohashi et al., 2010, 2011). Expression of Notch1 and β-catenin is increased in $CD44^+$ and $CD133^+$ HNSCC cells, respectively (Chikamatsu et al., 2012; Zhang et al., 2010). $CD133^+$ CSCs also upregulate expression of the stem cell–associated gene, hTERT (Zhang et al., 2010). Concurrently, markers of squamous epithelial differentiation such as involucrin and CK18 are typically decreased in CSC populations (Chen, Wu, et al., 2011; Huang et al., 2009; Lim et al., 2011; Zhao et al., 2011).

4. EPITHELIAL TO MESENCHYMAL TRANSITION AND STEMNESS IN SCCs

EMT diversifies cell types during embryogenesis and also allows epithelial cells to acquire a migratory, mesenchymal-like phenotype during wound healing. There is accumulating evidence that a similar EMT contributes to invasion and metastasis of carcinoma cells (Singh & Settleman, 2010; Yang & Weinberg, 2008). The relevance of EMT to CSCs was first defined in $CD44^+C24^-$ breast cancer cells, which exhibit a prominent mesenchymal-like gene expression profile (Mani et al., 2008; Morel et al., 2008).

Currently defined SCC CSCs also possess mesenchymal-like traits, and inducing EMT in HNSCC cells correlates with the emergence of CSCs and vice versa. Gene expression profiling of $ALDH^{high}$ HNSCC cells demonstrated an EMT–associated expression signature (Chen et al., 2009). Overexpression of stem cell surface protein CD133 in HNSCCs similarly induces expression of mesenchymal markers vimentin and fibronectin while downregulating epithelial specific antigen (ESA) (Chen, Wu, et al., 2011). Sphere-forming HNSCC cells express increased levels of Snail, Twist, α-SMA, and vimentin and possess a more invasive phenotype (Chen, Wei, et al., 2011). Similarly, $CD44^{high}$ ESA^{low} cells within HNSCC cell lines possess fibroblast-like morphology and express high mesenchymal markers vimentin, Snail, Twist, and Axl, versus low E-cadherin (Biddle et al., 2011). Accordingly, inducing EMT by adding TGFβ enriches for these $CD44^{high}$ ESA^{low} CSCs.

Modulation of EMT-related genes can affect CSC populations in HNSCC. Lo et al. found overexpression of the metastasis-promoting gene S100A4 to drive EMT and stemness in HNSCC cell lines (Lo et al., 2011). Likewise, silencing of S1004A simultaneously inhibited sphere formation, Oct4 and Nanog expression, and xenograft tumor formation. Inhibition of Snail in $ALDH^{high}$ HNSCC cells also suppresses the CSC phenotype, evidenced by decreased sphere formation and tumorigenicity (Chen et al., 2009).

5. THERAPY RESISTANCE IN CSCs

The critical function of adult stem cells in normal tissue homeostasis necessitates their resistance to diverse stressors, including hypoxia, nutrient deprivation, radiation, and chemical toxins. The CSC hypothesis predicts that CSCs possess comparable resistance to chemotherapy and radiation and thus serve as a reservoir for tumor repopulation posttherapy. Limited studies of CSCs in SCCs show evidence of such enhanced therapy resistance (Table 8.3), while mechanistic understanding in this area remains to be fully developed.

5.1. Resistance to Chemotherapy

Some studies have used cell viability assays (MTT, MTS) to assess the sensitivity of SCC CSCs defined by functional readouts such as sphere-forming capacity, SP status, and ALDH activity to a variety of

Table 8.3 Therapy Resistance in Squamous Cell Carcinoma CSCs

Treatment	CSC Population(s) with Increased Resistance	References
5-Fluoruracil	Tumor spheres, SP, $CD44^+$	(Lim et al. 2011; Okamoto et al. 2009; Tabor et al. 2011; Yajima et al. 2009; Yanamoto et al., 2011)
Cisplatin	Tumor spheres, SP, $CD44^+$	(Li et al., 2011; Lim et al., 2011; Okamoto et al., 2009; Sun & Wang, 2011; Yajima et al., 2009)
Carboplatin	SP, $CD44^+$	(Okamoto et al., 2009; Yajima et al., 2009)
Paclitaxel	Tumor spheres, $CD44^+$, $CD133^+$	(Lim et al., 2011; Okamoto et al., 2009; Zhang et al., 2010)
Docetaxel	Tumor spheres, $CD44^+$	(Lim et al., 2011; Okamoto et al., 2009)
Bortezomib	SP	(Li et al., 2011)
Mitoxantrone	SP	(Loebinger et al., 2008)
Radiation	Tumor spheres, $CD44^+$, $ALDH^{high}$	(Chen et al., 2009; Chiou et al., 2008; Chikamatsu et al., 2012)

chemotherapeutic drugs. Cells dissociated from primary HNSCC-derived spheres showed greater resistance to multiple cytotoxic drugs relative to HNSCC cells grown under differentiating conditions (Lim et al., 2011). SP cells isolated from HNSCC cell lines display increased survival compared with non-SP cells after treatment with the cytotoxic drug 5-fluorouracil (5-FU) (Tabor et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011). SPs from HNSCC and ESCC lines have also shown enhanced viability relative to non-SPs upon treatment with conventional and targeted drugs including platinum compounds (Li et al., 2011; Yajima et al., 2009) and bortezomib (Li et al., 2011). Sensitivity to the drug taxol can be increased in primary HNSCC-derived $ALDH^{high}$ cells through siRNA-mediated silencing of the stemness gene Bmi1 (Chen et al., 2010). SPs from HNSCC lines were shown to maintain increased colony formation compared with parental cells when cultured in the presence of the topoisomerase I inhibitor mitoxantrone (Loebinger et al., 2008).

Other studies have tested drug resistance in CSCs defined by cell surface markers. For example, a $CD44^{high}$ subpopulation from the HNSCC Gun-1 line showed modestly increased viability, measured by MTS assay, compared with $CD44^{low}$ cells after treatment with a panel of cytotoxic drugs (5-FU, docetaxel, paclitaxel, cisplatin, and carboplatin) (Okamoto et al., 2009).

Drug treatment has also been shown to enrich for populations of SCC CSCs. Treatment of HNSCC cells with 5-FU and paclitaxel enriches for SP and $CD133^+$ cells, respectively (Yajima et al., 2009; Zhang et al., 2010). A subpopulation of $p75^{NTR+}$ cells is increased in ESCC when exposed to cisplatin (Huang et al., 2009). c-Met^+ CSCs are markedly enriched in HNSCC-xenografted mice treated with cisplatin (Sun & Wang, 2011). These cisplatin resistant tumor cells also have enhanced secondary tumor growth, supporting a role for c-Met^+ CSCs in disease relapse. Interestingly, HNSCC cells selected for cisplatin resistance were found to possess several CSC properties including increased proliferation, sphere-forming capacity, colony formation, and invasion compared to the drug sensitive parent cells (Tsai et al., 2011). Cisplatin resistant HNSCC cells also express high levels of stem cell surface markers (CD133 and c-Kit) as well as stemness markers Oct4, Nanog, Nestin, and Bmi1.

5.2. Resistance to Radiation

Radiation resistance has been shown to increase within CSC subpopulations in SCC cell lines and primary tumors. Sphere-forming cells derived from an HNSCC cell line were less sensitive to up to 10 Gray of ionizing radiation than parental cells (Chiou et al., 2008). $ALDH^{high}$ and $ALDH^{high}/CD44^+/CD24^-$ populations sorted from primary HNSCC tumors displayed a comparably decreased radiation dose response relative to parental or $ALDH^{low}$ cells (Chen et al., 2009). Two cell lines containing small subsets of $CD44^{hi}$ cells were also used to show a modest increase in survival of this subpopulation following exposure to 10 Gray (Chikamatsu et al., 2012). Sensitivity to irradiation may be restored through silencing of genes involved with CSC maintenance; knockdown of Bmi1 or GPR78 restored sensitivity to ionizing radiation in primary $ALDH^{high}$ HNSCC cells and $GPR78^{mem+}$ cells from HNSCC lines, respectively (Chen et al., 2010; Wu et al., 2010). Importantly, how CSCs respond to the radiation dosing and fractionation regimens used clinically for HNSCC remains unknown.

5.3. Mechanisms of Drug Resistance in CSCs

Acquired drug resistance in clonal populations of tumor cells can arise by both induction of epigenetic changes and selection of spontaneous genetic variants that confer survival advantage during treatment. Based on the CSC hypothesis, drug therapy may selectively enrich intrinsically resistant CSCs and/or promote acquired resistance by inducing epigenetic shifts that drive

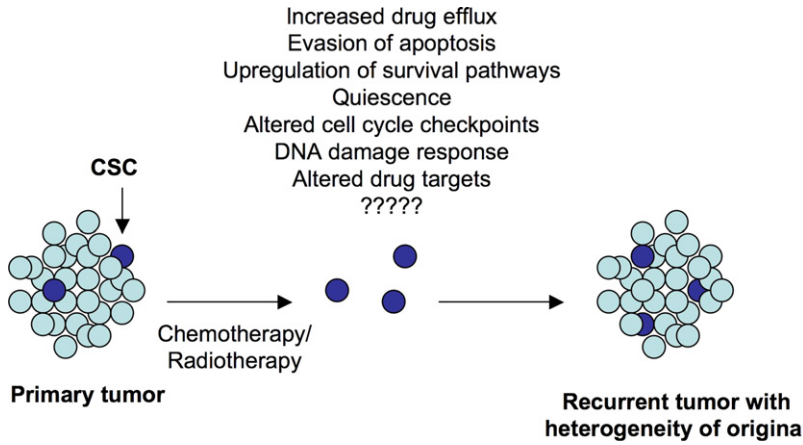

Figure 8.2 Model and proposed mechanisms of CSC-mediated therapy resistance. For color version of this figure, the reader is referred to the online version of this book.

differentiation to a stem-like state. Resistance in CSCs likely derives from multiple factors including quiescence, resistance to DNA damage/capacity for DNA repair, and expression of adenosine triphosphate-binding cassette (ABC)-transporter pumps and anti-apoptotic proteins (Fig. 8.2).

5.3.1. Multidrug Efflux Proteins

Alteration of effectors that regulate the accumulation of drugs within cells is one of the most studied mechanisms of multidrug resistance. ABC transporters, a class of multidrug efflux pumps, are known to be associated with cancer drug resistance. Normal stem cells express high levels of specific ABC transporters, which function to protect them from certain damaging agents (Moitra et al., 2011; Scharenberg et al., 2002). Similarly, CSCs can express higher levels of these efflux proteins that afford protection to some chemotherapeutic drugs.

5.3.1.1. ABCG2

ABCG2 is an ABC-transporter that homodimerizes at the plasma membrane and actively effluxes a range of substrates, including both cytotoxic compounds and fluorescent DNA binding Hoechst dyes (Sarkadi et al., 2004). It is therefore not surprising that CSCs selected based on their enhanced ability to exclude Hoechst (SP cells) often show increased resistance to chemotherapeutic drugs. SCC SP cells have been reported to display increased ABCG2 expression and/or activity (Li et al., 2011; Sun

et al., 2010; Tabor et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011), which may mediate resistance to diverse cancer drugs including platinum compounds, bortezomib, and 5-FU (Li et al., 2011; Sun et al., 2010; Tabor et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011). In support of a role for ABC family proteins in CSC drug resistance, SCC SP cells can be sensitized to chemotherapy upon general inhibition of ABC transporters by the calcium channel blocker verapamil (Loebinger et al., 2008).

Tumor spheres generated from primary HNSCC specimens showed increased ABCG2 expression (Chiou et al., 2008; Lim et al., 2011) as well as a higher fraction of SP cells compared with the same cells maintained in differentiating media (Lim et al., 2011). Furthermore, these spheres displayed less sensitivity to paclitaxel, cisplatin, 5-FU, and docetaxel than their differentiated counterparts. CSCs defined by expression of the cell surface markers CD44 and CD133 or by ALDH1 activity also express elevated levels of ABCG2 and can be more resistant to various therapies (Chen et al., 2009, 2010; Okamoto et al., 2009; Zhang et al., 2010; Zhao et al., 2011). Finally, SCC cells with a CSC-like phenotype selected for cisplatin resistance show enhanced ABCG2 expression (Tsai et al., 2011).

5.3.1.2. Other ABC Transporters

ABCB1, also known as MDR1 or P-glycoprotein, can bind a variety of hydrophobic compounds including the anticancer drugs doxorubicin, vinblastine, and taxol (Gottesman et al., 2002). High expression of ABCB1 is found in SCC CSC populations selected by multiple methods, including SP analysis and ALDH1 activity (Chen et al., 2009; Li et al., 2011; Yajima et al., 2009). $CD44^+$ cells from ESCC specimens, which are enriched upon treatment with 5-FU or cisplatin, show increased expression of ABCA5 in addition to ABCG2 (Zhao et al., 2011). $ALDH^{high}$ and $ALDH1^{high}/CD44^+/CD24^-$ populations of HNSCC cells were shown to express multiple efflux pumps including ABCG2, ABCB1, and ABCC1 (or MRP1) (Chen et al., 2009). SP cells from ESCC tumors were shown to have high expression of a number of different ABC transporters (ABCB1, ABCG2, ABCA3, ABCC1), as well (Li et al., 2011).

5.3.2. Resistance to Apoptosis

Evasion of apoptosis as a pro-survival strategy is a hallmark of both cancer and stem cells (Hanahan & Weinberg, 2011; Kruyt & Schuringa, 2010); thus, activation of anti-apoptotic pathways likely plays a role in resistance of CSCs to therapy. Differential expression of apoptosis-related genes, most

commonly B-cell lymphoma/leukemia-2 (Bcl-2) family genes, is described in SCC CSCs. The BCL2 oncogene product suppresses apoptosis through inhibition of caspase activation (Ola et al., 2011). SP cells from an HNSCC line showed increased levels of BCL2 and BCL2A1 gene expression (Yajima et al., 2009) along with high expression of another pro-survival gene, CFLAR. $CD44^{high}$ CSCs within certain HNSCC lines have decreased basal levels of apoptosis and display an increased resistance to the apoptotic inducing stimuli TNF-alpha, anti-Fas, and TRAIL (Chikamatsu et al., 2012). This $CD44^{high}$ subpopulation also upregulated Bcl-2, Bcl-2 family genes BCL2A1 and BCL2L1, and inhibitor of apoptosis protein (IAP) family genes BNIP1 and NAIP. Manipulation of mediators that regulate SCC CSC dynamics can restore sensitivity to apoptosis. Knockdown of Bmi1 in $ALDH^{high}$ primary HNSCC cells increased apoptosis along with sensitivity to taxol (Chen et al., 2010). Similarly, silencing of GRP78 induced the expression of pro-apoptotic molecules Bax and Caspase 3 in CSCs in HNSCC cell lines (Wu et al., 2010).

5.3.3. EMT

EMT has received considerable attention for its emerging role in intrinsic and acquired drug resistance (Singh & Settleman, 2010). We have previously demonstrated that a low-turnover, mesenchymal-like subpopulation within HNSCC cell lines and PDXs resists both cytotoxic and EGFR-targeted therapy (Basu et al., 2010; Basu et al. 2011). Although mechanisms underlying this EMT-based resistance are incompletely defined, they likely overlap with those attributed to CSC populations, which can share a mesenchymal-like gene signature (Section 4).

5.3.4. Other Potential Mechanisms of Drug Resistance

Diverse additional mechanisms likely underlie intrinsic therapy resistance in CSCs. For example, Akt activity is enhanced in SP cells from primary ESCC tumors (Li et al., 2011). Activation of the pro-survival PI3K/Akt pathway is associated with chemoresistance, and inhibition of this pathway induces apoptosis and decreases growth of drug-resistant tumor cells (Abdul-Ghani et al., 2006; Cordo Russo et al., 2008; García et al., 2009; Lee et al., 2004). Inhibition of Akt in these SP cells decreased ABCG2 activity, thus linking this pathway to drug efflux mechanisms (Li et al., 2011). $CD133^{+}$ HNSCC cells display increased activation of the tyrosine kinase Src, which continues to hold interest as a potential target for overcoming cytotoxic drug resistance (Chen, Wu, et al., 2011; Grant & Dent, 2004). $p75^{NTR+}$ ESCC CSCs

express low levels of the major copper influx transporter CTR1, which has been shown to mediate cisplatin uptake, potentially contributing to the resistance of this population to the drug (Huang et al., 2009). Future analyses of SCC CSCs will likely identify other targetable signaling components as regulators of their drug resistance.

6. CSCs IN SCC CLINICAL SAMPLES AND PROGNOSIS

Identifying CSCs in human tissues is limited by the inability of any single current marker to accurately select all the cells of interest while excluding other phenotypes. Still, some markers associated with SCC CSCs in preclinical investigations have been validated in clinical specimens, and, in some cases, correlated with disease grade and/or prognosis.

Normal stem cells reside in the basal layer of mucosa in the upper aerodigestive tract (Janes & Watt, 2006) and thus CSCs may also be found in the basal compartments of those SCC tumors retaining a stratified architecture. Accordingly, an HNSCC PDX showed $CD44^+$ staining to be most intense in the basal layer of a well-differentiated tumor (Prince et al., 2007). CD44 costained with the basal cytokeratin CK5(14) in this primary HNSCC, with involucrin staining being mutually exclusive with these markers. Cells that were positive for CD44 and nuclear Bmi1 were also mainly localized to basal regions; co-expression of CD44 and nuclear Bmi1, however, was most prominent in poorly differentiated tumors (Prince et al., 2007). Sterz et al. observed that CD44 co-localized with the matrix metalloproteinase, MMP-9 within a basal-cell-like compartment at the invasive front of HNSCC tissues (Sterz et al., 2010). CD44 also showed strongest staining in the basal layer of well-differentiated ESCC specimens (Zhao et al., 2011). Interestingly, Krishnamurthy et al. observed that in primary HNSCC tumors, $ALDH^{high}$ cells were found mainly in close proximity to blood vessels, suggesting a potential perivascular CSC niche (Krishnamurthy et al., 2010).

Coexpression of CSC markers Oct4 and Nanog is increased in cisplatin-resistant tumors (Tsai et al., 2011). Increased expression of Oct4, Nanog, and CD133, individually or in combination, was observed in association with higher grade in HNSCC (Chiou et al., 2008). Importantly, expression of one or more of these markers more strongly correlated with poor prognosis, which in HNSCC is not clearly associated with grade, and co-expression of all three predicted the worst overall survival. It was further noted that $CD133^+$ cells in tumors are not consistently positive for either Oct4 or

Nanog (Chiou et al., 2008), which likely reflects the inability of surface markers in current use to fully capture CSCs.

S100A4 expression also appears to have prognostic significance, correlating with moderate to poor differentiation and worse overall survival in HNSCC (Lo et al., 2011). S100A4 is also found co-expressed with Oct4 and Nanog (Lo et al., 2011). GRP78 similarly correlates with poor prognosis in HNSCCs, and co-expression with Nanog further increases its negative prognostic value (Wu et al., 2010). ABCG2, which is often highly expressed in SCC CSCs, may also be an independent prognostic factor associated with poor survival in ESCC (Tsunoda et al., 2006).

7. DISCUSSION

It is evident that CSC populations with self-renewal and differentiation capacities exist within head and neck and esophageal SCCs. To date, few studies go further than isolating such populations and supporting their stemness based on sphere formation and clonogenicity *in vitro*, tumorigenicity in xenograft models, and gene expression profiling. A deeper understanding of the mechanisms that govern the dynamics of SCC CSCs may be critical for deciphering their roles in SCC progression and for targeting for therapeutic benefit. Key questions include (1) the extent to which CSCs as currently defined contribute to intrinsic drug resistance, relative to subpopulations in the non-CSC pool, (2) the detailed intrinsic resistance mechanisms in CSCs, (3) developmental relationships between CSCs and non-CSCs that determine the outcome of successful CSC targeting, and (4) developmental relationships between CSCs and other potentially related subpopulations with therapy resistance, including those defined by hypoxia, autophagy, and/or quiescence.

The diverse methods and markers discussed here provide tools for studying CSCs but likely fail to capture all (or the only) tumor-propagating cells within a population. CD44, to date the most broadly applicable marker of CSCs in primary human SCCs, is not without its caveats. The frequency of $CD44^+$ cells varies greatly between tumors, with reported frequencies up to 80% in aggressive primary HNSCCs (Joshua et al., 2012), making it unlikely to reflect the heterogeneity most relevant to determining the treatment response in these tumors. In addition, the level of ERK1/2 activation in a given HNSCC directly regulates CD44 surface expression, *in vitro* growth, and engraftment efficiency (Judd et al., 2012) in a manner that may not necessarily be linked to CSC frequency.

Though evidence supports CD44 as a CSC marker in SCCs, it is unlikely that a $CD44^+$ subpopulation is comprised exclusively of highly tumorigenic stem-like cells. Indeed, $CD44^+$ populations exhibit heterogeneity in expression of other CSC markers, proliferation, and tumor formation/propagation and can be subdivided using additional markers such as ALDH1 and c-Met to enhance tumorigenicity and stemness (Krishnamurthy et al., 2010; Sun & Wang, 2011). The apparent heterogeneity of SCC CSCs presents the challenge of systematically delineating the transition between these subpopulations and their individual roles in progression and therapy resistance. This complexity has been nicely illustrated by Biddle et al., who subdivide $CD44^{high}$-expressing HNSCC cells by ESA level to reveal two biologically distinct phenotypes: an epithelial-like CSC population ($CD44^{high}/ESA^{high}$) and a mesenchymal-like CSC population ($CD44^{high}/ESA^{low}$). Moreover, ADLH activity could predict the bipotent capacity of the $CD44^{high}/ESA^{low}$ population; $CD44^{high}/ESA^{low}/ALDH^{high}$ cells were bipotent, whereas $CD44^{high}/ESA^{low}/ALDH^{low}$ cells were not (Biddle et al., 2011). These data support a model of cell type regulation in HNSCC with a dynamic component. Biddle et al. propose that these HNSCC tumor cells exhibit a phenotypic plasticity in which $CD44^{high}/ESA^{high}$ CSCs can self-renew, produce terminally differentiated $CD44^{low}$ cells, or undergo EMT generating $CD44^{high}/ESA^{low}$ CSCs. The mesenchymal-like CSCs ($CD44^{high}/ESA^{low}$) with high ALDH1 activity can, in turn, self-renew, differentiate to a unipotent $ALDH^{low}$ state, or undergo mesenchymal to epithelial transition, regenerating the epithelial-like CSCs ($CD44^{high}/ESA^{high}$).

Further evidence of nonhierarchical differentiation by CSCs is provided by the capacity of both $CD44^+$ and $CD44^-$ HNSCC cells from primary tumors to form spheres, suggesting that the negative population can also enter a self-renewing state (Lim et al., 2011). In addition to highly tumorigenic $CD49f^{high}/ALDH^{high}$ cells in a HNSCC cell line, the $CD49f^{low}/ALDH^{low}$ population was shown to have latent tumorigenic potential (Bragado et al., 2012). Existence of phenotypic plasticity between CSCs and non-CSCs has garnered increasing support in other tumor types (Gupta et al., 2011; Quintana et al., 2010; Roesch et al., 2010).

The resistance of SCC CSCs to chemotherapy and radiation remains to be precisely defined at the mechanistic level; several inherent properties of stem cells appear to play a role, including altered expression of drug transporter molecules, evasion of apoptosis, and EMT-based shifts in gene expression. Altered cell signaling, including those mediated by the PI3K/

Akt pro-survival pathway and others, may confer resistance to cytotoxic and targeted therapies. For instance, a recent study using cutaneous SCC cell lines found CSC features localizing to a subpopulation (1.3%) with low cell surface EGFR expression (Le Roy et al., 2010), suggesting potential resistance to the EGFR-targeted therapies in current clinical use. Additional hallmarks of stem cells, such as alteration of DNA repair pathways or maintenance of a quiescent state, may play important roles in the survival of therapy-resistant CSCs.

Normal epithelial tissues contain slow-cycling stem cells that divide asymmetrically, giving rise to new stem cells that retain their quiescence as well as actively cycling transit-amplifying cells. Similarly, quiescence has been linked to CSCs in various tumor types (Moore & Lyle, 2011). Label-retaining methods, in which low-turnover cells are identified by retention of a fluorescent vital membrane dye that dilutes as a cell divides, have identified subpopulations of cells with tumor-forming and/or propagating capacities in melanoma, glioblastoma, and ovarian, breast, colon, and pancreatic cancers (Deleyrolle et al., 2011; Dembinski & Krauss, 2009; Fillmore & Kuperwasser, 2008; Kusumbe & Bapat, 2009; Moore et al., 2011; Roesch et al., 2010). Like CSC populations, label-retaining cells (LRCs) can possess an inherent resistance to chemotherapy (Dembinski & Krauss, 2009; Fillmore & Kuperwasser, 2008; Kusumbe & Bapat, 2009; Moore et al., 2011). Little is known about slow-cycling subpopulations of cells in head and neck and esophageal SCCs. A recent study revealed that a slow-cycling subpopulation of HNSCC cells, defined by retention of the fluorescent label CFSE, displayed enhanced proliferative potential and produced heterogeneous tumors in xenografted mice (Bragado et al., 2012). These LRCs are also enriched for CD49f, a marker of normal stem cells. Investigation into how these quiescent cells as well as CSCs defined by other methods differentially regulate cell cycle progression may provide important insight into their roles in tumorigenesis and drug resistance. Furthermore, using quiescence to identify CSC subpopulations in SCCs may shed light on the heterogeneity of CSCs and offer novel markers for these cells.

8. CONCLUSION

Significant evidence supports a role for CSCs in intrinsic SCC therapy resistance, though the specific mechanisms by which these subpopulations escape treatment are not currently understood. In this regard, there exist

a number of ongoing challenges. Current CSC models are limited in their ability to encompass all drug-resistant SCC cells with a single molecular state or marker. Going forward, the detailed methods used to identify SCC CSCs also merit increased attention, as differences in engraftment host and assay conditions can greatly impact *in vivo* tumorigenicity. Understanding the roles of SCC CSCs in regulating tumor heterogeneity and therapy resistance is increasingly complicated by evidence for multidirectional state transitions between CSC and non-CSC subpopulations. Nevertheless, advancing understanding of CSC biology in SCC is likely to ultimately impact the development of novel therapeutic strategies.

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ABBREVIATIONS

ABC Adenosine triphosphate binding cassette
ALDH Aldehyde dehydrogenase
AML Acute myeloid leukemia
CSC Cancer stem cell
EGFR Epidermal growth factor receptor
EMT Epithelial to mesenchymal transition
ESA Epithelial specific antigen
ESC Embryonic stem cell
ESCC Esophageal squamous cell carcinoma
5-FU 5-fluorouracil
HNSCC Head and neck squamous cell carcinoma
SCC Squamous cell carcinoma
SP Side population

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CHAPTER NINE

Targeting the Tumor Stroma as a Novel Therapeutic Approach for Prostate Cancer

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Abstract

Interactions between epithelium and the surrounding stroma are required to maintain organ function. These interactions provide proliferative and migratory restraints that define anatomical and positional information, mediated by growth factors and extracellular matrix components. When cancer develops, transformed cells lose these constraints while stroma adapts and coevolves to support the "function" of the tumor. The prostate is a good example of an organ that relies on its surrounding stroma during normal development and cancer progression. Carcinoma-associated fibroblasts (CAFs) constitute a substantial volume of the tumor stroma and play a pivotal role in tumor maintenance, dissemination, and even drug resistance. The origins of CAF and the exact mechanisms by which they promote tumor progression are still debated. CAF acquire an activated phenotype quite similar to the one seen during wound repair in sites of injury. Here, we describe the CAF ontogeny, the similarities with activated fibroblasts during physiological wound repair, and potential pathways that can be targeted to prevent their appearance in tumors and their protumorigenic functions in cancer progression. A strategy to identify aspects of stromal cell biology for therapeutic targeting is becoming increasingly plausible, driven by the increased understanding of the complex interplays between the cells and tissues of which tumors are comprised. Several preclinical and clinical studies show that targeting the stroma may be a promising and attractive therapeutic option for the treatment of cancer and has the potential to play an increasingly prominent role in future treatment strategies.

1. INTRODUCTION

With an estimated 241,740 new cases in 2012, prostate cancer is one of the most commonly diagnosed malignancies in American men. Although the trend in cancer mortality for prostate cancer is decreasing, this disease is still the second leading cause of cancer death in males, exceeded only by lung cancer. In the United States, an estimated 28,170 men will die from prostate cancer in 2012 (Siegel et al., 2012). An aggressive form of the disease is

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particularly prevalent among African–Americans. The therapeutic success rate for prostate cancer can be tremendously improved if the disease is diagnosed early. Thus, a successful therapy depends on reliable clinical indicators (biomarkers) for early detection of the presence and progression of the disease, as well as for prognosis following clinical intervention. The current clinical biomarkers for prostate cancer are not ideal. There is a need for biomarkers that can consistently and specifically distinguish between those patients who should be treated with definitive surgery to stop the aggressive form of the disease and those who should avoid overtreatment of the more indolent form of the disease (Boorjian et al., 2012).

While the death rate from prostate cancer has fallen, this success can be attributed mostly to improved detection and treatment (Abdollah et al., 2011). For the majority of patients, for whom surgical intervention represents overtreatment, there is still a need for an effective low impact medical approach to eradicate or at least impair tumor progression. Such an approach could be used as an adjuvant to watchful waiting/active surveillance, giving a level of comfort to both patients and clinicians. In order to provide the biological understanding needed to achieve such a goal, a trend over several years has been to consider cancers as resembling a developing organ. The uncontrolled growth of the tumor cells themselves is seen in the context of a complex organ in which all the constituent parts (including stromal tissues such as fibroblasts and muscle, blood vessels, immune/inflammatory cells, nerves, and extracellular matrix [ECM]) are in constant communication (crosstalk) and contribute to the aggressive nature of the disease. This scenario mimics specific aspects of organ development, an area that has been studied in much detail in many organs including the prostate gland and which has been the focus of many groups in recent decades.

1.1. Stromal–Epithelial Interactions During Normal Development and Disease

The interplay between mesenchymal fibroblasts and epithelial cells is known to be essential during embryonic and postnatal development (Cunha, 2010; Cunha et al., 2004). These interactions result in the harmonized development of tissues in correct spatial orientation with their surrounding anatomical neighbors, and in the timely expression of the genes required for function consistent with physiological demands during different phases of postnatal life. Epithelial–mesenchymal interactions continue during adulthood, playing a homeostatic role in the maintenance

of epithelial and stromal differentiation and growth quiescence. One of the organs in which these phenomena have been well studied is the prostate. The prostate develops from the embryonic urogenital sinus under the influence of circulating androgens (Cunha et al., 2004). During prostatic development, the urogenital mesenchyme (UGM) specifies urogenital epithelial identity, induces epithelial bud formation, and promotes growth and differentiation of a secretory epithelium (Marker et al., 2003; Staack et al., 2003). As the epithelium differentiates it, in turn, induces the UGM not only to undergo differentiation into smooth muscle, but also directs the spatial patterning of the smooth muscle (Cunha et al., 1992; Cunha et al., 1996; Hayward et al., 1996; Hayward et al., 1998). In the adult prostate, androgens act upon both the epithelium, to regulate differentiated function, specifically the expression of secretory proteins, and on the smooth muscle of the prostate. Androgenically driven interactions between the muscle and epithelium maintain glandular prostatic morphology. At a functional level, the contraction of the smooth muscle allows the excretion of the glandular secretions, necessary for the survival of the sperm. It has been shown recently in the rat prostate that contractility of the smooth muscle cells is positively influenced by circulating testosterone and regulates downstream effectors through the cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase-1 (cGKI)/phosphodiesterase 5 (PDE5) pathway (Zhang et al., 2012). Changes in the stromal compartment are critical components of benign proliferative conditions such as benign prostatic hyperplasia (BPH) and also play a role in the regulation of malignant tumor progression (Chung, 1995; Hayward et al., 1997; Ronnov-Jessen et al., 1996). Interestingly, the prostate is an organ that continues to grow after puberty and throughout adulthood resulting in a high incidence (>80%) of BPH in men older than 85 years (Bushman, 2009; Price et al., 1990). BPH is a benign condition and is not considered to be either premalignant or a precursor to prostate cancer. The condition is characterized by a progressive, but discontinuous, hyperplasia of both glandular epithelial and stromal cells leading to expansion of the prostate gland and clinical symptoms, prominently including constriction of the urethra and consequent difficulties with voiding. One of the pioneers of the role of the stroma during BPH pathogenesis was the pathologist John McNeal who proposed that changes observed in BPH are attributed to the "reawakening" of the adult stromal cells acquiring inductive properties reminiscent of mesenchymal cells in early stages of development (McNeal, 1978). The exact mechanisms that

control these events, as well as the pathological changes associated with BPH are essentially unknown.

Disruption of the coordinated interactions between the stromal and epithelial tissue compartments during carcinogenesis led pathologist G. Barry Pierce to promulgate the concept that "Neoplasia is a caricature of differentiation," and certainly it seems reasonable to describe a tumor as a caricature of a functional organ (Pierce et al., 1978). In many cases, the pathways that lead to normal development and growth are subverted in carcinogenesis to support a less organized and more invasive structure. This concept is also evident in the metastatic progression of local tumors. In 1889, Stephen Paget in his paper titled, "Distribution of secondary growths in cancer of the breast," introduced the concept of ***"seed and soil"*** in relation to cancer metastasis, although his comments were preceded by those of Fuchs who observed that certain organs may be "more predisposed" because they could provide the proper environment (***soil***) for tumor cells (***seeds***) to grow (Fuchs, 1882; Paget, 1889). This concept is also consistent with observations made during carcinogenesis and local tumor invasion. In a carcinoma, the ***seeds*** reside within the tumor epithelium, whereas the composition of the ***soil*** (tumor stroma) is heterogeneous and more complex. There are two major components of the stroma: the tumor ECM, which provides the connective-tissue framework of the tumor, and the cellular components such as muscle, fat, fibroblasts, immune and inflammatory cells, nerves, and blood vessels. The most abundant cell type within the stroma immediately adjacent to many prostate tumors is the fibroblast. The components of the tumor stroma resemble that of the granulation tissue formed during wound healing, and as a result of this phenotypic and functional similarity Hal Dvorak described a tumor as a "wound that never heals" (Dvorak, 1986). "Activation" of the stromal fibroblasts during wound healing and their transformation into myofibroblasts results in the secretion of growth factors and remodeling enzymes, as well as the physical contraction, necessary for tissue repair (Tuxhorn et al., 2001). The infiltration of blood vessels is an event that follows the conversion of fibroblasts into myofibroblasts, a phenomenon that causes tumor expansion favoring progression. The origin and functions of myofibroblasts during normal wound healing and pathological states will be discussed in detail in the following sections.

Another active cellular player in the tumor stroma is the recruited immune/inflammatory component. These cells were once thought to be protective but the situation is now understood to be far more complex. In

1850, Rudolph Virchow described the positive effect of chronic inflammation in tumor promotion. Examples of this association can be found in several malignancies including stomach, colon, cervix, breast, and prostate (Castellsague et al., 2002; Kornfeld et al., 1997; Mantovani et al., 2008; Nelson et al., 2002). During the transition from acute to chronic inflammation, monocyte-derived macrophages or tumor associated-macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) constitute the major component of the leucocytes recruited to the tumors (Mantovani et al., 2009). These cells are attracted via cytokines/chemokines produced by tumor cells and their surrounding stroma. Heterogeneity of TAMs and MDSCs and the diversity of actions attributed in different stages of cancer progression is the focus of current research to better understand their function (Mantovani, 2010; Qian and Pollard, 2010). For example, the presence of TAMs can be used to predict prostatic specific antigen (PSA) failure or prostate cancer progression after hormonal therapy (Nonomura et al., 2011). An interesting feature of atrophic prostatic glands, which are commonly seen in the aging prostate, is an increased inflammatory response surrounding these areas. Compared with normal epithelium, a large fraction of epithelial cells proliferates in these focally atrophic lesions. These two main components (atrophy - + inflammation) led pathologists propose the term proliferative inflammatory atrophy (PIA) for most of these atrophic lesions. The clear transition from PIA to prostate intraepithelial neoplasia [PIN], a likely precursor of prostate cancer) suggested that PIA should be considered a precursor of prostate cancer and highlights the role of inflammatory cells during prostatic carcinogenesis (De Marzo et al., 1999). For a more detailed description of PIN and its role during prostate cancer progression, excellent recent reviews have been published (Epstein, 2009; Montironi et al., 2011). The recent identification of macrophages expressing high levels of the proinflammatory interleukin 17 (IL-17) in PIA lesions have reinforced the idea of inflammation in prostate cancer progression (Vykhovanets et al., 2011).

The development of malignant tumors includes a series of changes in the interactions between the cells and tissues comprising the tumor, resulting in the formation of a complex, growing structure with metastatic capability. Understanding the role of each cellular component of this complex structure will enable us to develop better treatment strategies to target not only the tumor cells, but also more importantly the host environment that plays an active role positively and negatively regulating tumor progression.

2. CAF TAXONOMY

The terms "carcinoma-associated fibroblasts," "peritumoral fibroblasts," "cancer-associated myofibroblasts," "reactive stroma (RS)," or simply "myofibroblasts" have often been used interchangeably to describe the most common cell type present in the stroma surrounding solid malignant tumors (Desmouliere et al., 2004). We would suggest that the descriptor "CAF" should be considered as defining a functional status rather than a specific cell type. To define a CAF, cells should be able to retain their two main effects on epithelial cells, the induction of growth and invasion. We have been able to demonstrate in a series of publications that a proper identification of CAF, in the prostate, can be achieved by a functional *in vivo* biological assay (Hayward et al., 2001). This has become the "gold standard" method to identify fibroblasts with "tumor-inductive" properties (iCAF) compared to noninductive tumor-derived fibroblasts (niCAF) or fibroblasts isolated from normal areas of the prostate or normal prostate fibroblast (NPF) (Fig. 9.1). To assay this activity, a nontumorigenic but genetically initiated cell line is converted to a tumorigenic state in the presence of iCAF, but not of niCAF or of NPF. The BPH1 cell line has been immortalized with the SV40T antigen with the consequent inactivation of the p53 and pRb tumor suppressor pathways, which makes them susceptible to malignant transformation (Hayward et al., 2001). We would anticipate that other epithelial cell lines with similar levels of genetic damage could also be used as reporters of CAF activity. We have performed similar studies in other tissues (notably breast cancer—Franco, unpublished data) and identified tissue-appropriate alternative reporter lines. However, alternative reporters for prostate cancer progression have not been described at this point. Recently, several normal prostate epithelial cell lines have been generated in different labs; however, most of these cannot be completely transformed under the influence of iCAF (data not shown). The advantage of BPH1 cells over the new cells is the ability of these cells to form benign structures when recombined with mesenchymal cells isolated from the urogenital sinus. Thus, our data based upon 15 years of experience would suggest that not all fibroblasts derived from tumors have tumor-inducing potential, but that fibroblasts derived from normal tissues never express this inductive phenotype. We have recently observed that fibroblasts from bladder and breast can also induce transformation of predisposed or "initiated" reporter cell lines (unpublished data) suggesting that this *in vivo* system has clear benefits over

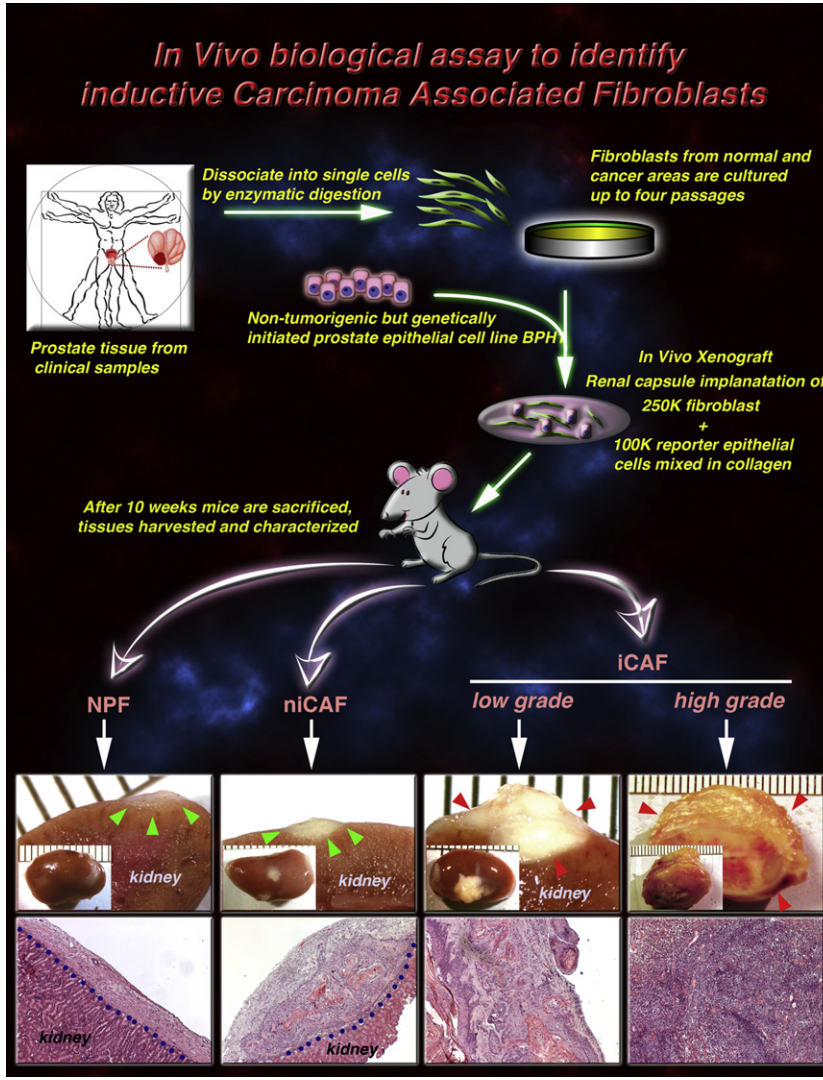

Figure 9.1 Isolation and assay of carcinoma-associated fibroblasts (CAFs). We have established an *in vivo* assay in which an "initiated" prostate epithelial cell line (BPH1) is transformed only in the presence of CAF cells with inductive potential (iCAF). This iCAF protumorigenic capacity can be classified as low- or high-grade based on the degree of invasion and growth. Some tumor-derived fibroblasts do not promote tumorigenicity (niCAF), similar to normal prostate fibroblasts(NPFs) isolated from benign areas of the prostate. Briefly, fibroblasts isolated from cancer patients are cultured *in vitro* for up to four passages before recombination with the epithelial cells. Xenografts are performed under the kidney capsule of immunocompromised mice and 10 weeks later retrieved for histological analysis. Once classified, cells can be used for additional experiments. For color version of this figure, the reader is referred to the online version of this book.

the standard assumption that all the fibroblasts surrounding tumors behave in the same manner.

A similar situation, in which some tumors progress and other, apparently similar lesions do not, is seen in the clinic. One factor that seems to play a major role in deciding tumor progression is the stroma. Two patients with the same histopathologic tumor grade may differ in their stromal response (RS). In the prostate, the majority of the stroma is composed of dense eosin-positive smooth muscle cells. Histologically, these cells are uniform in size and shape, with an ample cytoplasm and rounded nuclei. In contrast, RS cells lose the majority of their abundant eosinophilic cytoplasm; the well-organized band pattern of smooth muscle is replaced by a disorganized pattern with deposition of collagen fibrils and ECM. The collagen fibers are irregular in thickness and length, and there is a delicate fibrillary background. The presence of this "stromogenic carcinoma" increases the risk of progression and can be used to predict patient prognosis (Ayala et al., 2003; Ayala et al., 2011; Tuxhorn, et al., 2002a). The identification of tumor stromal components is of paramount importance to target appropriate cell types. The complexity of the fibroblast population in the tumor stroma is represented by the cellular overlap between commonly used markers, such as smooth muscle actin (αSMA) and vimentin, fibroblast-specific protein 1 (FSP1; also known as S100A4), and platelet-derived growth factor receptor beta (PDGFRβ) (Sugimoto et al., 2006). Functional identification of different fibroblast populations within the RS has not been properly addressed. We have recently proposed for the first time a model to study prostate stroma heterogeneity using an *in vivo* system of human-derived prostate stromal cell line BHPrS1. In this model, abrogation of the transforming growth factor type II (TGFβRII) expression in a subpopulation of normal fibroblasts resulted in the transformation of adjacent epithelial cells (mimicking loss of TGFβRII in some stromal cells adjacent to human prostate tumors) (Franco et al., 2011; Kiskowski et al., 2011; Placencio et al., 2008). The repertoire of cytokines and chemokines expressed by the heterogeneous stroma were similar to those observed in CAF derived from cancer patients.

The stroma is a heterogeneous mixture of different cell lineages including, not only just fat, muscle, fibroblasts, and immune/inflammatory cells but also the cells that compose the vasculature (endothelial cells and pericytes), lymphatics, and nerves. The function of these diverse cell types in a normal state is to maintain homeostasis and control epithelial cell polarity and also to ensure organ health and function. While the lineage of these cells

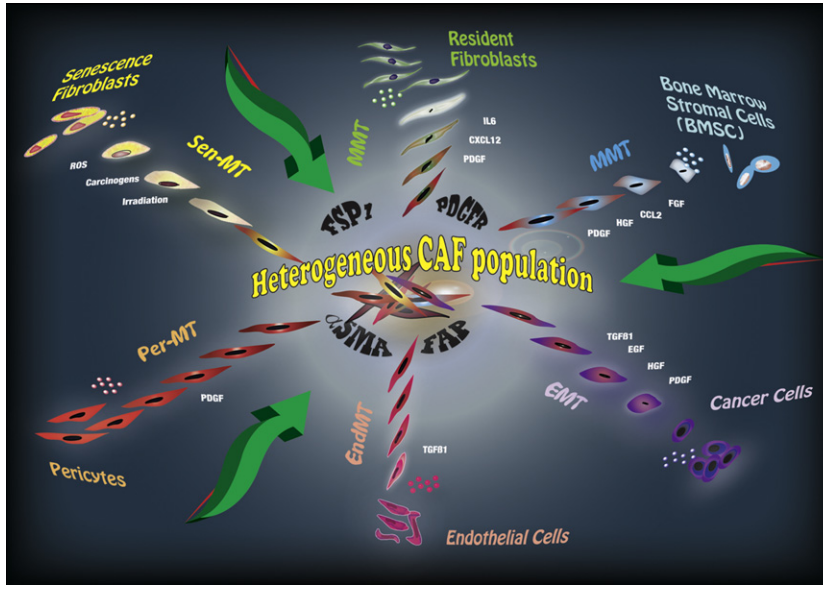

Figure 9.2 Potential origins of carcinoma-associated fibroblasts (CAFs). CAFs are key players in cancer progression and play a central role in the modulation of cancer growth. CAFs are heterogeneous and composed of a mixture of fibroblasts, which likely have different origins. Local host fibroblasts or bone marrow-derived cells may be recruited into the developing tumor and undergo mesenchymal to mesenchymal transition (MMT) to adopt a CAF phenotype under the influence of the tumor microenvironment. CAFs have also been suggested to originate from epithelial (EMT) or endothelial cells (EndMT). Other potential sources include senescence cells and pericytes. When activated, these cells interact with cancer cells and express several mitogenic and proinvasive factors that create a favorable milieu for immune/inflammatory cell recruitment and for tumor cells to proliferate and invade into the surrounding tissue. The potential for multiple origins of CAF confer a unique cellular heterogeneity that seems to be essential for their inductive properties. For color version of this figure, the reader is referred to the online version of this book.

is well established, the origin of the fibroblasts that compose the CAF populations is under intensive investigation and discussion, and several different concepts have been proposed (Fig. 9.2). We now discuss these concepts in detail.

2.1. Mesenchymal-Mesenchymal Transition

Perhaps, the most accepted idea for the origin of CAF is the suggestion that local or resident fibroblast "convert" or "transform" into an activated state through a process commonly identified as mesechymal-mesenchymal transition (MMT) (Cat et al., 2006a). This transdifferentiation is potentially

facilitated by the very high levels of growth factors present within tumors, produced either by cancer cells or by recruited inflammatory cells. One factor that has a likely role is the profibrotic transforming growth factor beta type I (TGFβ1). Autocrine TGFβ1 in conjunction with stromal-derived factor 1 alpha (SD1α or CXCL12) has been shown to be involved in the acquisition and maintenance of the myofibroblast phenotype in breast cancer (Kojima et al., 2010). Other cancer cell–secreted factors such as PDGFα/β, basic fibroblast growth factor (βFGF or FGF-2), and IL-6 can induce MMT in resident fibroblasts (Giannoni et al., 2010a; Okada et al., 2000; Shao et al., 2000). Some studies have suggested that this activation occurs via the generation of reactive oxygen species (ROS). Early work in skin tumors showed that dermal myofibroblasts respond to TGFβ1 stimulation by increasing ROS leading to the downregulation of gap junctions between CAFs with the subsequent promotion of tumor progression (Cat et al., 2006a). Loss of Caveolin-1 in CAF triggers nitric oxide (NO) overproduction, mitochondrial dysfunction, and oxidative stress via ROS production, with hypoxia-inducible factor 1α (HIF-1α) upregulation (Martinez-Outschoorn et al., 2010). More recently, it has been shown that in prostatic stromal cells TGFβ1-mediated fibroblast-to-myofibroblast differentiation is driven via induction of NOX4/ROS signaling. NOX4/ROS induce the phosphorylation of c-jun N-terminal kinase (JNK). Elevated ROS signaling is supported by the concomitant downregulation of selenium-containing ROS-scavenging enzymes and the selenium transporter SEPP1. Selenium supplementation restored expression of selenium-containing ROS scavengers, increased thioredoxin reductase 1 (TXNRD1) activity, depleted NOX4-derived ROS levels, and attenuated differentiation. These effects have clear therapeutic implications and show the potential clinical benefit of selenium supplementation and/or local NOX4 inhibition in stromal-targeted therapy (see the following sections) (Sampson et al., 2011).

2.2. Recruitment of Fibroblasts from Distant Organs

A second potential source for progenitors of CAF is represented by bone marrow-derived mesenchymal stem cells or MSCs. MSCs have been characterized by flow cytometry analysis based on the expression of several characteristic surface markers including CD44, CD71 (transferrin receptor), CD73 (SH3, SH4, or ecto 5′-nucleotidase), CD90 (THY-1), CD105 (SH2 or endoglin), and CD271 (low-affinity nerve growth factor receptor) and

the lack of expression of hematopoietic markers (Bernardo et al., 2009; Uccelli et al., 2008). Evidence of the high rate of MSC recruitment to tumors comes from studies performed in mouse models of gastric cancer in which MSCs can contribute as much as 25% of the total CAF population (Quante et al., 2011). MSCs are multipotent stromal cells and have the capacity to differentiate into multiple cell lineages including bone, cartilage, fat, and fibrous connective tissues under appropriate inductive conditions. They contribute to many physiological and pathological processes (Bergfeld and DeClerck, 2010). MSCs have the particular characteristic of mobilization to injury sites in many settings such as tissue repair, inflammation, and neoplasia. Several cytokines and growth factors produced by tumor cells or their activated stroma such as vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), hepatocyte growth factor (HGF), βFGF, PDGF, and chemokine (C-C motif) ligand 2 (CCL2) have been proposed to mediate the recruitment of MSCs to cancer tissues, in a manner that seems to mirror the activation of inflammatory cells during tissue remodeling (Dwyer et al., 2007; Feng & Chen, 2009; Spaeth et al., 2008). Some *in vivo* studies using labeled MSCs have shown that these cells can be recruited from not only the bone marrow but also from other tissues where they stay in a dormant state. Once within the tumor mass, they differentiate into CAF and acquire *de novo* expression of several characteristic markers phenotypically associated with tumor progression such as αSMA, fibroblast activated protein (FAP), tenascin-C, and thrombospondin-1 markers (Spaeth et al., 2009). However, the role of recruited MSCs within the tumor microenvironment is still controversial. MSCs can impact negatively or positively to tumor progression, through immunomodulatory and proangiogenic properties, depending on the source of MSC and the tumor model used (Kidd et al., 2008). Interestingly, in the prostate, CD90hi-CAF has been shown to express increased levels of cancer-promoting genes while increasing the resistance to apoptosis of prostate epithelial cells. The ability of MSC to home to areas of tissue damage and the potential of these cells as a therapeutic tool to efficiently deliver exogenously expressed soluble factors has been tested (Niess et al., 2011). Thus, MSC are potentially an option to target the tumor stroma. Previous studies have suggested that human MSC populations, *in vitro* and *in vivo*, are morphologically and functionally heterogeneous; thus, identification is based on the expression of several cell surface markers. Basically, these cells can be grouped into two categories: fast- and slow-growing clones based on the time taken by the individual clones to reach 20 population doublings (Mareddy et al., 2007). However,

all these putative mesenchymal markers are not uniquely expressed in stem cells and the search to find typical markers that determine the fate and function of MSCs still remains active. Thus, a more detailed functional analysis of the role of MSC subtypes during tumor progression is necessary to modulate the factors involved in their protumorigenic properties before they can be introduced in the clinic.

2.3. Other Sources of CAF

2.3.1. Endothelial Cells

Recently, endothelial to mesenchymal transition or EndMT, another type of cellular transdifferentiation, has emerged as a possible source of CAF in pathological situations such as fibrosis or cancer. Transdifferentiation is a process in which a "terminally differentiated" nonstem cell transforms into a different type of cell. In EndMT, an endothelial cell type present in mature vasculature is able to acquire a smooth muscle or myofibroblast phenotype. During this process, endothelial cells lose cell–cell junctions, become invasive or migratory, lose endothelial markers such as CD31 (also known as platelet endothelial cell adhesion molecule-1 or PECAM-1), and gain mesenchymal markers such as FSP1 or αSMA. While this transformation is a common feature during heart development, EndMT can also be observed postnatally in several pathological disorders including fibrosis and cancer (Stresemann et al., 2006; Zeisberg, Tarnavski, et al., 2007). Several studies have shown that cardiac fibrosis is closely associated with EndMT. For example, in a cardiac fibrosis model using external aortic constriction, LacZ-expressing endothelial cells under the control of the endothelial-specific promoter Tie 1 accumulate at injury sites. *In vitro* studies showed that TGFβ1, acting through Smad3 signaling, induced endothelial cells to differentiate into mesenchymal cells. Bone morphogenic protein 7 (BMP-7) prevented endothelial cells from undergoing EndMT (Zeisberg, Tarnavski, et al., 2007). This process recapitulates the transdifferentiation of endothelial cells that leads to the formation of cardiac valves during embryonic development (Goumans et al., 2008). A similar mechanism, via activation of TGFβ signaling, has been proposed to be responsible in the EndMT observed during kidney fibrosis in an experimental model of diabetic nephropathy (Li et al., 2010). The authors suggested that blockade of EndMT using inhibitors of the TGFβ pathway, such as the specific Smad3 inhibitor SIS3, may provide a new strategy to retard the progression of diabetic nephropathy and other fibrotic processes (Li et al., 2010). EndMT is

a common event observed in other organs, such as lung, undergoing fibrotic changes and in idiopathic portal hypertension in the noncirrhotic liver and can lead to organ failure due to retrograde hypertension (Hashimoto et al., 2010; Nakanuma et al., 2009). In regard to cancer, the occurrence of EndMT in tumors was reported in a recent study that investigated two different mouse models of melanoma and pancreatic cancer demonstrating that a substantial proportion of CAFs arise through EndMT. These CAFs were identified as a unique population of cells that coexpress the endothelial marker CD31 along with one of the mesenchymal markers, FSP1 or αSMA. Approximately, 40% of CAF expressing FSP1 and 11% of αSMA expressing CAFs were found to coexpress the endothelial marker CD31 possibly indicating an endothelial origin (Zeisberg et al., 2007). While this phenomenon has not been shown in other organs, it is very likely to be present in other types of cancer with high stromal response such as the prostate in which CAF cells play an important role during tumor progression. Angiogenesis is a hallmark of tumors; therefore, this endomyofibroblast subpopulation may have the fundamental role of supporting and directing the intrinsic minivasculature necessary for cancer cells. The molecular mechanisms responsible for EndMT have been studied in more detail in fibrosis with a less clear picture currently available in cancer. TGFβ family members are implicated in EndMT acting both via the canonical Smad2/3 and noncanonical participation of important kinases, including the c-Abl protein kinase (c-Abl), protein kinase Cδ (PKC-δ), and glycogen synthase kinase 3β (GSK-3β). These events result in a marked increase in the transcriptional effects of Snail1 and eventually in the expression of mesenchymal cell-specific proteins such as αSMA (Li & Jimenez, 2011).

Recent studies have linked TGFβ-induced EndMT to microRNA (miRNA) action, including effects of miR-125b and miR-21 (Ghosh et al., 2012; Kumarswamy et al., 2012). miRNAs are small noncoding RNAs that bind to mRNA targets, resulting in repression of target expression by translational inhibition or degradation of target mRNAs. Aberrant expression of selected miRNAs has been linked with various pathological conditions including cancer. The potential therapeutic advantages of targeting these pathways are evaluated in the following sections.

2.3.2. Epithelial or Tumor Cells

Epithelial to mesenchymal transformation (EMT) is an epigenetic transcriptional program observed during development, in which epithelial cells gain mesenchymal features, reduced cell–cell contact, and increased

motility. In cancer, this is proposed to allow cells to escape the primary tumor and metastasize at a distant organ where they undergo a mesenchymal to epithelial transformation (MET) (Acloque et al., 2009; Thiery et al., 2009). EMT and its relationship to human cancer are controversial and in many circles not well accepted. While much of the work in this area has been done using *in vitro* systems, some *in vivo* studies have suggested that phenotypic changes of tumor cells acquiring mesenchymal properties are required during the metastatic process (Ao et al., 2006; Lyons et al., 2008; Scheel and Weinberg, 2011; Thompson et al., 2005). In addition, CAF may arise directly from carcinoma cells through EMT (Radisky et al., 2007). However, genetic studies do not show chromosomal rearrangements in CAF and thus have not supported this possibility (Haviv et al., 2009). EMT can be induced by several growth factors including PDGF, TGFβ, EGF, and HGF and is mediated by the activation of characteristic transcription factors including Snail, Slug, Twist, and FOXC2 (Kalluri & Weinberg, 2009; Medici et al., 2008). While the role of EMT during breast cancer progression has received more attention, recent studies including our own have shown that some stromal cells present in prostate cancer tumors may contribute to EMT of cancer cells (Orr et al., 2011). For example, CAF-induced EMT in PC3 cells (a prostate cancer cell line) leads to enhanced tumor growth and the development of metastasis (Giannoni et al., 2010b). The full spectrum of signaling agents that contribute to EMT of carcinoma cells is not clear. One suggestion is that the genetic and epigenetic alterations undergone by cancer cells in primary local tumors make the cells especially susceptible to EMT induced by heterotypic signals originating in the tumor-associated stroma. However, this contradicts the observation of "pristine" genomes found in tumor-associated stroma (Haviv et al., 2009; Scheel & Weinberg, 2011). Among the signals proposed to mediate these events, activation of the TGFβ pathway deserves special consideration. In normal tissues, a major TGFβ function is to prevent uncontrolled epithelial proliferation, thus acting as a tumor suppressor. However, it is now clear that TGFβ may also serve as a positive regulator of tumor progression toward metastasis (Bierie & Moses, 2006). *In vitro* studies have clearly demonstrated that TGFβ can induce an EMT in certain types of cancer cells (Ao et al., 2006; Song, 2007). Two possible signaling pathways have been identified as mediators of TGFβ-induced EMT: the canonical pathway with activation of Smad3 and the noncanonical pathway involving the phosphatidylinositol-3-kinase–Akt and signaling through RHOA and p38 MAPK (Derynck et al., 2001; Kalluri & Neilson, 2003). While activation of Smads, specifically

Smad3, has been the main mechanism suggested for EMT, we have shown recently that tumorigenic cell lines may have a different response to TGFβ. Our *in vitro* and *in vivo* studies, consistent with other observers, suggest that cells with constitutively high levels of Akt modulate the response to TGFβ by blocking the nuclear translocation of Smad3 and p21 proteins (Conery et al., 2004; Remy et al., 2004). This mechanism allows cells to escape cell cycle arrest. Then, Akt induces the expression of EMT marker vimentin while gaining the ability to invade the surrounding tissues (Ao et al., 2006; Chen et al., 2012; Wu et al., 2011). It is clear that EMT is context-dependent *in vivo* and is influenced by several factors offered by the host environment. A recent study using a mouse model and clinical samples suggest that androgen-deprivation therapy may have a direct role promoting EMT in cancer cells through a negative feedback loop between the transcription factors androgen receptor (AR) and Zeb1 (Sun et al., 2012).

It has been proposed that stromal cells might acquire somatic genetic alterations similar to those observed in malignant epithelium. A number of studies in breast, head, and neck cancers suggest that the acquisition of somatic mutations observed by some workers in CAFs might be associated with tumorigenesis. For example, frequent somatic mutations in classic tumor suppressor genes, such as PTEN and TP53, have been reported in fibroblasts associated with breast carcinomas (Henneman et al., 2008; Hill et al., 2005; Kiaris et al., 2005; Weber et al., 2007; Zhang et al., 2008). Unfortunately, some technical aspects of this body of work raised serious questions as to whether these apparently frequent somatic mutations were authentic (Campbell et al., 2009; Eng et al., 2009). This may suggest that any genetic changes observed in the stroma may be attributed to epithelial cell contamination or perhaps EMT cancer cells; however, this point is not definitively settled.

While EMTs are considered precursors of metastatic cells that gain the ability to migrate from the local tumor site toward the blood stream, it is still unclear what the interactions between EMT, and local fibroblasts might be and whether or not these cells are active players supporting other cancer cells to mobilize or are simply responding to microenvironmental signals.

2.3.3. Senescent Fibroblasts

The term senescence was once used to describe an aging process. However, cellular senescence is a particular phenomenon associated with replicative exhaustion. Normal diploid differentiated cells lose the ability to divide and enter a state of permanent growth arrest in the G1/G0 cell-cycle phase

(Povysil et al., 2008). Within the stromal compartment, fibroblasts have been subdivided into subtypes that share the same lineage being derived from fibrocytes. These fibrocytes can be considered a progenitor cell, and depending on the stimulation they receive, can continue to replicate, become senescent, or differentiate into myofibroblasts (Coppe et al., 2008; Untergasser et al., 2005). Contrary to what the word may indicate, senescence is a physiological state in which cells do not replicate, instead they are resistant to apoptosis, do not respond to mitogens, but remain metabolically active. Senescence can be triggered by several stressful conditions, such as exposure to ROS, irradiation, carcinogens, and more recently to telomere shortening with the resultant replicative exhaustion. Activation of the p16, p21, and p53 genes and inactivation of pRB prevents cell-cycle progression during senescence (Povysil et al., 2008; Roninson, 2003). Microscopically, these cells exhibit a typical flat and enlarged cellular morphology with increased granularity. The paradox of senescence in tumors is based on the concept that although this mechanism is a way that cells have to escape malignant transformation, accumulating data suggest that over time, these cells can become part of the tumor stroma and may contribute to the carcinogenic process promoting tumor progression. For example, prostate fibroblasts isolated from older (>60 yo) compared to younger men (<50 yo) showed an increased expression of proinflammatory chemokines. In this study, BPH1 prostate epithelial cells, either in direct coculture or exposure to conditioned media from senescent fibroblasts were able to grow better compared to controls using their nonsenescent cells (Eyman et al., 2009). Because senescent fibroblasts derived from lung, breast, or foreskin secrete similar cytokines to those seen in the prostate, the term "senescence associated secreted proteins or SASP" has been proposed to refer to the contribution from these cells (Coppe et al., 2008). While there is a clear association of senescence fibroblasts during cancer progression, it is not known whether this represents a particular CAF subpopulation or a transient state between fibroblasts and myofibroblasts.

2.3.4. Pericytes

Perivascular cells also known as pericytes have recently been proposed to be a source of differentiated intermediates potentially contributing to CAF activity. Pericytes express a large number of cell markers in common with CAF including PDGFRβ, Thy-1, and NG2. However, these markers are found not only in the perivascular position where the pericytes reside, but also deep within the tumor mass (Sugimoto et al., 2006). These cells may

represent a subpopulation that is mobilized depending on the needs of tumor cells. Evidence of the tumor-promoting role of pericytes comes from analysis of the effects of suppressing PDGF receptor signaling in a mouse model of cervical carcinoma (Ma et al., 2008). While these models are undeniably intriguing, a clearer understanding would come from animal model experiments indicating the transdifferentiation of pericytes from the mesenchymal precursor into the stromal compartment and compared to the native CAF population present in tumors.

3. MYOFIBROBLAST CONVERSION DURING NORMAL AND PATHOLOGICAL CONDITIONS

To better understand the therapeutic opportunities offered by CAF, identification of the essential pathways involved during normal and pathological myofibroblast conversion is of paramount importance. The normal wound healing process is now briefly presented and compared to the changes that occur during pathological conditions.

3.1. Normal Healing Wound (Benign Myofibroblasts)

Normal wound healing process has been extensively studied in the skin. So much of the knowledge about this process has come from work related to the restoration of the dermis and epidermis. Wound healing comprises a cascade of three overlapping dynamic phases. The *inflammatory phase* occurs immediate after tissue damage, more specifically injured capillaries, which leads to the activation of the coagulation cascade and results in the formation of a blood clot composed of fibrin and fibronectin. This temporary matrix has the role of filling the tissue defect and permits the influx of the cellular components. Platelets that are present in the clot secrete multiple cytokines that participate in the recruitment of inflammatory cells, fibroblasts, and endothelial cells (Ma, Cheng, et al., 2008). This inflammatory phase is followed by a *proliferative phase*. In this phase, angiogenesis creates new capillaries, allowing nutrient delivery to the wound site; this rich environment supports fibroblast proliferation. Fibroblasts present in granulation tissue are activated and acquire smooth muscle cell-like characteristics and become myofibroblasts, which are major players during wound healing. Myofibroblasts are characterized by an abundant rough endoplasmic reticulum (ER), in contrast to the well-developed ER and oval nucleus present in normal fibroblasts. Myofibroblast function is twofold. Biochemically,

they synthesize and deposit ECM components including collagen (mainly collagen type III) and elastin that replace the provisional matrix and giving the ECM strength and elasticity. They also neoexpress bundles of microfilaments with dense bodies similar to those found in smooth muscle cells. These bundles of microfilaments, also known as stress fibers, are the framework of the organized αSMA that confer the mechanical contractile properties of myofibroblasts. This feature suggests that myofibroblasts are the main cell type responsible for the production of the force determining wound contraction. Fibronectin serves as a fundamental anchor for the myofibroblasts during the tissue repair. Differentiation of fibroblastic cells into myofibroblasts appears to begin with the appearance of the protomyofibroblast, whose stress fibers contain only β- and γ-cytoplasmic actins (Hinz & Gabbiani, 2003). Protomyofibroblasts may evolve into fully differentiated myofibroblasts containing only α-smooth muscle actin stress fibers depending on the stimulus. Accumulation of protomyofibroblast occurs in the first phase of wound healing *in vivo*. The third *scar formation phase* involves gradual remodeling of the granulation tissue and reepithelialization. A process mediated by proteolytic enzymes, especially matrix metalloproteinases (MMPs) and their inhibitors (TIMPs, for tissue inhibitors of metalloproteinases). Evidence for the role of these enzymes comes from work in animal models in which reepithelialization is significantly delayed and wound contraction and myofibroblast formation are reduced in KO mice for MMP2 and -13 (Fang et al., 2004). At the end of this phase, type III collagen, the main component of granulation tissue, is gradually replaced by type I collagen, and elastin, which was lost at the initial phase reappears to give back the elasticity to the tissue. In the *resolution phase*, apoptosis of vascular cells and myofibroblasts or dedifferentiation toward the quiescent form normalizes the cellular density required for normal function (Desmouliere et al., 1995). Myofibroblasts orchestrate and direct the dynamics of the wound healing process, so special attention to the molecular events that regulate the life cycle from the quiescent states until the disappearance of the wound need to be discussed. These mechanisms may illustrate the pathways that can be targeted during pathological scenarios. Several cytokines and growth factors have been studied for their regulation in the differentiation of fibroblasts into myofibroblasts (Rhee et al., 2010; Werner & Grose, 2003). PDGF was the first growth factor shown to be chemotactic for cells, such as neutrophils, monocytes, and fibroblasts, migrating into the healing skin wound. In addition, PDGF enhances proliferation of fibroblasts and production of ECM by these cells. Perhaps, the most potent inducer of

myofibroblast differentiation is TGFβ1. TGFβ1 acts directly on granulation tissue formation and fibrogenic cell activation and induces the expression of αSMA. Under the influence of TGFβ1, cells deposit large amounts of ECM (particularly fibrillar collagen and fibronectin) and at the same time reduce their expression of the TIMPs. The induction of myofibroblast differentiation from fibroblasts by TGFβ1 requires the ectodomain A (ED-A) sequence of cellular fibronectin (Serini et al., 1998). It has been shown that fibronectin can bind to the α4β7 integrin receptor and activate the MAPK kinase pathway to induce myofibroblast differentiation in fibroblasts expressing this particular receptor (Kohan et al., 2010). Binding to these receptors allows myofibroblasts to migrate and attach to the ECM. Adherens and gap junctions between myofibroblasts maintain these cells' interconnections to themselves and also to the ECM by a complex structure called the fibronexus. This involves a series of intracellular microfilaments closely related with the extracellular fibronectin fibers (Eyden et al., 2009). Thus, when the actin microfilaments in the myofibroblast contract, the fibronexus transmits this force to the surrounding ECM, and allows the wound to contract acting as a mechanotransducer of stress in the extracellular milieu (Ingber, 2008). As the wound becomes epithelialized and the scar forms, there is a striking decrease in myofibroblast population. During this process, there is a condensation and fragmentation of the nucleus and several modifications of the cytoplasmic organelles compatible with the cell going through apoptosis. Apoptotic cells are removed by phagocytosis either by macrophages or by other neighboring cells (Darby & Hewitson, 2007). Reduction in growth factor expression, increased ECM turnover, and nitric oxide generation may result in apoptosis seen during the rapid remodeling of tissue. However, retention of the myofibroblast phenotype during fibrosis has been suggested to result from imbalanced cytokine signals. A vicious cycle is created when high levels of TGFβ are present in the tissues resulting in conversion of fibroblasts to myofibroblasts. Thus, myofibroblasts contribute to their own continued survival by secreting more activated TGFβ thus closing the cycle. At the end of the normal healing process, IL-1β-induced apoptosis in fibroblasts through inducible NO synthase can be blocked by TGFβ (Zhang & Phan, 1999). Any condition that causes elevated levels of TGFβ can inhibit myofibroblast death and result in fibrosis. While all cells recruited to wounds are capable of expressing TGFβ, elevated levels in fibrosis can be provided by eosinophils (Minshall et al., 1997). A hyaluronan-rich ECM can retain TGFβ and serve as a sustained reservoir for myofibroblasts, which in turn secrete more TGFβ, thereby

closing the autostimulatory cycle and preventing apoptosis. More recently, using a mouse model of diabetes—with focus on fibroblasts—has been shown that apoptosis may be the result of high levels of tumor necrosis factor-alpha (TNF-α) and activation of the proapoptotic transcription factor FOXO1 (Siqueira et al., 2010). More studies are needed in this area because inappropriate delay of apoptosis with the subsequent increased survival of myofibroblasts may be a crucial factor determining the fate of the normal versus the pathological wound seen in tumors.

3.2. Pathological Wound Healing (Malignancy-Associated Myofibroblast)

Myofibroblasts can survive in at least three different pathological settings: in response to injury (fibrosis), in nonneoplastic proliferative conditions such as those encountered in fibromatosis and BPH, or in regions surrounding tumors (Schmitt-Graff et al., 1994). A common example can be seen in patients with extensive burns in which scars can lead to severe functional and aesthetic defects. These scars are composed mainly by myofibroblasts expressing high levels of αSMA with a disarranged pattern of contraction that contributes to the appearance of the scars. It has been shown that cells with high levels of Akt can inhibit the apoptotic pathways leading to an accumulation of these cells during the proliferative phase (Aarabi et al., 2007). Another feature of fibrosis is the excessive accumulation of ECM that causes a disruption of the normal tissue architecture, thus affecting its normal function. The matrix composed of fibrin, fibrinogen, and fibronectin that serves as a bed for fibroblasts during the initial phase of wound healing is stabilized when the myofibroblasts secrete hyaluronan and proteoglycans (Johnson et al., 2007). This scaffold, rich in hyaluronan and versican, is important for cells to change their shape and facilitate division and migration. Maintenance of the myofibroblast phenotype has also been linked to the presence of hyaluronan. The association of hyaluronan with CD44 influences the positioning of TGFβ receptors, with downstream effects on TGFβ signaling. For example, blocking the synthesis of hyaluronan in fibroblasts inhibits the increase in αSMA expression induced by TGFβ during the fibroblast to myofibroblast conversion (Wang & Hascall, 2004). Hyaluronan formation can also come from activation of TLR-3 receptors. These receptors are commonly activated by viruses and have been linked to the induction of myofibroblast phenotype and to induction of TGFβ expression (Sugiura et al., 2009). In contrast to fibrotic scars, keloid scars are

devoid of mature αSMA positive cells, with an accumulation of proto-myofibroblasts, which are responsible for excessive deposit of ECM but unable to develop the forces to contract the lesion even though some αSMA-expressing cells can be found in keloid lesions. The collagen fibers in keloid scars are thinner than those in fibrotic lesions pointing to an imbalance in the MMP/TIMP system (Verhaegen et al., 2009).

While these changes are observed in adult tissues, an interesting phenomenon is seen in fetal wounds in which fewer αSMA myofibroblasts are present but retain their contractility potential. The net effect is that fetal wounds do not scar or contract (Estes et al., 1994; Moulin et al., 1997). These differences compared to the adult tissue can be attributed to the low levels of TGFβ1 and TGFβ2 ligands and the lack of TGFβ3 response during fetal injury. Another important fetal feature is the rapid ECM remodeling resulting from high MMP and TIMP levels compared to adult tissues (Cowin et al., 2001; Dang et al., 2003). This likely reflects the rapid and constant remodeling that is ongoing during fetal development. Understanding what controls these changes in the fetus may shed some light into targeting specific pathways.

The origins of myofibroblasts were discussed in the previous section; however, some differences to those present in normal wound healing and those in malignancy should be addressed.

In general, fibroblasts adjacent to neoplastic cell nests express significant amounts of α-smooth muscle actin, as seen in the stroma of several types of cancer including breast, melanoma, pancreas, and some myeloproliferative diseases. Thus, fibroblastic cells of the RS are predominantly myofibroblasts. It has been shown that myofibroblasts represent an extremely heterogeneous and multifunctional cell population exhibiting different phenotypes. Other smooth muscle markers like desmin and smooth muscle myosin have only been documented in a minority of such myofibroblasts (Skalli et al., 1989). In prostate cancer, myofibroblasts coexpress αSMA and the mesenchymal marker vimentin with loss of late-stage smooth-muscle differentiation markers (desmin, calponin) (Ayala et al., 2003). However, it has been shown that not all fibroblasts present in these tumors show smooth-muscle differentiation, and because the origin and function of each subpopulation is not completely understood, we prefer to refer them collectively as CAF to designate the inductive nature of this heterogeneous tumor fibroblast population. Nevertheless, this pattern supports the notion that myofibroblasts may correspond to modified fibroblasts rather than to smooth muscle cells. This fibroblast diversity may be due to the influence exerted by

neighboring tumor cells that required specific subset of myofibroblasts for tumors to progress. Evidence for these paracrine effects can be found in αSMA-positive mesenchymal cells surrounding noninvasive breast ductal carcinoma and in cervical intraepithelial neoplasia suggesting that epithelial/stroma signaling may be fundamental even before the onset of invasion. Also, increased expression of vimentin and synthesis of collagen I were observed in activated periacinar fibroblasts adjacent to the premalignant lesion PIN (Tuxhorn et al., 2002b). Once activated, myofibroblasts can promote tumor progression by different mechanisms including the secretion of several growth factors and cytokines. Myofibroblasts are the main cell type responsible for resolution of wounds, it has been proposed, based upon data acquired using an *in vitro* system, that these cells can escape apoptosis through a process called nemosis (Vaheri et al., 2009). Nemosis is a model for stromal fibroblast activation. Occurring when normal human fibroblasts are deprived of growth, the process is characterized by clustering, giving rise to multicellular spheroids. This results in increased expression of a number of proinflammatory markers, including cyclooxygenase-2 (COX-2) and prostaglandins, as well as proteinases, cytokines, and growth factors. Fibroblasts activated by nemosis induce wound healing and also seem to be involved in tumorigenic responses (Enzerink et al., 2010; Enzerink et al., 2009a). The changes in gene expression in nemotic fibroblasts resemble those known to promote cancer progression (Sutherland, 1988). For example, high levels of HGF have been found to be expressed by these clustered fibroblasts. HGF secreted by nemotic fibroblasts affects not only the proliferation of cancer cells but has also an additive effect on their motility. These changes can be abrogated blocking the c-Met binding ability of the HGF ligand (Kankuri et al., 2005). The proangiogenic properties of HGF are enhanced by the increased expression of VEGF in nemosis suggesting that nemotic fibroblasts could further stimulate tumor growth and metastasis through enhanced angiogenesis *in vivo*. Nemotic fibroblast spheroids secrete substantial amounts of several proinflammatory cytokines (IL-1, IL-6, IL-8, LIF, GM-CSF) and chemokines (MIP-1α, RANTES, and IL-8). MIP-1α and RANTES acting through the receptor CCR1 have been shown to attract monocytic THP-1. Neutrophil migration has been shown to be dependent on IL-8 levels in nemosis (Enzerink et al., 2009b). Low levels of the endogenous inhibitor IκBα and associated increased DNA-binding activity of NF-κB have been found in nemotic fibroblasts indicating a role for NF-κB in regulating the induction of proinflammatory cytokines and chemokines in nemosis. The induction of COX-2 and secretion of

prostaglandins (markers of nemosis) are observed in nemotic fibroblasts, suggesting that the recruitment of inflammatory cells may be a key consequence of nemosis. Several proteinases including MMP-1 (interstitial collagenase), MMP-10 (stromelysin-2), and MT-MMP-1 (membrane type MMP-14) are strongly induced both at mRNA and protein levels in nemosis. In contrast, most MMPs and tissue inhibitors of MMPs (TIMPs 1–3) are downregulated. Nemosis may represent a mechanism by which myofibroblasts escape apoptosis and perpetuate the inflammatory reaction that provides the signals needed for survival.

4. CAF HETEROGENEITY

Several studies suggest the presence of multiple fibroblast cell types in the tumor microenvironment. While conversion to myofibroblasts has been seen as the hallmark of the tumor stroma compartment, the role of the normal fibroblasts in genesis of the CAF phenotype has received little attention. Our recent observations suggest that these "normal fibroblasts" are not just mere witnesses, but rather active players that contribute to the transformation of normal epithelial cells. For example, loss of TGFβ receptor II function in stromal cells increases the expression of TGFβ ligand (>5 fold), a defensive mechanism that fibroblasts have to control stromal proliferation, promotes epithelial proliferation but does not have an effect on the transformation of epithelial cells. However, when these TGFβ function-deficient cells are in the presence of fibroblasts with functional TGFβ signaling, TGFβ expression increases >50-fold concomitantly with protumorigenic chemokines such as SDF1α (or CXCL12) and several growth factors that promote epithelial proliferation. The heterogeneous stromal population (composed of native and TGFβ-deficient fibroblasts) induces transformation of prostate epithelial cells. These results correlate with the heterogeneous activation of Smad2 in stromal cells observed in the tissues of prostate cancer patients. Other paracrine mechanisms such as HGF and activation of the Stat3 signaling by Wnt3 have been proposed to contribute to the protumorigenic properties of the heterogeneous stroma (Franco et al., 2011; Kiskowski et al., 2011; Li et al., 2008). Apart from their direct effects on tumor cells, heterogeneous stromal cells in tumors have proinflammatory properties and secrete high levels of PGE2 and IL-6 inducing not only tumor growth, but also promoting the expansion of cancer stem-like cells (Rudnick et al., 2011). Thus, the signaling present within the stromal

compartment between different fibroblast subpopulations seems to be an important component of CAF functionality. Better understanding of the role of normal fibroblasts in this complex system is needed to develop strategies that can modulate their behavior to exploit its potential therapeutical benefit.

5. TARGETING THE INDUCTIVE PROPERTIES OF CAF

Tumors are complex three-dimensional structures composed of multiple cell lineages, which, as described, represent a caricature of an organ. The main malignant component is represented by the cancer cells themselves, which by definition in a carcinoma are epithelial. Apart from the surgical removal of the primary tumor, efforts to prevent or palliate the uncontrolled growth, which centered exclusively on the epithelial component role, have been largely unsuccessful. The efficacy of anticancer agents has been hampered by our incomplete understanding of the complex interactions between tumor cells and their surrounding stroma, which can constitute up to 50% of the tumor mass. Over the past decade, several studies have demonstrated that the tumor microenvironment plays a critical role in supporting and even promoting the cancer phenotype (Mueller & Fusenig, 2004; Orimo & Weinberg, 2006). Evidence of the essential contribution of the stroma during cancer progression has diverted many groups to look for alternatives to suppress tumor growth. CAF (as probably the most abundant cell type) has a central role and acts as a translator between cancer cells and the host responses orchestrating many or most of the "afferent" and "efferent" signals from and to the tumor. Targeting CAF cells offers several advantages: (1) these cells (despite the caveats discussed earlier) are generally considered to be genetically stable, thus the occurrence of mutations that may lead to resistant to drug treatments are minimal compared to the genetically unstable cancer cells; (2) as discussed, during the wound healing process, fibroblasts are the main source of the desmoplastic reaction and the deposition of ECM proteins which can restrict diffusion and thus access of therapeutic agents to the center of tumors; (3) survival of tumors is achieved by the proper provision of nutrients through CAF-induced neovascularization; and (4) tumor–CAF interactions have a positive effect on survival and invasiveness of cancer cells.

The repertoire of signals present within the stroma is complex and dynamic with cells moving into and out of tumors. The differentiation status

of each of these components varies depending upon the stage of the tumor, adding to this complexity. This means that many routes can be targeted and approaches explored.

5.1. Targeting CAF Differentiation/Recruitment

We have presented several potential sources for CAF cells, and because the presence of these cells is considered a key event during carcinogenesis, it is natural to believe that preventing the differentiation of normal fibroblasts into the activated myofibroblasts state may have a therapeutic effect.

A number of growth factors are associated with myofibroblast differentiation, including PDGF, angiotensin II, CTGF, and TGFβ1. TGFβ1 is the factor most frequently associated with the αSMA myofibroblast phenotype and has been determined to be the preeminent growth factor responsible for fibroblast activation and matrix synthesis *in vitro* and during fibrosis. Also, TGFβ1 has an essential role in myofibroblast conversion. Other growth factors, including PDGF and angiotensin II, exert their effects by directly stimulating TGFβ1 production. TGFβ can be considered a double-edged sword because of its dual roles on epithelial cells during cancer progression. On one side, it exerts a growth inhibition function in premalignant cells eradicating potential transformation of normal cells, but TGFβ also promotes tumor progression and metastasis in later stages of cancer. The mechanisms underlying this dual role of TGFβ are complex (Massague, 2008). Diverse autonomous tumor-cell signaling pathways have significant roles with changes in the signal intensity and connectivity of Smad-dependent and Smad-independent pathways (Ikushima & Miyazono, 2010). Smad-dependent pathways might mediate growth-inhibitory effects of TGFβ signaling, whereas Smad-independent pathways could mediate the tumor-promoting effect of the TGFβ signaling. Smad-independent pathways might synergize with the amplification of oncogenes such as MYC and activating mutations of RAS, along with inactivating mutations in retinoblastoma or cyclin-dependent kinase inhibitors especially in tumor cells. However, the effects of TGFβ on fibroblasts are much clearer. *In vitro* and *in vivo* studies have shown that TGFβ1 stimulates phenotypic switching of fibroblasts to myofibroblasts, regulates expression of ECM components, and stimulates angiogenesis (Peehl & Sellers, 1997). Because TGFβ is overexpressed in several human carcinomas, including prostate cancer, it seems likely that TGFβ promotes the formation of RS (Eastham et al., 1995). Worse, TGFβ also induces the expression of more TGFβ in myofibroblasts that can perpetuate the presence

of activated fibroblasts. Thus, appropriate targeting of this pathway might represent an important step in therapy development. Recently, several approaches acting on different levels of the TGFβ signaling have been explored to inhibit oncogenic properties of the ligand.

To target the myofibroblast conversion and the pleiotropic actions of TGFβ, several preclinical and clinical trials using anti-TGFβ strategies—mainly against fibrotic disease, but with a potential scope on cancer—have been carried out. In one study using dermal fibroblasts, abrogation of ALK-5 kinase activity by SB431542 blocked the TGFβ response in fibroblasts by preventing Smad phosphorylation and nuclear translocation (Mori et al., 2004). The net effect was the prevention of myofibroblast conversion with the addition of abrogation of TGFβ-induced stimulation of collagen, fibronectin, plasminogen activator inhibitor 1, connective tissue growth factor gene expression, and also TGFβ autoinduction. SB431542 can also prevent the myofibroblast-induced conversion of adipose tissue-derived MSCs by tumor-derived exosomes (Cho et al., 2012). In addition to kinase inhibitors, preclinical experiments using an anti-TGFβ antibodies such as CAT-152 (Lerdelimumab), a fully human neutralizing antibody with high affinity for TGFβ2 and some cross-reactivity to TGFβ3 was shown to be capable of inhibiting scarring after glaucoma surgery in rabbits (Mead et al., 2003). Although initial clinical trials with CAT-152 indicated possible effects in reducing scar formation in glaucoma patients, these results were neither confirmed in larger phase III clinical trials nor has this agent been used in cancer models (Grehn et al., 2007; Khaw et al., 2007). The utility of soluble TGFβ receptors have also been evaluated. Adenovirally expressed soluble TGFβ-RII can inhibit liver fibrosis, with a dramatic reduction of collagen type-I expression and inhibition of hepatic stellate cell activation, a key process in liver fibrosis (George et al., 1999). Also, a soluble TGFβ-RII construct was shown to attenuate apoptosis, injury, and fibrosis in bleomycin-induced lung fibrosis in mice (Yamada et al., 2007). P144 is a synthetic peptide, which was derived from the ligand-binding domain of betaglycan and is capable of reducing the number of αSMA positive myofibroblasts, hence the occurrence of liver and skin fibrosis when used in mice (Ezquerro et al., 2003; Santiago et al., 2005). Currently, P144 is being tested in clinical trials for (skin) fibrosis (Clinical trial identifier NCT00781053). More recently, a different TGFβ peptide inhibitor P17 was shown to reduce the accumulation of myofibroblasts in lung fibrosis and when combined with P14 was able to enhance the efficacy of anticancer immunotherapies *in vivo* (Arribillaga et al., 2011; Llopiz et al., 2009).

PGDF stimulates fibroblasts to contract collagen matrices and differentiate into myofibroblasts *in vitro* (Jinnin et al., 2005). The PDGFβ receptor inhibitor imatinib mesylate (Gleevec®/Glivec®) can reduce the myofibroblast numbers and expression of fibronectin ED-A and collagen type I. It has been proposed that the main mechanism for this decrease in myofibroblasts is by blocking pericyte migration (Rajkumar et al., 2006). In preclinical studies, other tyrosine kinase inhibitors dasatinib (Spyrcel®) and nilotinib (Tasigna®) potently reduced the number of myofibroblasts in a dose-dependent manner while the drugs were well tolerated (Akhmetshina et al., 2008). Other factors such as CTGF/CCN2 can act as downstream cofactors for TGFβ, inducing the expression of collagen type I and αSMA. Drugs targeting the action of CCN2, such as small interfering RNAs or neutralizing antibodies, are currently under development (Brigstock, 2009). Other approaches such as inhibition of DNA methyltranferase 1 by 5-aza-2-deoxycytidine or the use of monoclonal antibodies (MABs) against FAP, a protein involved in the myofibroblast differentiation, have shown promising results in clinical trials (Scott et al., 2003). Similarly, fibroblast activation of skin cancer fibroblasts in response to TGFβ can be abolished by antioxidant treatment using trolox or selenite (Cat et al., 2006b).

5.2. Targeting the Secretion of Soluble Factors by CAF Cells

The stimulation of tumor progression by CAF cells has been attributed to a large list of factors identified using array analysis of *in vitro* cultured cells as well as patient samples. Due to the many targets available, we focus on those that have attracted most attention and summarize additional candidates in Table 9.1.

CAF support growth and invasion directly or indirectly by promoting angiogenesis and modifying the inflammatory/immune response. Apart from the described promyofibroblastic actions of TGFβ ligands, activation of the canonical and noncanonical pathways during different stages of tumor progression makes it a good candidate for therapy. Antisense oligonucleotides that can bind to the mRNA have been designed. For example, the phosphorothioate oligodeoxynucleotide AP12009 (trabedersen) can reduce TGFβ2 secretion up to 73% when glioma cells are treated *in vitro*. *In vivo* AP12009 can reduce the proliferation of cancer cells and counteract the immunosuppressive effects of TGFβ2 (Hau et al., 2009). Studies in a phase IIb trial showed that a 10 μM dose of AP12009 can stop tumor growth and have a better control in patients with high-grade gliomas and a trend toward

Table 9.1 Summary of Potential Targetable Molecules in CAF

	Target	Drug, Class	Effects On	Stage
Differentiation/ recruitment	ALK-5	SB431542, GW788388 kinase inhibitors	Myofibroblast, MSC recruitment	Preclinical
	TGFβ1	LY238770, TGF-β1 neutralizing antibody	Myofibroblasts conversion	Phase II (diabetes kidney disease) NCT01113801
	TGFβ2, TGFβ3	CAT-152 (Lerdelimumab) anti-TGFβ antibody	Inhibit scarring	Phase III completed (trabeculectomy patients)
	TGFβ ligand	Soluble TGFβ-RII	Reduction in Collagen-I, stellate cells activation	Preclinical (liver fibrosis)
	TGFβRIII (betaglycan)	P144, P17 (soluble TGFβRII)	Myofibroblasts conversion	P144: Phase II (skin fibrosis) NCT00781053 P17: preclinical
	CCN2	Small interfering RNAs or neutralizing antibodies	Myofibroblasts conversion	Preclinical (cardiac fibrosis)
	PDGFβ receptor	Tyrosine kinase inhibitors: Imatinib mesylate (Gleevec/Glivec), dasatinib (Spyrcel) and nilotinib (Tasigna)	Myofibroblast, fibronectin, collagen type I, pericyte migration	Imatinib and dasatinib: Phase II (non−small cell lung cancer NSCLC) Nilotinib: treatment of chronic myelogenous leukaemia

	FAP	Anti-FAP (Sibrotozumab), 5-aza-2-deoxycytidine	Fibroblast activation	Phase III (metastatic cancer) NCT00004042
	FAP	FAP-activated peptide protoxins from bee venom	Blood vessel density, collagen deposition and disruption of the MSC-mediated immunity	Preclinical (breast and prostate cancer)
Secretion of soluble factors	TGFβ	Trolox or selenite	Myofibroblast activation	Selenite (prostate cancer) NCT01155791
Secretion of soluble factors	TGFβ2	Antisense oligonucleotides AP12009 (Trabedersen)	Angiogenesis	Phase II (High-grade glioma) NCT00431561
	TGFβ1	Antisense oligonucleotides AP11014	Angiogenesis	Preclinical (prostate, NSCLC and)
	TGFβ1, 2, 3	Neutralizing antibody 2G7 or 1D11, GC1008 (Fresolimumab)	Increases NK cells, cytotoxic T-lymphocyte activity, decreases MDSC	2G7 and 1D11: preclinical GC1008: Phase II (glioma) NCT01401062
	TGFβRII	Anti-TβRII antibodies TR1	Suppresses metastasis and primary tumor growth	Preclinical (breast cancer)
	TGFβ	Soluble TβRII/TβRIII or betaglycan and TβRII:Fc fusion	Increases TGFβ-driven apoptosis	Preclinical (breast cancer)

(*Continued*)

Table 9.1 Summary of Potential Targetable Molecules in CAF—cont'd

	Target	Drug, Class	Effects On	Stage
	HGF	NK4, anti-HGF Abs, anti-MET Abs, and small-molecule MET tyrosine kinase inhibitors	Tumor growth, metastasis	NK4: Preclinical (prostate cancer) Anti-MET: Phase II (breast, melanoma, myeloma, NSCLC, and lymphoma)
	VEGF	Bevacizumab (Avastin)	Angiogenesis	Phase IV (breast, NSCLC, and colon cancer)
CAF-induced inflammation	COX2	Inhibitors (Celecoxib, Refecoxib)	EMT, recruitment of inflammatory cells	Celecoxib: Phase III (prostate cancer) NCT00136487 Refecoxib: Phase III (prostate cancer) NCT00060476 and other cancers
	SDF1/CXCR4	CXCR4 antagonist AMD3100	Decreases BMDC	Phase II and III in several cancers
	IL-6	Ligand-blocking antibody (CNTO-328)	Macrophage infiltration, angiogenesis, and subsequent tumor growth	Phase II (prostate cancer) NCT00385827 and other cancers

	IL-6R	Blocking antibody (Tocilizumab)	Castelman's disease and rheumatoid arthritis	Phase IV (rheumatoid arthritis) NCT01119859
	Jak1/2	Inhibitor AZD1480		Phase I (solid tumors) NCT01112397
Other	DNMT1, DNMT3a, and DNMT3b	Methyltransferase inhibitors (5-azacytidine, 5-aza-2′ -deoxycytidine and zebularine procaine, procainamide, EGCG, and RG108)	Methylation	Phase I up to Phase IV trials
	Insulin resistance	Metformin	Metabolism (diabetes)	Phase IV trials (diabetes, hepatitis, metabolic syndrome, and other nonmalignant diseases)

better survival compared to higher dose of AP12009 (80 μM) or standard chemotherapeutic treatment (Bogdahn et al., 2011). Because of the success and few side effects observed, AP12009 is currently evaluated in a large phase II trial on glioma patients (Clinical trial identifier NCT00761280). This observation led other investigators to assess the efficacy of AP12009 in a phase I trial on melanoma, colorectal, and pancreatic cancers (Clinical trial identifier NCT00844064). A similar antisense approach targeting TGFβ1 mRNA (AP11014) is currently being tested in preclinical trials for non–small cell lung cancer, colorectal cancer, and prostate cancer.

Another approach is the use of neutralizing antibodies to minimize the interactions between ligands and receptors, preventing the phosphorylation of downstream effectors. For example, treatment with the pan-TGFβ neutralizing antibody 2G7 or 1D11 (Genzyme, Inc. Cambridge, MA) significantly suppressed lung metastasis of basal cell–like cells through increased natural killer cell and cytotoxic T-lymphocyte activity as well as decreased numbers of Gr-1 + myeloid cells in the tumor microenvironment (Ganapathy et al., 2010; Zhong et al., 2010). The human analog of the 1D11 antibody, GC1008 (Fresolimumab), is currently also tested for the treatment of several cancers (Clinical trial identifier NCT00899444/NCT01112293). Anti-TβRII antibodies TR1 (antihuman) and MT1 (antimouse) suppress primary tumor growth and metastasis (Zhong, et al., 2010). In addition to neutralizing antibodies, soluble TβRII/TβRIII or betaglycan and TβRII:Fc fusion proteins prevent the ligand–receptor interactions reducing local growth and metastasis (Bandyopadhyay et al., 2002; Rowland-Goldsmith et al., 2002; Yang et al., 2002). However, none of these agents has entered clinical trials, partially because of safety concerns.

Activation of the MET receptor in cancer cells has been shown to be important for epithelial transformation and enhanced invasion. Large amounts of the HGF ligands secreted by CAF cells can confer resistance to conventional tyrosine kinase inhibitors against EGF receptor in breast cancer. Preclinical studies targeting NK4, which competes with Met, as well as anti-HGF monoclonal antibodies showed promising results by decreasing tumor growth and metastasis, which makes them good candidates for human studies (Kim et al., 2007). More recently, foretinib, an oral, small molecule multikinase MET inhibitor that targets members of the HGF and VEGF receptor tyrosine kinase families has shown activity in several types of cancer including lung, renal, and hepatocellular carcinoma. New anti-HGF antibodies, anti-MET antibodies, and small-molecule MET TKI inhibitors are in various stages of development (Table 9.1).

Neoangiogenesis is a hallmark of developing tumors. High levels of VEGF are produced by the tumor stroma and because of the side effects and the emergence of resistance to conventional antiangiogenic therapies, questions remain regarding how to best combine angiogenesis inhibitors. Angiogenesis targets include VEGF, FGFR, PDGF, Notch/Delta-like ligand 4 (DLL-4) signaling, and Tie2/angiopoietin signaling. Part of the resistance can be due to sustained expression of VEGF by CAF-derived PDGF-C. Thus, targeting PDGF-C may be useful in order to inhibit angiogenesis in tumor refractory to anti-VEGF therapy (Crawford et al., 2009). More recently, efforts have been made to develop multitargeted tyrosine kinase inhibitors. For example, Cediranib that targets both the VEGF and PDGF receptors has been evaluated for clinical efficacy. Recently, a proangiogenic FAP protein expressed at high levels in activated fibroblasts has been evaluated as a potential target. For example, after FAP activation, protoxins generated from bee venom have shown antitumor properties against both breast and prostate cancer xenografts by decreasing blood vessel density, collagen deposition, and disruption of the MSC-mediated immunity (LeBeau et al., 2009).

5.3. Targeting Proinflammatory Molecules

Proinflammatory cytokines such as interleukins, interferons, and members of the TNF family produced by CAF influence tumor growth. CAFs have a double role in the tumor microenvironment first helping cancer cells to evade immunosurveillance and second promoting a chronic inflammatory environment, which in turn maintains the protumorigenic status of the stromal cells. A key CAF mediator in the inflammatory process is COX-2. For example, COX-2 expression increases when stromal cells are cocultured with cancer cells. Upregulation of COX-2 regulates VEGF and MMP14 production *in vivo*, and facilitates invasion and cancer progression (Hu et al., 2009; Sato et al., 2004). EMT in prostate cancer has been linked to the presence of COX-2 (Giannoni et al., 2010b). Thus, the addition of COX-2 inhibitors such as Celecoxib or Rofecoxib may be beneficial in high-risk patients based on their CAF-induced inflammatory response. The appearance of cardiovascular disease in some patients treated with COX inhibitors and the potential increased risk in several tumors has decreased the enthusiasm and hampered the widespread use of this approach in cancer patients (Dogne et al., 2006; Vinogradova et al., 2011). A recent report has found a proinflammatory signature in prostate cancer stroma driven by the nuclear factor-κB (NF-κB). These cells have increased levels of SDF1, IL-6, and

IL-1β that promote macrophage infiltration, angiogenesis, and subsequent tumor growth. Blocking the SDF1/CXCR4 axis with the CXCR4 antagonist AMD3100 has shown promising results by inhibiting tumor growth and decreasing the recruitment of bone marrow-derived cells (BMDC) (Erez et al., 2010). IL-6 has pleiotropic functions activating numerous cell types expressing the gp130 receptor and the membrane-bound IL-6 receptor (Culig, 2011). Given the importance of IL-6 signaling in driving Jak/Stat3 activation in cancers, blocking IL-6 using ligand-binding antibodies or receptor-blocking antibodies have been tested pre-clinically, with positive results when used either alone or in combination. Clinically, an IL-6 ligand-blocking antibody (CNTO-328) is being tested in a number of phase I/II clinical trials in transplant-refractory myeloma and castrate-resistant prostate cancer (Dorff et al., 2010; Wallner et al., 2006). An IL-6R blocking antibody (tocilizumab) was approved for Castelman's disease and rheumatoid arthritis and will likely be tested in cancers (Garnero et al., 2010; Nakashima et al., 2010). Based on the success in preclinical trials in several cancer models including breast, ovarian, and prostate cancer, the role of Jak inhibition using the Jak1/2 inhibitor AZD1480, is now being tested in phase I clinical trials for solid tumors (Hedvat et al., 2009).

5.4. Other Targets

Targeting epigenetic alterations (DNA methylation) may also be an interesting opportunity to inhibit the function of CAFs. For example, changes in DNMT1 expression in CAF cells suggest that these stromal cells may be more susceptible to hypomethylating drugs like 5aza-dC (5-aza-2′-deoxycytidine). Thus, epigenetic interventions that target DNA methylation can be achieved by using methyl donor modifiers (folate, betaine, and choline) or methyltransferase inhibitors such as nucleoside inhibitors (5-azacytidine, 5-aza-dC, and zebularine) or nonnucleoside inhibitors (procaine, procainamide, EGCG, and RG108) (Stresemann et al., 2006). Silencing of TGFβ receptor occurs in prostate cancer stroma and is commonly associated with epigenetic mechanisms, suggesting that these inhibitors may offer a unique strategy to target stromal cells. The role of methyl donors in carcinogenesis is an area of some controversy showing a correlation between methyl donor deficiency and cancer, while others suggested an acceleration of carcinogenesis following supplementation.

Due to the low expression of DNMT1 in CAFs compared to cancerous epithelium, anti-DNMT targeted therapy may be more effective against

stromal cells. Thus, inhibition of DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) may offer a better opportunity to inhibit CAFs.

Recent studies have proposed that the effects of the metabolic activity in tumors are bidirectional between CAF and tumor cells. CAF cells support tumor growth and progression by altering metabolic processes in the microenvironment, including the production of nutrients such as lactate and pyruvate through aerobic glycolysis and high levels of the tumor-promoting ROS (Pavlides et al., 2009). During this "Reverse Warburg Effect," lactate produced by the fibroblasts provides energy to tumor cells. It is hypothesized that cancer cells may induce oxidative stress in fibroblasts, which can act as a metabolic and mutagenic driver of DNA damage and aneuploidy in cancer cells (Martinez-Outschoorn et al., 2010). CAF cells have high expression of lactate dehydrogenase and PKM2 with decreased expression of caveolin-1. Multiple epidemiologic and clinical studies have shown that overweight and obesity associated with the modern Western lifestyle increase diabetes and hyperinsulinemia, which in turn are linked to increased cancer incidence and poor outcome. Treatment with the antidiabetic agent metformin is associated with decreased breast cancer incidence and breast cancer-related mortality in patients with type 2 diabetes. These results indicate that drugs targeting the metabolic interactions between CAF and cancer cells can provide novel therapeutic opportunities for treating and preventing human cancers.

6. CONCLUSION

Recent advances in the understanding of the contribution of carcinoma-associated fibroblasts to tumor progression suggest pathways that can be targeted to restrict cancer growth. However, much work is still needed to unravel the full range of essential biological and pathological cellular interactions, and determining how these can best be used for clinical interventions. Identification of CAF markers that can distinguish high/low-risk patients will also be beneficial when selecting appropriate therapeutic approaches.

ABBREVIATIONS

CAF carcinoma-associated fibroblasts
BPH benign prostatic hyperplasia

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CHAPTER TEN

Resistance to Chemotherapy: Short-Term Drug Tolerance and Stem Cell-Like Subpopulations

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Abstract

Personalized medicine in cancer treatment has been a major goal for decades. Recently, the development of several therapies that specifically target key genetic alterations in different malignancies has dramatically improved patient outcome and brought the goal of personalized medicine closer to practicality. Despite the improved specificity of these treatment options, resistance to targeted therapy is common and remains a major obstacle to long-term management of a patient's disease. Often patient relapse is a result of the positive selection of cells with certain genetic alterations that result in a bypass of the therapeutic intervention. Once this occurs, patient relapse is inevitable and further treatment options are limited. The time to relapse is often quite rapid indicating that cancer cells may be primed for adapting to cytotoxic stimuli. Recently, it has been suggested that small subpopulations of cells allow resistance to occur more rapidly. It is thought that these cells are capable of surviving strong apoptotic stimuli until more permanent mechanisms of long-term resistance are developed. In order to decrease the rate of patient relapse, more studies are required in order to identify these subpopulations of cells, understand the mechanisms underlying their drug tolerance, and develop strategies to prevent them from evading treatment.

1. INTRODUCTION

Decades of cancer research have clearly laid a foundation for understanding the common characteristics that are shared between different types of cancer. Generally, these hallmark characteristics include uncontrolled proliferation, apoptotic evasion, and increased invasive potential (Hanahan & Weinberg, 2011). Current research continues to expand our knowledge of these traits in order to increase prevention, detection, and treatment of malignancies. A fundamental step toward this goal is a better understanding of the mechanisms through which a tumor acquires these characteristics.

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Studies continue to identify abnormalities in signaling pathways that contribute to the malignant phenotype of cancer cells.

Generally, these abnormalities are the result of alterations in key regulatory genes. Alterations of these genes disrupt normal cellular function and are often referred to as driver mutations because they are crucial for the malignant progression of the disease. The ability to identify driving mutations in patient samples and cell lines has been aided by the development of high-throughput assays, such as whole exome sequencing. This may lead to an encyclopedia of cancer mutations that can be used to develop specific targeted therapies. The utilization of targeted therapies that are linked to the mutational makeup of individual patients should lead to more personalized care and improved patient outcome.

Over recent decades, the theoretical possibility of targeted therapy and personalized medicine has become a reality for some malignancies. For example, chronic myelogenous leukemia (CML) is commonly characterized by t(9;22) chromosomal translocation, which results in the formation of the p210 form of the breakpoint cluster region-c-abl oncogene (BCR-ABL) fusion protein. This fusion protein lacks the negative regulatory domain of the ABL protein and, therefore, has constitutive tyrosine kinase activity (Davis et al., 1985). The identification of this fusion protein as a driving mutation of the disease led to the development of imatinib and other second generation inhibitors of this fusion protein. Treatment with these drugs has improved 5-year survival rate of patients with CML to more than 85% (O'Brien et al., 2003).

Mutations are also found in epidermal growth factor receptor (EGFR) family receptors in several different types of cancer. This family of receptors includes EGFR (v-erb-b erythroblastic leukemia viral oncogene homolog—ERBB1), ERBB2/HER2, ERBB3/HER3, and ERBB4. High expression of these proteins is seen in epithelial cancers such as breast and non–small cell lung cancer (NSCLC). Inhibitors such as the small molecule inhibitors, gefitinib and erlotinib, and the monoclonal antibody, herceptin have shown success in treating malignancies dependent on these receptors.

Another key example of targeted therapy has recently been developed for patients with metastatic melanoma. The development of this therapy was driven by the realization that around 50% of melanomas contain mutations in the serine/threonine kinase, v-raf murine sarcoma viral oncogene homolog B1 (B-RAF) (Davies et al., 2002). Less than a decade after this discovery, the mutant B-RAF inhibitor, vemurafenib/PLX4032/zelboraf, was introduced into the clinic. This drug is now the FDA-approved standard

of care for patients with mutant V600 B-RAF positive metastatic melanoma and has shown improvement overall and progression-free survival in naïve patients (Chapman et al., 2011).

However, despite an increased ability to target mutant gene products, most patients still experience tumor relapse over time. Various mechanisms of resistance have been identified in these patients that enable their tumors to bypass targeted therapy. These mechanisms usually result in reactivation of the original pathway targeted by the therapy or activation of new pathways that compensate for the inhibition of the targeted pathway (Fig. 10.1).

The high frequency of patient relapse with targeted therapies suggests that most tumors have inherent plasticity that allows for adaptation to cytotoxic stimuli. One possible explanation for this underlying plasticity is the presence of small subpopulations of cells within a tumor. These highly adaptable subpopulations of cells are often referred to as stem cell-like or

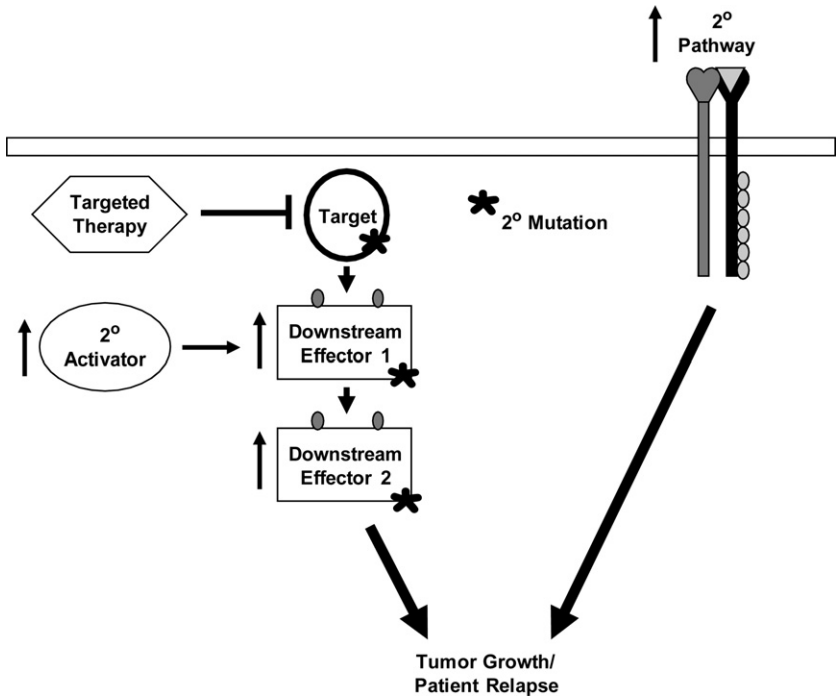

Figure 10.1 ***Canonical model of drug resistance.*** Drug resistance is generally thought to occur through permanent adaptations in signaling pathways that promote regrowth of the tumor. Often, this occurs through increased expression of secondary activators or downstream effectors, acquired mutations in pathway components, or increased activity of secondary pathways.

cancer stem cells (CSCs) due to the presence of certain cell surface molecules and the increased expression of stem cell factors in these cells. Studies examining the stem cell subpopulations of tumors have found that they have tumor initiating capabilities and are often resistant to chemotherapeutic treatment. The rate of resistance to targeted therapy suggests that the plasticity of tumor cells has been underestimated and requires more study. Current treatments are still not capable of coping with the evolution of most tumors. This chapter summarizes the latest findings about resistance to targeted therapies with a bias toward studies performed in melanoma.

2. LONG-TERM MECHANISMS OF RESISTANCE TO TARGETED THERAPY

Although treatments targeting various mutations in different cancer types have been developed over the past 10–15 years, a permanent cure for these malignancies still remains elusive for the majority of patients. This is due to emergence of resistance mechanisms in the tumor cells. Once resistance develops, cells undergo positive selection pressure and rapidly expand in order to reestablish the tumor cell population. As this occurs, patients relapse and are no longer responsive to the initial targeted therapy. Subsequent treatment options for such patients are limited.

2.1. Mechanisms of Pathway Reactivation/Therapeutic Bypass

As described earlier, therapeutic resistance is usually associated with either reactivation of the originally targeted pathway or activation of alternative pathways that compensate for the loss of the targeted pathway. One type of resistance mechanism that can lead to pathway reactivation and therapeutic bypass is the development of mutations in the gene that is targeted by the therapeutic intervention. Often, these mutations occur at what are known as "gatekeeper" residues. Mutations at these residues have been shown to interfere with the action of ATP-pocket-binding drugs and, thus, decrease drug efficacy. One study found that mutations in BCR-ABL were present in 29 of 32 patients who relapsed following imatinib treatment (Shah et al., 2002). Of these 29 patients, 10 had developed a mutation at the gatekeeper residue (T315I) (Shah et al., 2002). In NSCLC patients, who were resistant to EGFR inhibitors such as gefitinib, 50% had gatekeeper mutations (T790M) (Oxnard et al., 2011). *In vitro* data also suggest that mutation of the

gatekeeper residue of B-RAF^{V600E} (T529) can confer resistance to RAF-inhibitors (Whittaker et al., 2010); however, mutations in the gatekeeper residue of B-RAF have yet to be found in vemurafenib-relapse samples taken from patients (Nazarian et al., 2010).

Additionally, genetic alteration of the targeted gene can occur at areas outside of the gatekeeper residue. For example, one mechanism found to lead to resistance to vemurafenib is the development of a splice variant in B-RAF. Because this splice variant lacks the N-terminal region of B-RAF, it is rendered constitutively active via elevated homodimerization of spliced B-RAF and is insensitive to vemurafenib (Poulikakos et al., 2011). This mechanistic explanation is supported by the fact that expression of a dimerization-deficient mutant form of the splice B-RAF variant displays sensitivity to vemurafenib treatment (Poulikakos et al., 2011).

Although bypass of targeted therapy often results from mutational events arising after treatment initiation, the original genetic makeup of a tumor may also lead to heightened pathway activation and resistance. Specifically, this occurs in melanomas that harbor mutations in neuroblastoma RAS viral oncogene homolog (N-RAS) but have wild-type B-RAF. Despite strong activation of downstream RAF-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK)1/2 via mutant N-RAS, melanomas with this type of mutational status showed paradoxical hyper-activation of the pathway in response to treatment with vemurafenib (Halaban et al., 2010; Heidorn et al., 2010; Kaplan et al., 2011; Poulikakos et al., 2010). This is thought to be due to increased membrane recruitment and C-RAF heterodimerization of the inhibited form of wild-type B-RAF (Heidorn et al., 2010). For this reason, vemurafenib is not approved for metastatic melanoma patients with wild-type B-RAF.

Patients who have relapsed while on targeted therapies also show pathway reactivation via amplification of the targeted gene. One study has shown that transformed hematopoietic cells cultured continuously in imatinib develop resistance characterized by increased BCR-ABL mRNA and a nearly 10-fold increase in BCR-ABL protein (Weisberg & Griffin, 2000). Additionally, resistance of colorectal cancer cell lines to RAF and MEK inhibition has also been characterized by amplification of B-RAF (Corcoran et al., 2010).

Alterations in other components of the targets downstream pathway may also account for pathway reactivation and resistance to therapy. Several studies have shown that in ERBB2–overexpressing breast cancer cell lines, resistance to EGRF inhibitors can be mediated by upregulation of ERBB3,

leading to activation of the same downstream targets originally affected by ERBB2 overexpression (Grovdal et al., 2012). Pathway reactivation has also been shown as a key resistance mechanism to vemurafenib both *in vitro* and in the clinic. For example, a subset of patients who relapsed while on vemurafenib showed mutations in N-RAS, which can activate the MEK-ERK1/2 pathway via other RAF isoforms (Nazarian et al., 2010). Elevation of v-raf-1 murine leukemia viral oncogene homolog 1 (C-RAF) has also been proposed as a potential mechanism of pathway reactivation that would bypass B-RAF inhibition (Montagut et al., 2008), but the data supportive of this notion are *in vitro* based. Additionally, downstream activation of ERK1/2 signaling pathway has been shown to occur in one resistant patient sample via a mutation in MEK1 (Wagle et al., 2011).

2.2. Compensatory Pathway Activation

Although many cases of patient relapse can be attributed to mechanisms that reactivate the targeted pathway, resistance to therapy can also result from increased activity of other oncogenic pathways that lie outside of the influence of the initial target. Patients that develop this type of resistance may still display inhibition of the targeted pathway. However, their disease has evolved in such a way that the tumor cells are no longer dependent on the action of the original driving mutation. Cells that lack this dependency are then capable of reestablishing the tumor population despite the continued action of the targeted therapy.

Some patients that develop resistance to vemurafenib show this type of compensatory mechanism. Elevated expression of platelet-derived growth factor receptor, beta (PDGFRβ) was detected in 4 out of 11 samples obtained from patients that experienced tumor relapse during treatment (Nazarian et al., 2010). Increased expression and activity of this receptor has been shown to enhance growth and survival in cell lines that have developed resistance to RAF inhibitors. Additionally, one study has shown that melanoma cell lines can also develop resistance to RAF inhibition through increased phosphorylation of insulin-like growth factor 1 receptor (IGF-1R) (Villanueva et al., 2010). The study also demonstrated enhanced activation of IGF-1R and/or downstream signaling to v-akt murine thymoma viral oncogene homolog 1 (AKT) in two of five patient samples analyzed.

The plethora of mechanisms resulting in resistance to targeted therapy is a startling realization. Although the identification of aberrations leading to

bypass of the target and progression of the disease is important, further investigation into the inherent ability of cancer cells to evade targeted therapy is needed in order to improve the clinical outcome of chemotherapeutic treatments.

3. RESISTANCE OF STEM CELL-LIKE SUBPOPULATIONS

Many investigators have now realized that there are inherent properties of malignant tumors that provide resistance to targeted therapy. This type of resistance is based on the idea that these tumors contain populations of cells with readily available mechanisms in place that prevent the cell from succumbing to strong apoptotic signaling. If the majority of cells in a tumor have mechanisms that provide inherent resistance to a chemotherapeutic treatment, a patient may not show a dramatic response. This has been shown in the clinical trials of vemurafenib where approximately 50% of patients that have mutant B-RAF positive melanomas show low levels of tumor regression or, in some cases, progressive disease following treatment (Chapman et al., 2011). Patients who fail to show significant response to targeted therapy may have tumors with multiple driving mutations that promote proliferation and survival. Therefore, targeting only one of the driving mutations is not sufficient to promote tumor regression.

However, many models of resistance to targeted therapies have found that inherent resistance to chemotherapy is often a result of small subpopulations of cells that survive treatment and eventually reestablish the initial population. This theory is similar to a recent model of bacterial resistance that involves the presence of "persisting" cells rather than drug-resistant mutants. The presence of these persisting cells in bacterial populations is caused by phenotypic heterogeneity most likely caused by epigenetic mechanisms that increase the probability that some of the individual cells will survive lethal stimuli such as antibiotic treatment (Dhar & McKinney, 2007). It is thought that resistant bacteria colonies develop through these persisting subpopulations. However, only recent data have supported the possibility that this model of resistance could play a role in tumor cells (Sharma et al., 2010).

The idea that a subpopulation of cells is capable of reestablishing an entire tumor population after chemotherapeutic treatment is related to the idea of adaptive resistance that was previously discussed. However, short-term drug tolerance focuses on immediate survival rather than permanent mechanisms

that undergo positive selection and ultimately reestablish the ability of the tumor to survive and propagate. For this reason, cancer cells that display this type of inherent resistance are often called drug-tolerant persisters (Sharma et al., 2010). Although the emergence of the adaptive responses to treatment discussed earlier plays a crucial role in promoting long-term resistance to targeted therapy, the role of short-term adaptations of subpopulations of tumor cells to cytotoxic stimuli remains unclear.

The fact that a tumor is heterogeneous and contains subpopulations of cells is fundamental to cancer biology. This concept suggests that there are different cell types within a tumor that play crucial and specific roles in maintaining malignant properties. For example, evidence suggests that there is a subpopulation of cells in melanoma that are slower cycling and are responsible for maintaining the rapidly proliferating cell population (Roesch et al., 2010). Several studies have found similar results suggesting that a small subpopulation of cancer cells is responsible for establishing the entire tumor cell population. This theory is called the CSC theory and has been the subject of intensive study.

The theory of CSCs assumes that tumors have a similar hierarchy of cellular organization as that seen in normal tissue. Cells are classified as CSCs if they can reestablish a tumor cell population. Subpopulations of CSCs have been identified in many cancer types (Frank et al., 2010) and are usually identified by unique markers that distinguish them from other cancer cells in the population. Some of the molecular phenotypes that have been associated with CSC subpopulations include $CD34^{+}CD38^{-}$ for acute myeloid leukemia (AML), $CD44^{+}CD24^{-}$ for breast cancer, $CD133^{+}$ for colon cancer and pancreatic cancer, $CD90^{+}$ for liver cancer, $CD44^{+}CD117^{+}$ for ovarian cancer, and $ABCB5^{+}$ for melanoma (reviewed in Frank et al., 2010).

One of the most salient features of the CSC theory is that these subpopulations of cells are responsible for the establishment of all other cancer cells within a tumor. This means that the bulk of tumor cells may represent differentiated forms of the original CSCs. These differentiated cancer cells are thought to have a limited life span and an increased sensitivity to immune detection, hypoxia, and chemotherapeutic treatments. However, because CSCs are believed to have unlimited self-renewal, the effects of cytotoxic stimuli on this subpopulation is believed to be mitigated. This is supported by the fact that tumors that are resistant to therapeutic interventions have maintained the presence of the CSC subpopulation (Reya et al., 2001; Scheck et al., 1996). The fact that CSCs are more resistant to cytotoxic stimuli has dramatic implications for cancer treatment.

Several studies have demonstrated that the CSC subpopulation displays decreased sensitivity to radiation treatment. Radiation therapy causes DNA damage in cells, which leads to mitotic catastrophe and cell death. It is believed that CSCs may be resistant to radiation by increasing DNA damage repair mechanisms. For example, in normal stem cells, the activation of the wingless-type MMTV integration site family, member (Wnt)/β-catenin pathway plays an important role in normal stem cells and has been shown to be involved in resistance to DNA damage (Eyler & Rich, 2008). Previous studies have also shown that sorted mammary CSCs have increased the activity of the Wnt/β-catenin pathway following irradiation (Chen et al., 2007; Woodward et al., 2007). Other studies have also implicated the involvement of Chk1/2 and Notch signaling as possible explanations for the decreased radiosensitivity of CSCs (Bao et al., 2006; Phillips et al., 2006).

Evidence also suggests that the CSC subpopulation is resistant to DNA-damaging chemotherapeutic agents. For example, one study has demonstrated that CSCs from gliomas show resistance to temozolomide, carboplatin, VP16, and Taxol (Liu et al., 2006). Because CSCs are less sensitive to DNA damage caused by radiation therapy, it is not surprising that this subpopulation of cells shows little effect upon exposure to these types of agents. However, it is thought that the resistance that CSCs show against DNA-damaging agents may lie outside of their ability to upregulate DNA repair mechanisms. Both normal stem cells and CSCs express high levels of drug pumps such as ATP-binding cassette (ABC) transporters. These channels transport agents out of the cell and diminish the likelihood of having efficacious doses of the chemotherapeutic agent reach crucial areas of the cell. It is thought that these channels play an important role in the ability of CSCs to gain resistance to systemic therapies, for example, amplification of the ABC transporter, ATP-binding cassette, subfamily G, and member 2 (BCRP) reduces efficacy of imatinib (Burger et al., 2004). Other ABC transporters, such as ATP-binding cassette, subfamily B, and member 1 (MDR1), have been shown to remove agents such as paclitaxel (Green et al., 2006).

In addition to the characterization of CSC subpopulations, investigations have also aimed to understand the regulation of stemness factors that may be inherently expressed in various malignancies. For example, POU class 5 homeobox 1 (Oct4) has been shown to be highly upregulated in bladder cancer tissue compared to normal tissue (Atlasi et al., 2007). Oct4 and Nanog expression have also been shown to enhance malignancy and epithelial-mesenchymal transition (EMT) properties of lung adenocarcinomas

(Chiou et al., 2010). Additionally, increasing the expression of Oct4 enhances invasiveness and drug resistance in colorectal cancer and lung cancer cell lines (Chen et al., 2008).

A stemness factor that appears to have an important role in melanoma is forkhead box D3 (FOXD3). FOXD3 is a forkhead transcription factor that is crucial for maintaining pluripotency and self-renewal in embryonic stem cells (Hanna et al., 2002; Liu & Labosky, 2008) possibly by regulating other stem cell factors such as Nanog and Oct4 (Pan et al., 2006). Recent studies have demonstrated that FOXD3 is upregulated in response to inhibition of mutant B-RAF in melanoma cells (Abel & Aplin, 2010). This upregulation of FOXD3 has been shown to provide resistance to cell death induced by RAF inhibitors, such as vemurafenib (Basile et al., 2011). However, preliminary data indicate that FOXD3 prevents cell death independent of changes in the expression patterns of many key regulators of apoptosis such

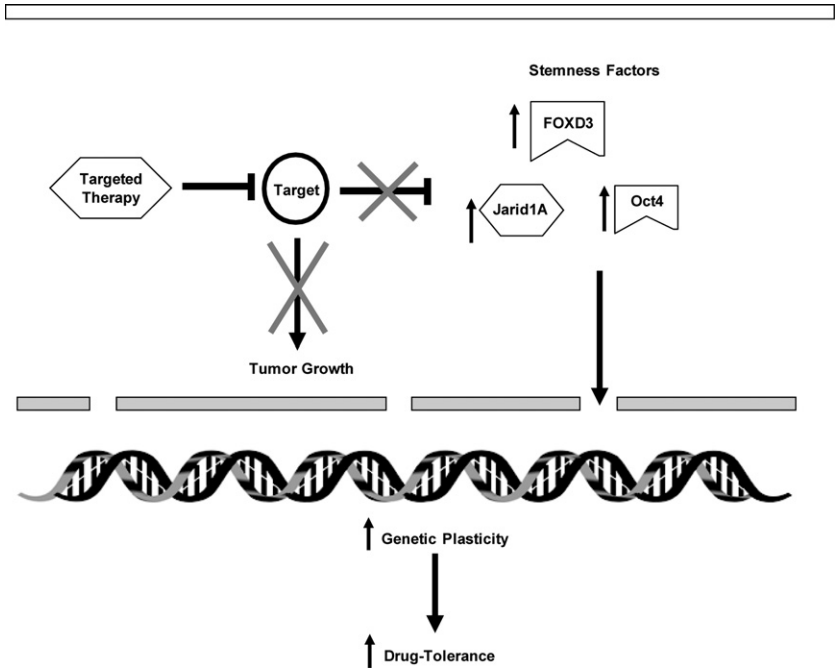

Figure 10.2 The role of stemness in drug resistance. Resistance to therapy is believed to be a complex, multistep process that begins with the short-term, inherent plasticity of subpopulations of cancer cells. The cytotoxic stimuli resulting from inhibiting a target can result in enhanced expression/activity of stemness factors in persisting subpopulations of cells. Expression of these stemness factors results in genetic plasticity that allows these cells to remain in a dormant, drug-tolerant state.

as Bim-EL, Bmf, and Mcl-1 (Basile et al., 2011). Instead, it is believed that FOXD3 is influencing other targets that provide compensation for the strong apoptotic stimuli that has previously been shown to be associated with loss of ERK1/2 signaling (Shao & Aplin, 2010).

The fact that FOXD3 is upregulated rapidly after inhibition of RAF/MEK in mutant B-RAF melanoma cells in order to prevent cell death suggests that these cells have an inherent ability to adapt to environmental stresses. Although the role of FOXD3 in melanoma seems to be complex, the increased expression of this stemness factor in response to inhibition of strong pro-survival signaling may be the result of an "emergency response" that the cells use to withstand a sudden change in the equilibrium of their malignant phenotypes. Because this response prevents cell death, these cells may be capable of going into a state of dormancy until they regain malignant properties through other mechanisms (Fig. 10.2).

4. PLASTICITY OF TRANSIENTLY DRUG-TOLERANT SUBPOPULATIONS

In order to eradicate these stem cell-like subpopulations of cancer cells, it is necessary to gain a better understanding of the inherent plasticity of cancer cells. Current research suggests that cancer cells are highly adaptable to a variety of stimuli. These adaptations occur quickly and efficiently as if the cancer cell itself is preprogrammed to respond to continuous environmental changes. Therefore, the root cause of resistance to therapeutic treatments is likely to lie within this adaptability.

Many mechanisms of adaptive resistance such as those discussed earlier are based on the idea that alterations in a single gene or pathway lead to resistance. However, it is unlikely that the majority of relapse cases can be explained this simply. Instead, many now believe that drug resistance is caused by widespread epigenetic changes. These epigenetic changes reflect an inherent plasticity of small subpopulations of cells that can alter complex signaling networks quickly and efficiently in order to survive cytotoxic stimuli.

It has previously been shown that several oncogenic pathways can influence epigenetic changes. For example, loss of adenomatous polyposis coli (APC) is known to result in dysregulation of DNA methyltransferases that promote undifferentiated states similar to stem cell-like populations (Rai et al., 2010). Transformed breast epithelial cells were also shown to

have higher levels of spontaneous conversion of non–stem cells to stem-like cells than their nontransformed counterparts (Chaffer et al., 2011). Additionally, oncogenic RAS can alter global histone modification (Pelaez et al., 2010).

Epigenetics has also been implicated in drug-resistant populations of cells. Originally, it was thought that methylation of certain genes would impact resistance to therapy. For example, studies of ovarian cancer have found that hypermethylation of the mismatch repair gene, mutL homolog 1 (MLH1), can confer resistance to DNA-damaging agents (Brown et al., 1997; Strathdee et al., 1999). However, recent evidence suggests that global epigenetic changes may contribute to the ability of cancer cells to develop resistance. This seems to be especially crucial for short-term survival of small subpopulations of persisting cells. Sharma et al. (2010) have found global chromatin alterations in drug-tolerant subpopulations of cancer cells that are associated with increased expression of the histone demethylase, jumonji/AT-rich interactive domain-containing protein 1A (JARID1A). This study found that increased expression of JARID1A was essential for the formation of drug resistance in their experimental systems. Therefore, results from this study would suggest that global epigenetic changes are crucial in the development of resistance to chemotherapeutic treatments.

The presence of stem cell-like subpopulations with global epigenetic modifications offers several key advantages that increase the tumor's ability to gain resistance to therapy. One advantage that this model of drug resistance offers is the efficiency by which global gene expression can be controlled. Increased activity of a few key epigenetic factors can change the expression patterns of hundreds of genes, whereas mutational changes that alter the same number of genes would take a much greater amount of time. Indeed, a previous study has shown that gene alterations by DNA methylation are far more common than mutations in genetic sequence (Bhattacharyya et al., 1994). Because resistance to therapy in the clinic can occur rapidly, it is likely that drug-tolerant subpopulations of cells may gain resistance using epigenetic mechanisms that can alter genetic expression more quickly than positive selection of permanent genetic mutations.

Another advantage that this model of drug resistance offers is the plasticity and reversibility of these drug-tolerant subpopulations of cells. Because epigenetic modifications can be removed, the changes to gene expression that accompany epigenetic alterations are capable of being transient. This reversibility would not occur with mutations in the genetic sequence, which would remain permanent unless there was a negative selection pressure.

Several studies have demonstrated the plasticity of subpopulations of cancer cells. For example, a study of melanoma cells found that there was a slow-cycling subpopulation of cells that was maintained at a very low frequency in the population (Roesch et al., 2010). This subpopulation had elevated expression of the histone 3 lysine 4 (H3K4) demethylase, JARID1B, which again indicates the use of epigenetic modification (Roesch et al., 2010). Interestingly, this study found that the JARID1B-positive cells were essential for continuous growth of the tumor cell population, and even if this subpopulation was removed, previously JARID1B-negative cells were capable of becoming JARID1B-positive cells (Roesch et al., 2010). This indicates that differing populations of melanoma cells are capable of reversing their JARID1B status, demonstrating a high level of plasticity.

Studies of drug resistance have also demonstrated increased plasticity of subpopulations of cells. Previously, it was mentioned that a study by Sharma et al. (2010) found that drug-tolerant subpopulations of cells had high expression of the histone demethylase, JARID1A. Additionally, this study found that the drug-tolerant status of this subpopulation was highly reversible. Drug-resistant subpopulations that were grown in the absence of treatment regained sensitivity to the inhibitor within 9–30 passages (Sharma et al., 2010). Spontaneous heterogeneity of this drug-tolerant subpopulation was also detected using CD133 as a marker (Sharma et al., 2010). This means that the drug-resistant state of these cells is transient and indicates high levels of plasticity influenced by the absence or presence of cytotoxic stimuli.

5. POTENTIAL STRATEGIES TO TREAT DRUG-TOLERANT SUBPOPULATIONS

Although original theories suggested that using multitargeted inhibitors may offer the best chance of overcoming resistance to therapy, new evidence suggests that cancer cells have complex levels of adaptability. This adaptability appears to be based on the plasticity of subpopulations of cells within the tumor that are capable of global epigenetic modifications. Therefore, overcoming resistance to targeted therapy may benefit from using chromatin-modifying agents, such as histone deacetylase (HDAC) inhibitors (Fig. 10.3).

HDACs are associated with transcriptional repression. This is accomplished by the removal of key acetyl groups on histone proteins. This removal leads to chromatin condensation, which prevents transcriptional

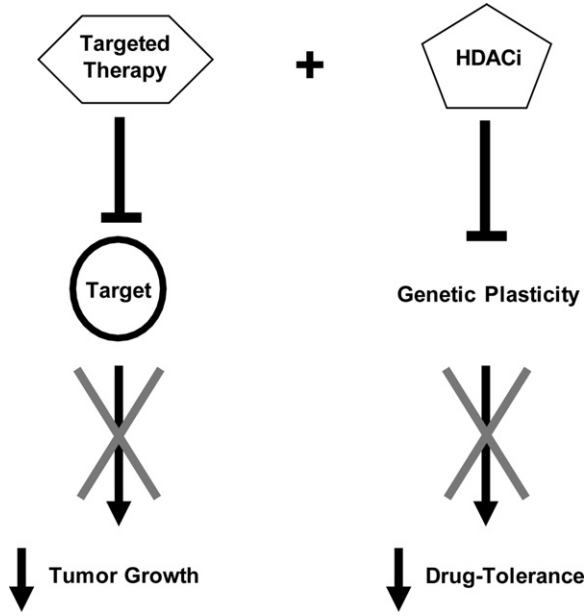

Figure 10.3 ***The proposed treatment modalities to overcome drug tolerance.*** Using targeted therapies will provide beneficial results by decreasing cell growth and increasing cell death. However, chromatin-modifying agents may be necessary to overcome the drug-tolerant state that results from the increased genetic plasticity of certain subpopulations of cancer cells exposed to cytotoxic stimuli. Therefore, the combination of these two agents may eliminate drug-persisting cells and decrease the rate of patient relapse.

machinery from associating with genomic regions. Most HDACs belong to three main classes. Class I HDACs reside exclusively in the nucleus, while class II HDACs shuttle between the nucleus and the cytoplasm (Monneret, 2007). Class III HDACs are classified based on sequence similarity to the yeast Sir2 homolog. Each HDAC differs in tissue specificity and substrate binding. For example, HDAC1 (Class I) is ubiquitously expressed in various tissue types and has substrates that include E2F1, while HDAC5 (Class II) is expressed in heart, smooth muscle, and brain tissue and has substrates that include SMAD7 (Dokmanovic et al., 2007).

In cancer, it is believed that dysregulation of HDACs might lead to altered function of key tumor suppressor genes, such as tumor protein 53 (p53) (Murphy et al., 1999). Therefore, many researchers have explored the feasibility of using HDAC inhibitors to treat various malignancies. Many HDAC inhibitors have been developed, and several of them have shown efficacy in both *in vitro* and *in vivo* cancer models. It is believed that HDAC

inhibitors work through several mechanisms, which are often independent of chromatin structure. This is because HDACs target a variety of nonhistone proteins such as transcription factors and DNA repair enzymes (Khan & La Thangue, 2011). In fact, it has been shown that HDAC inhibitors may affect the mRNA levels of only 5–10% of genes based on microarray studies (Peart et al., 2005). Despite the ambiguities of their mechanism, HDAC inhibitors have shown to be a promising avenue for cancer therapy.

For example, vorinostat/suberoylanilide hydroxamic acid (SAHA) is an FDA-approved HDAC inhibitor used to treat cutaneous T-cell lymphoma (CTCL). Prior to its approval, vorinostat was assessed in phase 1–2 trials. In these trials, vorinostat was well tolerated in patients and gave a response in around 30% of CTCL patients (Duvic et al., 2007). Currently, more clinical trials are investigating other HDAC inhibitors as well as combination treatment of vorinostat and other chemotherapeutic agents for various types of malignancies.

Because resistance to chemotherapy is likely to rely on chromatin modifications, it may be beneficial if certain chemotherapeutic treatments were used in combination with HDAC inhibitors. Combination treatment of HDAC inhibitors such as vorinostat with kinase inhibitors such as erlotinib or sorafenib has shown to be more effective at preventing the establishment of drug-tolerant colonies versus treatment with single-agent therapies (Sharma et al., 2010). The addition of HDAC inhibitors was also able to decrease resistance to nonspecific DNA-damaging agents such as cisplatin (Sharma et al., 2010).

Despite dramatic *in vitro* results, clinical data supporting the efficacy of using HDAC inhibitors in combination with other chemotherapeutic treatments have yet to be shown. A recent clinical trial exploring the efficacy of erlotinib in combination with vorinostat in 16 patients with NSCLC was prematurely terminated due to serious adverse events and a lack of efficacy (Clinical Trial Identifier: NCT00251589). This suggests that more specific chromatin modifying agents may be needed to increase efficacy and prevent toxicity resulting from combinatorial therapies.

6. CONCLUSION

Despite improved treatment options for many types of cancer, long-term management still remains elusive for the majority of patients. New evidence suggests that resistance to chemotherapy occurs in a step-wise

process that begins with the inherent plasticity and stem cell-like phenotype of small subpopulations of cells within the tumor. These drug-tolerant subpopulations persist through strong apoptotic stimuli but remain in a dormant state of growth inhibition. Eventually, more permanent mechanisms of resistance are developed and result in therapeutic bypass and reacquired growth potential of the tumor. At this point, the patient presents with relapse of their original disease despite the presence of their targeted treatment. Further study is needed in order to clarify the mechanisms of inherent resistance shown by these small subpopulations of cells and identify new treatment options that may potentially target this type of resistance.

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ABBREVIATIONS

ABC ATP-binding cassette
ABL c-abl oncogene
AKT v-akt murine thymoma viral oncogene homolog 1
AML Acute Myeloid Leukemia
APC adenomatous polyposis coli
BCR breakpoint cluster region
BCRP ATP-binding cassette, subfamily G, member 2
B-RAF v-raf murine sarcoma viral oncogene homolog B1
CML chronic myelogenous leukemia
C-RAF v-raf-1 murine leukemia viral oncogene homolog 1
CSC cancer stem cell
EGFR epidermal growth factor receptor
EMT epithelial-mesenchymal transition
ERBB v-erb-b erythroblastic leukemia viral oncogene homolog
ERK extracellular signal-regulated kinase
FOXD3 forkhead box D3
H3K4 histone 3 lysine 4
HDAC histone deacetylase
IGF-1R insulin-like growth factor 1 receptor
JARID jumonji/AT-rich interactive domain-containing protein
MDR1 ATP-binding cassette, subfamily B, member 1

MEK mitogen-activated protein kinase
MLH1 mutL homolog 1
N-RAS neuroblastoma RAS viral oncogene homolog
NSCLC non–small cell lung carcinoma
OCT4 POU class 5 homeobox 1, p53 tumor protein 53
PDGFRβ platelet-derived growth factor receptor, beta
SAHA suberoylanilide hydroxamic acid
WNT wingless-type MMTV integration site family, member

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CHAPTER ELEVEN

Intratumoral Heterogeneity as a Therapy Resistance Mechanism: Role of Melanoma Subpopulations

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Abstract

Malignant melanoma is an aggressive form of skin cancer whose incidence continues to increase worldwide. Increased exposure to sun, ultraviolet radiation, and the use of tanning beds can increase the risk of melanoma. Early detection of melanomas is the key to successful treatment mainly through surgical excision of the primary tumor lesion. But in advanced stage melanomas, once the disease has spread beyond the primary site to distant organs, the tumors are difficult to treat and quickly develop resistance to most available forms of therapy. The advent of molecular and cellular techniques has led to a better characterization of tumor cells revealing the presence of heterogeneous melanoma subpopulations. The discovery of gene mutations and alterations of cell-signaling pathways in melanomas has led to the development of new targeted drugs that show dramatic response rates in patients. Single-agent therapies generally target one subpopulation of tumor cells while leaving others unharmed. The surviving subpopulations will have the ability to repopulate the original tumors that can continue to progress. Thus, a rational approach to target multiple subpopulations of tumor cells with a combination of drugs instead of single-agent therapy will be necessary for long-lasting inhibition of melanoma lesions. In this context, the recent development of immune checkpoint reagents provides an additional armor that can be used in combination with targeted drugs to expand the presence of melanoma reactive T cells in circulation to prevent tumor recurrence.

1. INTRODUCTION

The American Cancer Society (ACS) predicts an increased incidence of all cancers in the United States for the current year (Siegel et al., 2012). This is also true for malignant melanoma which continues to rise worldwide. According to current ACS estimates, ~76,000 new cases of melanomas

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(~5% of all cancers) will be diagnosed in the United States in 2012 and about 9000 patients will die of metastatic disease (Siegel et al., 2012). Thus far, the reasons for the higher incidence of melanoma remain unclear but increased exposures to sun or ultraviolet radiation are some of the major risk factors. Family history of melanoma, genetic susceptibility, environmental factors, and age-related immunosuppressions are also some of the contributing factors that could influence the incidence rates (reviewed in de Souza et al., (2012); Miller and Mihm (2006)).

In many cases, melanoma begins with the transformation of a benign nevus that develops into a dysplastic lesion before progressing into a radial- and vertical-growth phase (RGP and VGP [primary melanoma]) that can invade into the dermis, regional lymph nodes, and from there disseminate to distant organs, leading to metastatic melanoma (reviewed in Koh (1991); Miller and Mihm (2006)). However, not all melanomas arise from nevus and many arise through direct transformation of normal skin cells (de Souza et al., 2012).

In the last decade, a number of important genetic alterations have been identified during various stages of melanoma progression leading to a better understanding and molecular classification of the disease (reviewed in Chin et al., (2006); de Souza et al. (2012); Fecher et al., (2007); Vidwans et al. (2011)). These studies have also provided in-depth analysis of cell-cycle regulation and alterations in signaling pathways during the progression of the disease. Unlike the older histological classification (Chin et al., 2006; Koh, 1991; Miller & Mihm, 2006), newer molecular approaches define melanoma as a more heterogeneous and rather complex neoplasm (de Souza et al., 2012; Koh, 1991; Miller & Mihm, 2006; Vidwans et al., 2011). Additionally, a better understanding of the aberrant signaling pathways in melanoma has led to the discovery of targeted therapies with drugs such as vemurafenib and a host of others that are either awaiting approval by the US Food and Drug Administration (FDA) or are in various stages of phase I–III clinical trials (Flaherty, Puzanov, et al., 2010; Friedlander & Hodi, 2010; Vidwans et al., 2011).

Although a large number of primary melanomas can be successfully treated through surgery, therapy of advanced stage metastatic melanoma patients remains challenging (de Souza et al., 2012; Fecher et al., 2007; Miller & Mihm, 2006). Melanoma patients undergoing chemotherapy or targeted therapy with small-molecule inhibitors aimed at blocking the most frequently mutated oncogene ($BRAF^{V600E}$) are known to develop drug resistance and experience tumor recurrence (Flaherty et al., 2010; Flaherty,

Puzanov, et al., 2010; Villanueva et al., 2011). Several molecular mechanisms underlying acquired drug resistance have been recently described (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2011; Villanueva et al., 2010); however, tumor recurrence can also be due in part to the presence and potential enrichment of tumor subpopulations that are inherently resistant to therapy (Frank et al., 2005; Monzani et al., 2007; Roesch et al., 2010). Like other malignancies, melanoma is a highly heterogeneous neoplasm, composed of subpopulations of tumor cells with distinct molecular and biological phenotypes (Boiko et al., 2010; Dick, 2009; Fang et al., 2005; Monzani et al., 2007; Roesch et al., 2010; Schatton et al., 2008; Zabierowski & Herlyn, 2008). These distinct subpopulations provide the cellular basis for the complex biology of the disease including phenomena such as self-renewal, differentiation, tumor initiation, progression, tumor maintenance, and therapy resistance.

Here, we discuss the heterogeneous nature of melanoma subpopulations, possible reasons of heterogeneity, its role in therapy resistance, and future approaches to targeted therapy.

2. MOLECULAR OVERVIEW OF MELANOMA

Melanoma arises through the transformation of melanocytes, a melanin producing cell (Koh, 1991; Miller & Mihm, 2006). These cells share a common origin with neural crest cells and during embryonic development migrate toward the skin where they reside in the basal layer of the epidermis (Koh, 1991; Miller & Mihm, 2006). Melanocytes are closely associated with epidermal keratinocytes, dermal fibroblasts, endothelial cells, and inflammatory cell types which regulate their functional homeostasis and controlled proliferation; any alteration in the function of these cells due to biological or genetic events can give rise to melanocytic nevi (Satyamoorthy & Herlyn, 2002). Benign nevi (comprised of neval melanocytes) are biologically stable precursor lesions of melanoma (Miller & Mihm, 2006). BRAF is a member of the mitogen-activated protein kinase (MAPK) pathway. It is mutated in about 50% of melanomas, with a glutamic acid for valine substitution at codon 600 (V600E) being the most frequent mutation (Davies et al., 2002; de Souza et al., 2012; Fecher et al., 2007; Vidwans et al., 2011). Mutant $BRAF^{V600E}$ is also found in ~80% of benign nevi (Davies et al., 2002; de Souza et al., 2012; Fecher et al., 2007; Vidwans et al., 2011). Cells expressing $BRAF^{V600E}$ usually have increased MAPK activity (Fecher

et al., 2007). The oncogene NRAS, mutated in ~20% of melanomas, can also cause hyperactivation of the MAPK pathway (Fecher et al., 2007; Vidwans et al., 2011). BRAF or NRAS mutations are more commonly present in nonchronic sun-exposed lesions and less common in chronic sun-exposed lesions or lesions of mucosal or acral or familial melanomas (de Souza et al., 2012; Friedlander & Hodi, 2010). Melanomas that do not express mutant $BRAF^{V600E}$ or mutant NRAS can have alterations in cell-cycle regulatory genes or proteins including Cyclin D1 [CCND1] (de Souza et al., 2012; Fecher et al., 2007), Cyclin-dependent kinases (CDK1, CDK2, CDK4, and CDK5) (Abdullah et al., 2011) or mutations in the proto-oncogene C-KIT (Fecher et al., 2007; Flaherty, Hodi, et al., 2010; Vidwans et al., 2011). However, a single oncogene cannot transform human melanocytes and additional genetic events are needed for malignant transformation (Bloethner et al., 2007; de Souza et al., 2012; Miller & Mihm, 2006). During the course of development and progression into melanoma, melanocytes tend to acquire additional genetic alterations (see Fig. 11.1). These alterations include loss or mutation of certain tumor suppressor genes such as phosphatase and tensin homolog (PTEN), p16INK4A (also known as cyclin-dependent kinase inhibitor [CDKN2a]), and inositol polyphosphate 4-phosphatase type II (INPP4b). Alterations in these genes are associated with activation of the phosphoinositide (PI)-3 kinase (PI3 K) pathway, increased proliferation, disease progression, and resistance to therapy (de Souza et al., 2012; Fecher et al., 2007; Gewinner et al., 2009; Miller & Mihm, 2006; Vidwans et al., 2011; Yuan & Cantley, 2008). Mutations in the p53 tumor suppressor gene, upregulation of the anti-apoptotic factors BCL-2 or MCL-1, or amplification of microphthalmia-associated transcription factor (MITF) are frequently observed in metastatic melanoma and have also been associated with chemoresistance (de Souza et al., 2012; Fecher et al., 2007; Vidwans et al., 2011).

3. THERAPEUTIC OVERVIEW

For many decades, metastatic melanoma was treated as a single disease entity; dacarbazine (DTIC), an alkylating agent, was the standard of care with temporary objective response rates below 15% (Koh, 1991; Miller & Mihm, 2006). Treatment of melanoma patients with temozolomide, a second-generation alkylating agent, also resulted in low response rates of about 10–12% (Fecher et al., 2007; Miller & Mihm, 2006; Vidwans et al.,

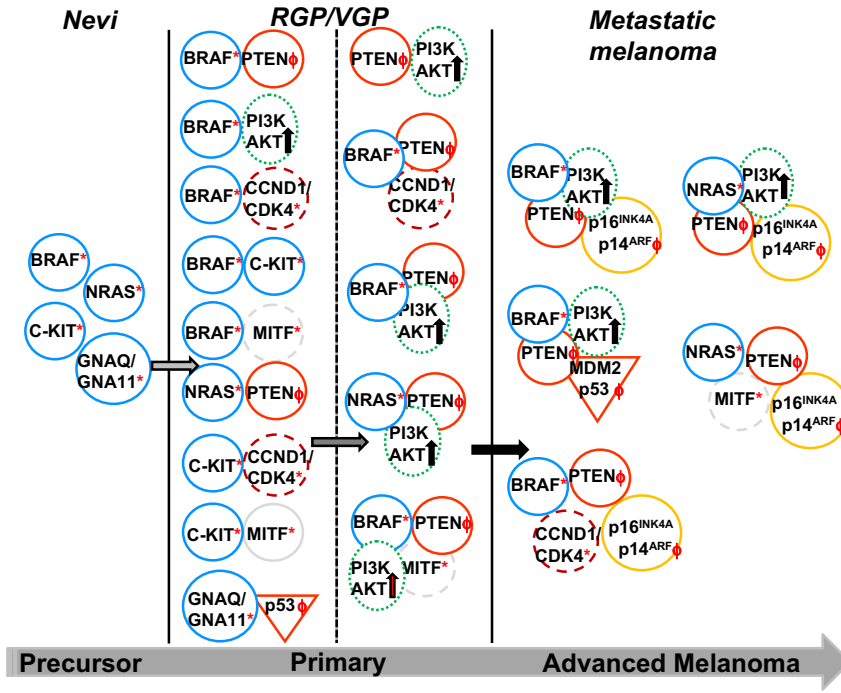

Figure 11.1 ***Molecular heterogeneity of melanomas.*** Precursor melanocytic lesions frequently harbor single gene mutations (*) such as BRAF, NRAS, C-KIT or GNAQ/GNA11 with a potential for neoplastic transformation. Additional oncogenic events (φ) such as deletions, mutations or loss of tumor-suppressor genes (PTEN, $p16^{INK4A}$/$p14^{ARF}$, p53), alterations in genes associated with cell-cycle regulation (CCND1/CDK4, MITF [dashed circle]), or activation (black arrow) of signaling pathways (PI3 K/AKT [dotted oval]; sometimes PI3 K/AKT mutations can also be found in low frequency) are needed for malignant transformation of benign nevi to primary tumor and then to progressive metastatic melanoma. The most frequent genetic alterations are depicted for simplicity. Mutations of tumor-suppressor genes ($p16^{INK4A}$, $p14^{ARF}$, and p53) may happen very early in the process of malignant transformation but there is no concrete evidence of their exact occurrence. Genomic instability further contributes to genetic heterogeneity. For color version of this figure, the reader is referred to the online version of this book.

2011). The use of adjuvant therapies such as interferon (IFN)-α or interleukin (IL)-2 has provided a modest improvement in patient survival (de Souza et al., 2012; Miller & Mihm, 2006). Additionally, these therapeutic modalities were associated with lingering toxicities, frequently leading to discontinuation of treatment. Many other forms of biological and immunological therapies have failed to go beyond the experimental stage. The recent FDA approval of anti-CTLA4 (also known as Ipilimumab or

Yervoy), an immune checkpoint agent, has shown some improvement in survival of melanoma patients and has created renewed interest in immunological therapies (Hodi et al., 2010). Another immune modulating agent, anti-program cell death (PD)-1, has provided favorable response rates in clinical trials (Brahmer et al., 2010; Kline & Gajewski, 2010). Additionally, recent advances developing engineered T cells designed to express chimeric-antigen receptor (CAR) with specificity against melanoma tumor cells has shown some promising response rates in a clinical trial involving adoptive T-cell therapies (Schmidt et al., 2009). The discovery of mutations such as $BRAF^{V600E}$ or NRAS and defects in cell-cycle regulatory genes or proteins has led to a more personalized targeted therapy approach for the treatment of melanoma. In this context, vemurafenib, a BRAF-selective kinase inhibitor recently approved by the FDA, has shown dramatic regression of metastatic melanoma lesions. Over 50% of BRAF-mutant melanoma patients respond to vemurafenib with a median progression-free survival of about 7 months (Chapman et al., 2011; Flaherty, Puzanov, et al., 2010; Sosman et al., 2012). Unfortunately, responses are transient and most patients develop resistance to treatment in the long run.

4. THERAPY RESISTANCE

Multiple mechanisms can mediate therapy resistance and the readers are referred to reviews that provide an excellent overview on drug-resistance pathways (Dean et al., 2005; Tredan et al., 2007). Drug resistance in tumor cells could be due to one or more distinct mechanisms, including some briefly described in the following sections.

4.1. Increased Drug Efflux Activity

Multidrug resistance in cancer is frequently linked to overexpression of the ABC (ATP-binding cassette) transporters, P-glycoprotein (ABCB1), multidrug resistance-associated proteins (MRP11/ABCC1 and MRP2/ABCC2), and breast cancer resistance protein (ABCG2/BCRP). Enhanced expression of MDR or ABC transporter proteins on the membrane of tumor cells can result in increased drug efflux activity resulting in lower than required intracellular concentration of drugs than is needed for inhibition of tumor cell growth. Several tumor cell types including leukemias, melanomas, and carcinoma cells obtained from brain, breast, colon, lungs, ovaries, pancreas, prostate, and renal express high levels of ABC transporter

proteins (Dean et al., 2005; Szakacs et al., 2004), which can collectively pump a multitude of chemical compounds and which lead to chemoresistance. For example, tumor-initiating cells or subpopulations in melanoma that express ABCB5 or ABCG2 proteins are highly resistant to chemotherapeutic agents and immune-mediated lysis (Schatton et al., 2008; Taghizadeh et al., 2011). These subpopulations are described in greater detail in section V.

4.2. Increased DNA Repair Activity

In vitro studies have shown that a subset of melanoma cell lines resistant to chemotherapeutic agents have increased or altered DNA repair mechanisms (Bradbury & Middleton, 2004; Kauffmann et al., 2008; Sarasin & Kauffmann, 2008). There are multiple pathways of DNA repair mechanisms, including direct repair, mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), and double-strand break recombination repair, which include both nonhomologous end joining (NHEJ) and homologous recombination repair (HHR) (Bradbury & Middleton, 2004; Sarasin & Kauffmann, 2008). Polyadenosine diphosphate-ribose polymerase (PARP), a BER DNA repair enzyme, is frequently upregulated in melanoma cells (Bradbury & Middleton, 2004; Kauffmann et al., 2008). Several reports have shown that melanoma cells resistant to temozolomide or DTIC have elevated levels of O6-methylguanine-DNA methyltransferase (MGMT), a protein that removes drug-induced alkylguanine adducts from DNA (Augustine et al., 2009; Bradbury & Middleton, 2004; Kauffmann et al., 2008; Rastetter et al., 2007). Similar to MGMT, BER plays an important role in repairing the cytotoxic methyl DNA adducts created by temozolomide, and consequently, high BER activity can confer tumor resistance to temozolomide (Augustine et al., 2009; Bradbury & Middleton, 2004; Kauffmann et al., 2008; Runger et al., 2000). Some clinical studies indicate that better response rates can be achieved in melanoma patients treated with a combination of PARP inhibitors and DTIC (Jones & Plummer, 2008; Plummer et al., 2008), further suggesting that DNA repair mechanisms are associated with chemoresistance.

4.3. Increased Existence of Slow Cycling Cells or Tumor Side Population

The presence within a tumor of nonproliferating cells or cells that proliferate very slowly (slow cycling cells) or a population of cells that excludes the

DNA-binding dye Hoechst 33342, called "side population," has also been linked to therapy resistance (Addla et al., 2008; Dembinski & Krauss, 2009; Hadnagy et al., 2006; Ho et al., 2007; Nishimura et al., 2002; Roesch et al., 2010; Scharenberg et al., 2002). This is likely due to the fact that chemotherapeutic agents are effective on fast dividing cells as they generally cause DNA alkylation or adduct formation and therefore, are less effective on slow cycling or nonproliferating cells.

4.4. Tumor Microenvironment-Induced Drug Resistance

It is well established that therapy can induce changes in the tumor microenvironment (TME); certain chemotherapeutic agents such as paclitaxel or carboplatin cause preferential accumulation of macrophages or other leukocytes in the tumor stroma, which can influence disease outcome (Zitvogel et al., 2008; Zitvogel et al., 2011). Tumor stromal-derived fibroblasts as well as tumor-associated macrophages (TAMs) can play a role in resistance to treatment by modulating the tumor phenotype (Brennen et al., 2012; Denardo et al., 2011; van Kempen et al., 2003). Inflammatory cytokines produced by the infiltrating cells can induce tumor phenotypic changes; they can induce changes in the surface expression of human leukocyte antigen class I or class II molecules and co-stimulatory molecules that are necessary for interactions with immune cells (Zitvogel et al., 2011). In addition, infiltrating inflammatory cells are a source of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that can cause epigenetic changes, DNA strand breaks, point mutations, and aberrant DNA cross-linking leading to genomic instability (Grivennikov et al., 2010; Schetter et al., 2009). Furthermore, chronic inflammatory conditions promote tumor initiation and increase tumor survival by activating anti-apoptotic pathways and inducing the expression of anti-apoptotic factors such as BCL-2, MCL-1, and survivin that are frequently associated with therapy-resistant cells (Grivennikov et al., 2010; Schetter et al., 2009). These findings have spurred new therapeutic combinatorial approaches targeting both tumor and stroma-derived macrophages or fibroblasts to curtail the negative influence of inflammatory cells on neoplastic growth. Recent pilot trials aimed at targeting both the tumor cells and the infiltrating macrophages or fibroblasts have shown improved therapy responses, indicating the beneficial effects of this new treatment strategy (Brennen, et al., 2012; Denardo et al., 2011; Korkaya et al., 2011a, b). Additional clinical trials will be needed to confirm these findings.

4.5. Epigenetic Changes After Therapy

Patients with small cell lung carcinoma show transient resistance to certain targeted drugs such as tyrosine kinase inhibitors (TKIs) (Sharma et al., 2010). Patients who acquire resistance to TKIs respond to retreatment after a "drug-holiday," indicating the transient nature of drug resistance (Sharma et al., 2010). This phenomenon is known as adaptive resistance due to drug-induced stress. Certain tumor subpopulations undergo epigenetic changes and acquire transient resistance to escape the effect of drugs. Upon drug withdrawal, the residual subpopulations can revert and become drug sensitive again. Settleman's group has shown that the histone demethylase JARID1A is responsible for transient drug resistance. In melanoma, *in vitro* studies have shown that tumor cells can undergo epigenetic changes leading to increased resistance to chemotherapeutic agents (Sharma et al., 2010). Likewise, methylation of certain DNA regions can alter signaling pathways, activating survival mechanisms in the tumor cells. For example, increased expression of BCL-2/MCL-1, activation of β-catenin/MITF, and silencing of tumor suppressor genes such as p53 or the invasive suppressor CD82 are some of the mechanisms that are known to occur following DNA methylation (Chung et al., 2011; Dean et al., 2005; Halaban et al., 2009; Howell et al., 2009; Taylor et al., 2000). Studies using tumor specimens obtained before and after therapy have confirmed the above *in vitro* results. Both chemotherapeutic agents and targeted drugs can indirectly recruit inflammatory cells that can cause epigenetic changes via cytokine mediators, which also stimulate increased expression or activation of anti-apoptotic proteins and alterations in cell signaling mechanisms promoting tumor cell survival.

4.6. Activation of Alternative Signaling Mechanisms after Therapy

Melanoma patients treated with newly discovered targeted drugs frequently develop resistance to therapy (Vidwans et al., 2011). Several studies have shown that tumor cells chronically treated with targeted drugs, such as BRAF-selective inhibitors, can activate alternate signaling pathways to promote proliferation and survival, and thus develop therapy resistance (Fecher et al., 2007; Vidwans et al., 2011; Villanueva et al., 2011; Villanueva et al., 2010). Multiple studies suggest that reactivation of the MAPK pathway in a BRAF-V600E-independent manner is commonly associated with resistance to BRAF-selective inhibitors. In addition to others, we have demonstrated that BRAF-V600E-mutant melanoma cells express somewhat

increased levels of CRAF or ARAF after prolonged exposure to BRAF inhibitors (Montagut et al., 2008; Villanueva et al., 2010). Furthermore, BRAF-V600E melanoma cells that acquire resistance to BRAF inhibitors no longer rely on BRAF for MAPK activation but rather use one of the other two RAF isoforms to sustain the MAPK signaling pathway (Villanueva et al., 2010). Some melanoma cells resistant to BRAF inhibitors also displayed increased NRAS activity or mutations in NRAS (Poulikakos et al., 2011), which can promote signaling via the MAPK and PI3 K pathways (Atefi et al., 2011; Vidwans et al., 2011). Reactivation of the MAPK pathway can also be mediated by overexpression or amplification of the serine threonine kinase COT/MAPK8 (Johannessen et al., 2010). More recently, Poulikakos et al. (2011) discovered that resistance to BRAF inhibitors and reactivation of the MAPK pathway can be mediated through the expression of a truncated form of BRAF, which lacks the RAS activation domain. In addition, resistance to BRAF inhibitors has also been linked to enhanced expression of receptor tyrosine kinases (RTK), including insulin-dependent growth factor (IGF)-1 or platelet-derived growth factor (PDGF) receptors, leading to altered receptor activity and signaling via the PI3 K/AKT pathway (Nazarian et al., 2010; Vidwans et al., 2011; Villanueva et al., 2011; Villanueva et al., 2010). Activation of the MAPK and PI3 K/AKT pathways results in increased expression of anti-apoptotic proteins such as MCL-1 that increases the survival of tumor cells (Vidwans et al., 2011). Interestingly, although about 10% of colon carcinoma patients express $BRAF^{V600E}$ only 5% of this patient cohort responds to vemurafenib (Villanueva 2012). Resistant tumors from these patients exhibit upregulation of the epidermal growth factor (EGF)-receptor pathway following inhibition of the MAPK pathway after treatment with BRAF inhibitors. In these patients, a combination strategy using vemurafenib and the EGFR inhibitor Erlotinib or the monoclonal EGFR antibody Cetuximab increased tumor response (Prahallad et al., 2012). The reported resistant mechanisms have been validated in tumor samples obtained from patients after tumor recurrence.

5. TUMOR HETEROGENEITY AND MELANOMA SUBPOPULATIONS: THEIR ROLE IN THERAPY RESISTANCE

Some patients with metastatic melanoma treated with chemo-, targeted-, or immunological therapies show mixed responses to treatment. While some

lesions undergo dramatic responses to therapy, even complete regression, other lesions in the same patient continue to progress or in some cases, new lesions develop, indicating the emergence of drug-resistant clones (see Fig. 11.2). Genotypic and phenotypic analyses of melanoma cells have revealed that the tumors are more heterogeneous than the original lesions. As melanoma progresses from primary to metastatic disease, the tumor acquires additional genetic and biologic properties that support tumor growth, invasion, and metastasis (see Fig. 11.1). It is known that some of these acquired properties are profoundly influenced by the TME. Using laser microdissection, Yancovitz et al. (2012) described both intra- and intertumor variabilities in $BRAF^{V600E}$ expression in tumor cells isolated from different regions of the primary lesions. In that study, the primary melanoma lesion likely harbored mutation positive $BRAF^{V600E}$ cells as well as mutation negative or wild-type (WT) BRAF; tumor cells with either genotype have equal ability to develop metastasis (Yancovitz et al., 2012).

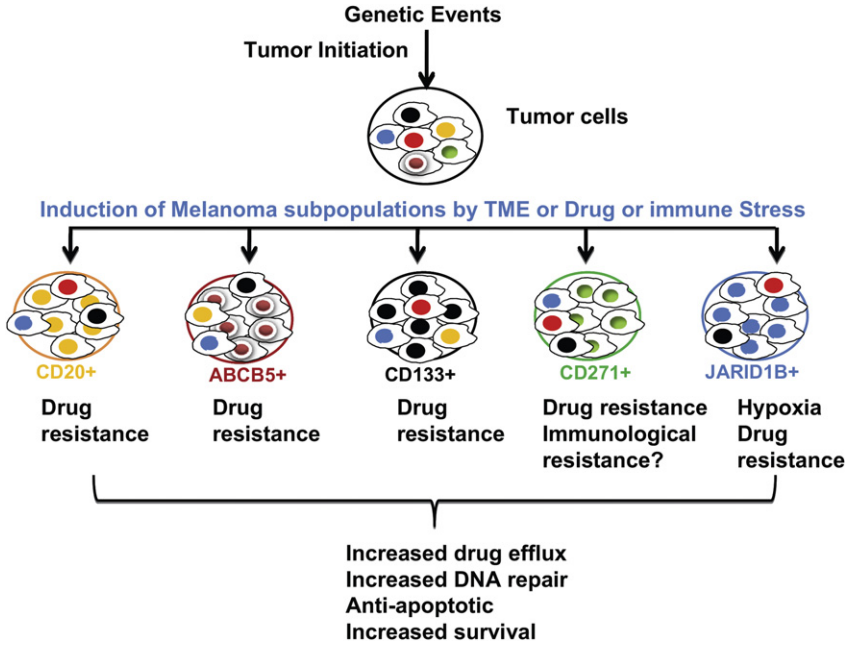

Figure 11.2 ***Induction of melanoma subpopulations: the role of TME, chemo- or targeted-therapy, and immune-related stress.*** TME niche and therapy-induced infiltration of leukocytes support and promote the induction of tumor subpopulations, which express increased levels of drug efflux proteins, DNA repair enzymes, and anti-apoptotic proteins resulting in activation of pro-tumor survival mechanisms. For color version of this figure, the reader is referred to the online version of this book.

This finding is further confirmed by the work of Sensi and colleagues on the heterogeneous genotypic expression of $BRAF^{V600E}$/WT-NRAS and WT-BRAF/$NRAS^{Q61R}$ in individual tumor cells (isolated after single-cell cloning) from the same lesion was shown (Sensi et al., 2006). Furthermore, Yancovitz et al. (2012) reported the presence of NRAS and BRAF mutations in different cells within the same primary lesion. $BRAF^{V600E}$ and NRAS mutations have long been considered as mutually exclusive (Fecher et al., 2007). However, Nazarian et al. (2010) have shown the presence of two different NRAS (Q61K and Q61R) mutations co-existent with BRAF-V600E in a nodal metastasis of a melanoma patient after therapy. In each study, the TME niche appears to play a critical role mediating the emergence of selective melanoma subpopulations with distinct genetic mutations. Similar observations were made in patients with advanced metastatic melanoma treated with immunological therapies. In patients who experienced mixed responses to therapy, their tumors had inter-and intra-lesional heterogeneity in the expression of melanoma-associated antigens (MAA), resulting in poor ability of T cells to bind and lyse the cancer cells (Campoli et al., 2009). Furthermore, many metastatic melanoma cells with low MITF expression have similar down modulation of MAA (Dissanayake et al., 2008). Given the implications of tumor heterogeneity in melanoma therapy, a better understanding of tumor subpopulations and their role in therapy resistance is required. In the following section, we will describe the most common melanoma subpopulations described thus far by us or others, which can mediate chemo-, targeted-, or immune-therapy resistance.

5.1.1. CD20

CD20 is a transmembrane protein, originally identified as a B-cell surface marker involved in Ca^{++} channeling, B-cell activation, and proliferation (Somasundaram et al., 2011; Tedder & Engel, 1994). Using gene expression profiling, CD20 has been identified as one of the top 22 genes in melanoma that defines the aggressive nature of the disease (Bittner et al., 2000). Our group has shown that a small proportion of melanoma cells express CD20 when grown as tumor spheroids under *in vitro* culture conditions (Fang et al., 2005). This $CD20^{+}$ population was previously considered to be a cancer stem-like cell or tumor-initiating cell as it fulfilled some of the criteria of "tumor stemness" by its ability to differentiate into multiple lineages including melanocytes, adipocytes, or chondrocytes (Fang et al., 2005). However, the concept of stem cells in melanoma has been challenged and remains controversial; along with Morrison's group, we have demonstrated

that any melanoma cell can be a tumor-initiating cell. Our unpublished observations indicate that melanoma cells that are resistant to chemotherapeutic agents such as cisplatin show higher expression of CD20. We and others have identified $CD20^+$ melanoma cells in metastatic tumor lesions; the significance of melanoma cells expressing CD20 under *in vivo* conditions is not yet clear and is currently under investigation (Pinc et al., 2012; Schmidt et al., 2011).

Recently, Schmidt et al. (2011) were able to target a small population of melanoma cells expressing CD20 using CAR-engineered T cells in a mouse xenograft model. They showed that by targeting a small subset of $CD20^+$ tumor cells with engineered T cells with redirected specificity for CD20, complete inhibition of tumor growth in mice could be achieved. Inhibition of tumor growth was long-lasting; furthermore, no tumor relapse in mice was observed for more than 36 weeks. Moreover, in a recent study, we reported that when advanced melanoma patients were treated with anti-CD20 antibody in an adjuvant setting, the majority of patients remained disease free during the 3-year period of observation (Pinc et al., 2012). Similarly, staged patients in historical controls showed less than 1 year of survival. In a single case study, Abken's group has confirmed the regression of metastatic melanoma lesions in a patient treated with anti-CD20 in a nonadjuvant setting (Schlaak et al., 2012). Overall, the above studies strongly suggest that a $CD20^+$ melanoma subpopulation could be a major driver of tumor progression and elimination of this subset could result in disease-free survival.

5.1.2. ABCB5/ABCG2/ABCB8

ABC transporters such as ABCB5, ABCB8, and ABCG2 are frequently reported to be present in various cancers including melanoma (Dean et al., 2005; Szakacs et al., 2004). Schatton et al. (2008) reported a subpopulation of melanoma cells that have high expression of ABCB5 with tumor-initiating properties. These cells were highly chemoresistant, and targeting of the ABCB5 subpopulation resulted in inhibition of tumor growth in immunodeficient nude mice. This group also reported that the expression of ABCB5 was higher in metastatic melanomas when compared to primary or melanocytic nevi tissues. Melanoma cells obtained from nodal metastatic lesions had higher expression of ABCB5 as compared to cells obtained from visceral metastasis. Using immunodeficient SCID mice, the authors showed that $ABCB5^+$ cells were more tumorigenic than ABCB5 negative melanoma cells. The $CD133^+$ melanoma subpopulation that is chemoresistant is

known to co-express ABCG2 (Monzani et al., 2007; Taghizadeh et al., 2011). Given the selective expression of ABCG2 in a minor subpopulation of $CD133^+$ cells, its expression in melanoma tissue sections has not yet been confirmed. *In vivo* xenograft studies indicate the aggressive potential of cells that co-express CD133 and ABCG2 cells (Monzani et al., 2007). In addition to ABCB5 and ABCG2, an *in vitro* study has shown the presence of an $ABCB8^+$ melanoma subpopulation that is resistant to drugs such as doxorubicin (Elliott & Al-Hajj, 2009). However, melanoma tumor tissue staining of ABCB8 has not been confirmed thus far.

5.1.3. CD133

CD133, a transmembrane glycoprotein also known as prominin-1, is normally expressed on undifferentiated cells including endothelial progenitor cells, hematopoietic stem cells, fetal brainstem cells, and prostate epithelial cells (Neuzil et al., 2007). CD133 has also been identified as a cancer stem cell marker with tumor-initiating properties (Monzani et al., 2007; Shmelkov et al., 2008). Various solid tumors including brain, breast, colon, liver, lung, pancreatic, and prostate cancers show expression of CD133 (Dembinski & Krauss, 2009; Liu et al., 2006; Ricci-Vitiani et al., 2007; Salmaggi et al., 2006; Shmelkov et al., 2008). A small proportion of melanomas and primary human melanocytes are known to express CD133 (Klein et al., 2007; Rappa et al., 2008). Klein et al. (2007) observed a significant increase in the expression of stem-cell markers CD133, CD166, and nestin in primary and metastatic melanomas compared with benign nevi. Aggressive melanomas were usually associated with greater expression of these markers. However, there are some discrepancies regarding immune detection of CD133 likely due to differences in the binding affinity of different antibody clones to the glycosylation sites of CD133 that vary between tumor and normal cells (Kemper et al., 2010). Some reports indicate that only $CD133^+$ melanoma cells are capable of forming tumors in immunodeficient NOD/SCID IL2Rγ_c (NSG) null mice, whereas $CD133^-$ cells failed to form tumors; these data imply that $CD133^+$ cells are key drivers of tumor cell repopulation under experimental conditions (Monzani et al., 2007). However, we find that both $CD133^+$ and $CD133^-$ melanoma cells are equally capable of forming tumors (unpublished). Drug-resistant tumor subpopulations that were obtained from breast, glioma, and lung tumors after chemotherapy frequently express CD133 (Levina et al., 2008; Liu et al., 2006; Visvader & Lindeman, 2008). Higher expression of CD133 has been associated with upregulation of anti-apoptotic proteins and

increased survival mechanisms. $CD133^+$ drug-resistant tumor subpopulations usually express increased levels of Nestin (NES) presence, which has been associated with de-differentiation and more aggressive behavior of the disease (Grichnik et al., 2006; Klein et al., 2007). NES co-expression is frequently observed in $CD133^+$ and $CD271^+$ tumor-initiating subpopulations of melanomas (Grichnik et al., 2006; Klein et al., 2007). As melanocytes share common lineage with neural crest cells, co-expression of nestin, CD133, CD271 (nerve growth factor receptor [NGFR]), and other embryonic markers in melanoma subpopulations is expected.

5.1.4. CD271 (NGFR, also Referred as p75 Neurotrophin Receptor)

CD271 or NGFR, a transmembrane protein, is found in a number of human neural-crest-derived tissues and in cancers from breast, colon, pancreas, prostate, ovaries, and melanomas. Boiko et al. (2010) have shown that $CD271^+$ melanoma subpopulations derived from patient tissues are more tumorigenic and aggressive than $CD271^-$ subpopulations when transplanted in immunodeficient $Rag2^{-/-}\gamma c^{-/-}$ mice. Many of the melanoma-associated antigens such as MART1, MAGE, and tyrosinase were lost or down modulated in $CD271^+$ cells (Boiko et al., 2010). These antigen losses in subpopulation variants are mostly likely linked to the selection of immunologically resistant melanoma cells *in vivo*. Civenni et al. (2011) found that the expression of CD271 correlated with higher metastatic potential and poor prognosis in an analysis performed in many biopsy specimens from melanoma patients. The authors have observed that $CD271^+$ subpopulations of melanoma cells frequently show higher expression of ABCB5 transport proteins and lower expression of MAA, indicating that these cells may have survived drug therapy and anti-melanoma reactive immune T cells.

5.1.5. JARID1B

We have identified a slow cycling subpopulation of melanoma cells representing ~1–5% of all cells in tumor lesions that have stem-like or cancer-initiating properties (Roesch et al., 2010). These cells show high expression of histone demethylases jumonji ARID (JARID, also referred as lysine demethylase 5 [KDM5]) 1B, known to be critically involved in regulating gene expression and transcriptional activities. Preliminary data indicate that JARID1B expression is influenced by the TME. In prostate cancer, JARID1B upregulation is usually associated with increased androgen receptor expression; activation of androgen receptors is known to confer resistance to

therapies. The expression of JARID1B in breast cancer cells is associated with increased proliferation due to specific repression of an anti-oncogene such as BRCA1 and members of the let-7 family of microRNA tumor suppressors (Mitra et al., 2011). We have shown that isolated JARID1B+ melanoma cells can give rise to a rapidly proliferating progeny that is again heterogeneous (JARID1B+ and JARID1B−) like the parental tumor cells (Roesch et al., 2010). Additionally, stable knockdown of JARID1B led to an initial acceleration of tumor growth followed by exhaustion, as determined by serial xeno-transplantation experiments in NSG null mice, suggesting that JARID1B has an essential role in continuous melanoma growth (Roesch et al., 2010). Notably, Settleman's group has recently reported that JARID1A, a close homolog of JARID1B, is required for drug resistance in non–small cell lung cancer cells (Sharma et al., 2010), suggesting that slow cycling cells can survive most conventional and targeted therapies and that this subpopulation needs to be selectively targeted.

6. NEW APPROACHES TO THERAPY

Melanomas are heterogeneous tumors, comprised of many genotypic and phenotypic subtypes. Given the complexity of the tumor cells, earlier therapeutic approaches designed to treat melanomas as a single disease using chemotherapeutic agents, such as DTIC or temozolomide, resulted in dismal response rates of <15%. Moreover, the majority of patients developed resistance to most available therapies very early during treatment. In the last decade, the identification of mutations in the genes involved in MAPK activation, including BRAF and NRAS, or alterations or mutations in cell-cycle regulatory genes/proteins such as CCND1/CDK4 or C-KIT, has led to the development of targeted therapy approaches using small-molecule inhibitors that are either approved (e.g., vemurafenib) or in late-stage clinical trials (e.g., the BRAF inhibitor dabrafenib, the MEK inhibitor trametenib) (Flaherty, Hodi, et al., 2010; Vidwans et al., 2011). BRAF-V600E^{+} melanoma patients treated with vemurafenib experienced dramatic tumor regression and improved survival compared to patients treated with conventional therapies (Flaherty, Puzanov, et al., 2010). Despite the impressive regression of bulky tumor lesions in patients treated with BRAF inhibitors, many of them eventually developed resistance to treatment (Vidwans et al., 2011). Resistance to targeted agents can be mediated by diverse mechanisms, including development of secondary mutations,

epigenetic changes in the target gene, and activation of compensatory signaling pathways that result in increased tumor survival (Vidwans et al., 2011; Villanueva et al., 2011; Villanueva et al., 2010). Several biological and chemical inhibitors are available to target multiple pathways that support proliferation and cell survival (Vidwans et al., 2011; Villanueva et al., 2011). For example, MEK inhibitors, which can block reactivation of the MAPK pathway, are in advanced stages of clinical investigation as single agents or in combination with BRAF inhibitors. RTK inhibitors or inhibitors of the PI3 K pathway could also be used to block compensatory survival mechanisms that usually become activated in drug-resistant tumors. A multimodal therapy approach that combines targeting multiple pathways that promote maintenance of the bulk of the tumor with targeting melanoma subpopulations with a panel of antibodies or inhibitors may be necessary to prolonged disease-free survival of melanoma patients (see Fig. 11.3). For this

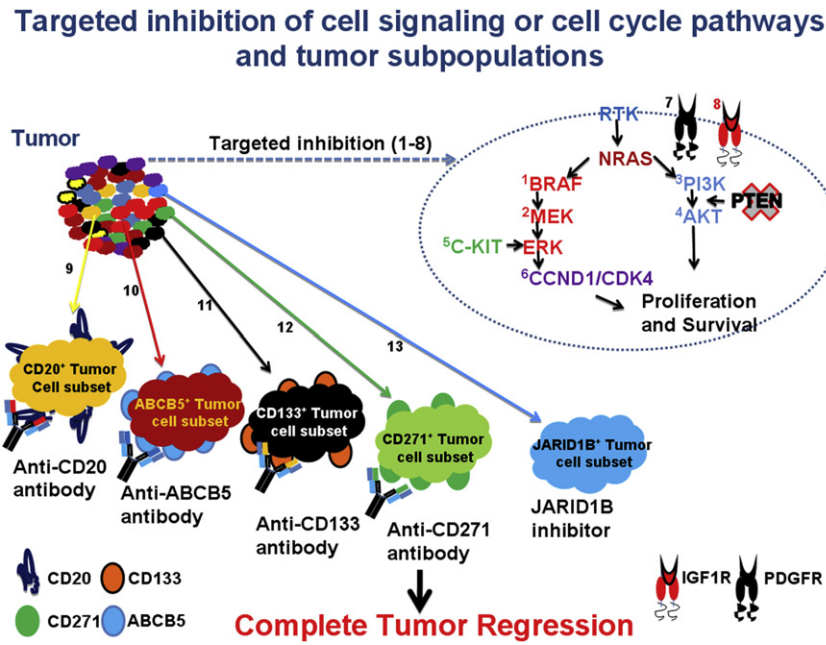

Figure 11.3 ***Potential new therapeutic approaches to target melanoma.*** A heterogeneous tumor such as melanoma will require multitargeted inhibition of signaling pathways (e.g., BRAF) or cell-cycle regulatory proteins (e.g., CDK inhibitors) (1–8) and depletion of minor subpopulations (e.g., CD20) that sustain the tumor using a combination of antibodies or inhibitors (9–13). This strategy will help prevent tumor recurrence and thus obtain long-lasting responses. For color version of this figure, the reader is referred to the online version of this book.

approach, each melanoma patient's tumor needs to be profiled before and after therapy to determine the best combination therapy approach to target each individual tumor. Drug-resistant tumor subpopulations that are frequently selected after therapy need to be analyzed for epigenetic and phenotypic changes in order to design a personalized targeted approach. Potentially, antibodies or drugs that neutralize IGF-1, PDGF, or other tyrosine kinase receptors could be used to target drug-resistant subpopulations that are known to have enhanced IGF1or PDFGF receptor signaling (Villanueva et al., 2011; Villanueva et al., 2010). An alternative approach is to use antibodies such as anti-CD20 or anti-CD133 or anti-CD271 or anti-ABCB5 to deplete respective minor drug-resistant subpopulations. JARID1B+ subpopulation can be depleted by use of inhibitors. This strategy will help prevent tumor recurrence and thus obtain long-lasting responses. Additionally, a marked increase in $CD8^+$ T-cell responses in regressing tumors after vemurafenib treatment (Wilmott et al., 2012) supports the recent strategy of combined use of immune checkpoint reagents such as anti-CTLA4 or anti-PD1 antibodies with vemurafenib. Preliminary results from these combination approaches, barring some skin sensitivity issues (Harding et al., 2012), are encouraging but it is still too early to know if this treatment modality will improve the overall survival of melanoma patients.

7. FUTURE DIRECTIONS

The recent development of advanced molecular techniques and their application to classify tumor subtypes based on gene signatures and protein expression profiles has revolutionized cancer treatment approaches. As described above, combination therapies targeting multiple signaling and cell-cycle pathways may be a useful approach to treat melanoma patients. This approach combined with immune checkpoint reagents using anti-CTLA4 and anti-PD1 antibodies will extend the expansion and retention of circulating anti-melanoma reactive cytotoxic T cells that are observed after targeted therapy. The presence of anti-melanoma reactive T cells could prevent recurrence of lesions after therapy withdrawal. Several recent reports suggest that primary or early stage lesions may have the genetic footprint for invasive potential of the neoplastic disease (Albini et al., 2008; Chin, et al., 2006; Ramaswamy et al., 2003). The metastatic potential of these tumors is supported by the stromal-derived cells such as macrophages, fibroblasts, or

other leukocytes. In this context, a combination approach targeting the tumor stromal–derived cells and the tumor may be beneficial, providing long-lasting responses and tumor regression.

8. CONCLUSION

Malignant melanoma, like other cancers, is a heterogeneous tumor comprised of many subpopulations with unique genotypic and phenotypic signatures. Single-agent therapies such as DTIC or temozolomide resulted in low (<15%) response rates that were frequently followed by drug resistance. Molecular identification of mutant $BRAF^{V600E}$ and other gene mutations has led to the development of a number of targeted therapy drugs that have shown dramatic response rates in patients. Unfortunately, the responses to targeted therapy drugs are also transient and many patients develop resistance. A personalized therapy approach of treating patients based on the genotype and phenotype of their tumors with a combination of targeted therapy drugs that inhibit multiple signaling and cell-cycle pathways will be necessary for long-lasting regression of melanoma lesions. Additionally, targeting tumor subpopulations that are generally drug resistant will be beneficial in preventing melanoma recurrence. Inclusion of immune checkpoint reagents such as anti-CTLA4 or anti-PD1 antibodies with targeted therapy drugs in the treatment regimen may provide additional benefits by expansion and retention of anti-melanoma reactive T cells that have a potential to prevent the emergence of drug-resistant tumor cells.

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ABBREVIATIONS

ACS American Cancer Society
ATP adenosine-5′-triphosphate
BCL B-cell lymphoma
BER base excision repair
CAR chimeric-antigen receptor
CCDND1 cyclin D1

CDK cyclin-dependent kinase
CDKN2a cyclin-dependent kinase inhibitor
CTLA4 cytotoxic T-lymphocyte antigen 4
FDA Food and Drug Administration
HHR homologous recombinational repair
IGF insulin-dependent growth factor
IFN interferon
IL interleukin
INPP4b inositol polyphosphate 4-phosphatase type II
MAPK mitogen-activated protein kinase
MCL myeloid cell leukemia
MDR multiple drug resistance
MGMT methylguanine-DNA methyltransferase
MITF microphthalmia-associated transcription factor
MMR mismatch repair
NER nucleotide excision repair
NES nestin
NHEJ nonhomologous end joining
NOD nonobese diabetic
NSG NOD SCID IL2 receptor gamma chain knockout
PARP polyadenosine diphosphate-ribose polymerase
PD programmed cell death
PDGF platelet-derived growth factor
PI phosphoinositide
PTEN phosphatase and tensin
RGP radial growth phase
RNI reactive nitrogen intermediate
ROS reactive oxygen species
RTK receptor tyrosine kinase
SCID severe combined immunodeficiency mice
TKi tyrosine kinase inhibitors
TME tumor microenvironment
VGP vertical growth phase
WT wild-type

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CHAPTER TWELVE

Chemoprevention of Melanoma

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Abstract

Despite advances in drug discovery programs and molecular approaches for identifying drug targets, incidence and mortality rates due to melanoma continue to rise at an alarming rate. Existing preventive strategies generally involve mole screening followed by surgical removal of the benign nevi and abnormal moles. However, due to lack of effective programs for screening and disease recurrence after surgical resection, there is a need for better chemopreventive agents. Although sunscreens have been used extensively for protecting from UV-induced melanomas, results of correlative population–based studies are controversial, with certain studies suggest increased skin cancer risk in sunscreen users. Therefore, these studies require further authentication to conclusively confirm the chemoprotective efficacy of sunscreens. This chapter reviews the current understanding regarding melanoma chemoprevention and the various strategies used to accomplish this objective.

1. INTRODUCTION

Chemoprevention is a strategy that was first proposed by Sporn et al., (1976). It was referred to the use of natural or synthetic agents to reverse, suppress, or prevent molecular or histologic premalignant lesions from progressing to invasive cancer (Sporn et al., 1976). The original definition also included treating patients who had undergone successful primary cancer treatment but were at increased risk of developing a second primary lesion (Sporn et al., 1976; Sporn et al., 1976). Cancer delay has been emphasized as yet another goal of chemoprevention (Lippman & Hong, 2002a,b). Chemopreventive agents that delay the onset of melanoma are extremely

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important as even small changes in the early melanocytic lesion size can significantly alter the 5-year survival rate (Balch et al., 2001; Lao et al., 2006). For example, a change in the Breslow's depth of 4 mm compared to 0.7 mm could decrease the 5-year survival rate by 40% (Balch et al., 2001; Lao et al., 2006). In breast and other cancers, chemoprevention has proven successful (Jordan, 2007). Tamoxifen, the first Food and Drug Administration (FDA)-approved chemopreventive agent, has been used effectively to reduce breast cancers (Freedman et al., 2003) (http://www.fda.gov/NewsEvents/Testimony/ucm115118.htm) (April 12, 2012). Similarly, the FDA-approved topical diclofenac and imiquimod were proven effective for actinic keratoses treatment (Weinberg, 2006).

Chemoprevention of melanoma is based on the principle that melanoma is a progressive disease, and various molecular events and pathways associated with different stages of the disease can be targeted using synthetic or naturally occurring chemical compounds (Demierre & Nathanson, 2003). However, chemoprevention of melanoma remains an underdeveloped area. One of the reasons for this under-exploration is the logistical and procedural difficulties associated with testing of chemopreventive agents in clinical trials. Even though ~30% melanomas are linked to exposure to UV radiation, risk factors responsible for about 60% melanomas are unknown (Husain et al., 1991; Madhunapantula & Robertson, 2011; Pathak, 1991; Robertson, 2005). Furthermore, the molecular basis for UV-mediated transformation of melanocytes to melanomas is also not fully understood (Abdel-Malek et al., 2010; Lund & Timmins; 2007; Quinn, 1997). Moreover, results of recent trials evaluating whether limiting or blocking sun exposure to reduce melanoma incidence and mortality rates are confusing and not encouraging (Barton, 2011; Goldenhersh & Koslowsky, 2011; Loden et al., 2011; Planta, 2011). Therefore, chemoprevention of melanoma remains a challenge to the scientific community. Recent studies have focused on identifying the molecular pathways triggering the transformation of melanocytes to melanomas when exposed to UV light, as well as genetic and nongenetic risk factors that could be targeted for chemoprevention (Afaq et al., 2005; Bennett, 2008a,b; Demierre & Nathanson, 2003; Walker, 2008; Wang et al., 2010). For example, Ras-signaling can be used as a chemoprevention target in UV-induced melanomas (Demierre & Merlino, 2004; Lluria-Prevatt et al., 2002). In addition, analysis of mutational data from the reported literature demonstrated high abundance of UVB signature mutations in CDKN2A, TP53, and PTEN loci in cutaneous melanomas compared to nonskin cancers (Hocker & Tsao, 2007).

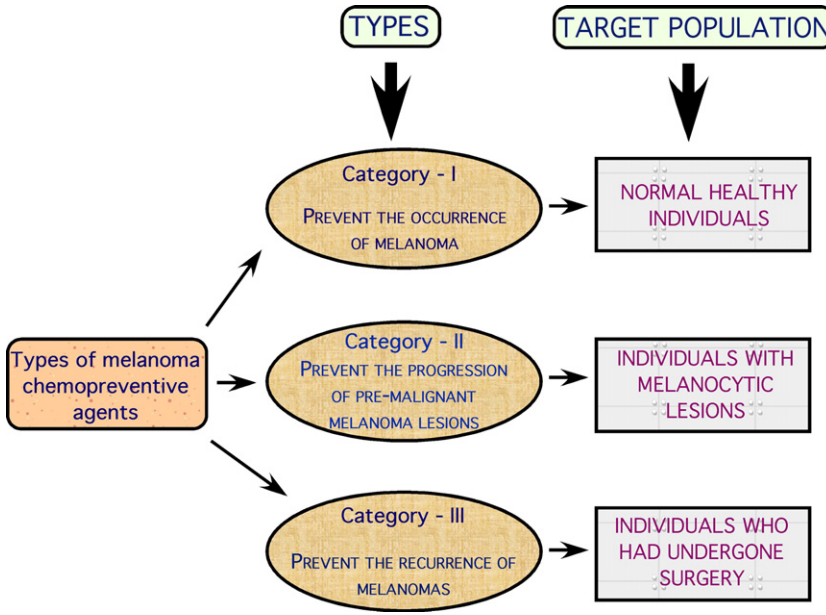

Figure 12.1 ***Types of chemopreventive agents used for preventing melanomas.*** Chemopreventive agents have been classified based on whether they prevent the occurrence of melanomas in normal healthy individuals (Category–I), or prevent the progression of already existing melanomas (Category–II), or inhibit the recurrence of melanomas after a surgical treatment (Category–III). For color version of this figure, the reader is referred to the online version of this book.

Broadly, three categories of melanoma chemopreventive agents exist (Lao et al., 2006; Manoharan & Balakrishnan, 2009) (Fig. 12.1). The first category prevents the occurrence of melanoma in healthy individuals, whereas, the second and third categories prevent the development in melanoma patients (Lao et al., 2006; Manoharan & Balakrishnan, 2009) (Fig. 12.1). Secondary chemopreventive agents would prevent premalignant lesions from developing into malignant melanomas (Lao et al., 2006; Manoharan & Balakrishnan, 2009) (Fig. 12.1). Tertiary chemopreventive agents would prevent melanoma recurrence after getting treated for melanomas (Lao et al., 2006; Manoharan & Balakrishnan, 2009) (Fig. 12.1).

An ideal chemopreventive agent should inhibit (a) oncogenic kinases inducing the transformation of melanocytes and (b) trigger apoptosis in damaged melanocytes (Demierre & Nathanson, 2003; Gupta & Mukhtar, 2001). In addition, chemopreventive agents should also induce DNA repair pathways so that UV-induced damage could be alleviated thereby preventing transformation (Nambiar et al., 2011; Nichols & Katiyar, 2010;

Rajendran et al., 2011). Therefore, chemoprevention strategies should consider the following key aspects while developing a particular compound for preventing melanomas: (a) molecular basis of melanoma genesis and tumor progression; (b) reasons for the failure of existing agents; (c) selection of appropriate *in vitro* and *in vivo* models representing different stages of tumor progression for testing the identified agents; and (d) better methods of drug delivery to reduce toxicity and release of the preventive agent at the site of action (Demierre & Sondak, 2005a,b).

2. MELANOMA MODELS FOR STUDYING THE EFFICACY OF CHEMOPREVENTIVE AGENTS

There is an urgent need to develop models for studying the efficacy of chemopreventive agents for melanoma. Since, not much information is available about the molecular or histological markers of the carcinogenic processes to be used as endpoints and prognostic as well as drug efficacy predictive indicators, development of potent chemopreventive agents for inhibiting melanomas has been hampered (Armstrong et al., 2003). Furthermore, testing the efficacy of existing agents in prevention studies in humans requires long periods and involves ethical, financial, as well as experimental difficulties (Demierre & Sondak, 2005a,b; Ming, 2011). Therefore, there is an urgent need to develop clinical research models to evaluate candidate chemopreventive agents for inhibiting melanoma development. The cell culture and animal models that are in wide usage for assessing chemoprevention include (a) laboratory-generated skin reconstructs with and without melanoma tumor nodules (Chung et al., 2011; Nguyen et al., 2011; Satyamoorthy et al., 1999); (b) use of human skins to test the drug permeability and safety; (c) xenografted melanoma tumor models combining topical or oral administration of chemopreventive agents (Chung et al., 2011; Nguyen et al., 2011; Satyamoorthy et al., 1999); and (d) use of spontaneous melanoma models (Becker et al., 2010; Dankort et al., 2009) (Fig. 12.2). Other models that have been developed to test chemopreventive agents include (a) transgenic hepatocyte growth factor (HGF)-scatter factor (SF) mouse models (Noonan et al., 2003); (b) transgenic mouse SV40 T antigen (Mintz & Silvers, 1993); (c) spontaneous and UV-induced xiphophorus fish model where melanoma progression from nevus to melanoma can be studied (Ha et al., 2005; Walter & Kazianis, 2001) (Fig. 12.2). Appropriate models also have to assess the suitability of

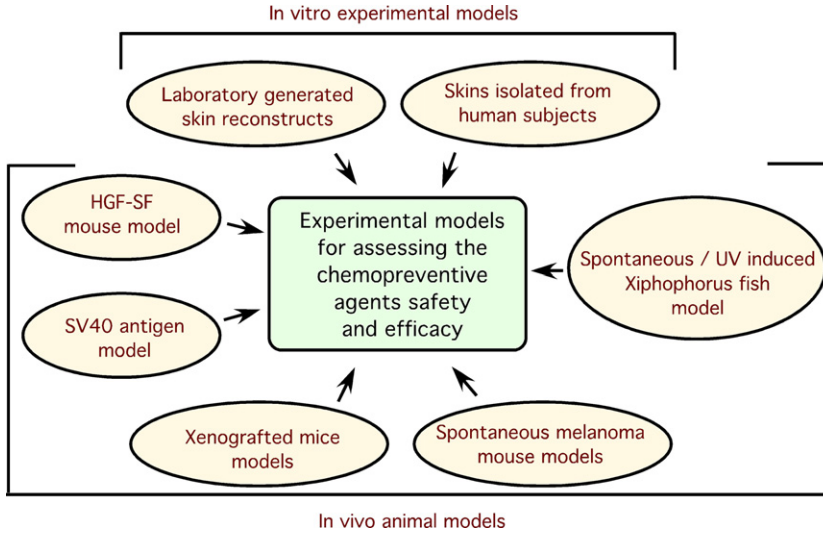

Figure 12.2 In vitro *and* in vivo *models for testing the efficacy of melanoma preventing agents.* Several *in vitro* and *in vivo* models have been developed and tested for their suitability to evaluate the safety and efficacy of a particular chemopreventive agent. Although *in vitro* skin reconstruct model is a good representative of human skins it is not an exact replica of *in vivo* situation, hence several *in vivo* models are also used for chemopreventive agents efficacy and safety testing. Both xenografted and spontaneous mouse models have been utilized by many research laboratories for chemopreventive agents' application. Additional models include transgenic mouse models and fish models. For color version of this figure, the reader is referred to the online version of this book.

administration of the particular agents, which can be a challenge for certain of these models.

3. CHEMOPREVENTIVE AGENTS THAT HAVE BEEN TESTED FOR PREVENTING MELANOMAS

3.1. Statins

Results of recent preclinical as well as Phase-I and Phase-II clinical trials and unanticipated secondary clinical observations from cardiovascular disease trials have led to enthusiasm regarding the use of statins for melanoma prevention (Bonovas et al., 2010; Curiel-Lewandrowski et al., 2011; Demierre et al., 2005; Hippisley-Cox & Coupland, 2010). Statins are antiproliferative, proapoptotic, angiostatic, anti-invasive, and immunomodulatory compounds known to inhibit Ras proteins (Demierre et al.,

2005). Mechanistically, statins inhibit key steps in the mevalonate pathway to decrease protein prenylation (Demierre et al., 2005; Khosravi-Far et al., 1992). Lack of this posttranslational modification of Ras and many other proteins impedes function, resulting in the prevention of melanoma cell proliferation and oncogenicity (Demierre et al., 2005; Khosravi-Far et al., 1992). A recent study showed inhibition of geranylgeranylation of RhoC and other small G-proteins by atorvastatin, which reverted the metastatic phenotype in human melanomas expressing this protein (Collisson et al., 2003) (Fig. 12.3).

While preclinical findings support the chemopreventive ability of statins for melanoma prevention, epidemiological data are yet to confirm this observation (Bonovas et al., 2010; Feleszko et al., 2002; Kidera et al., 2010; Lao et al., 2006). A meta-analysis of randomized controlled trials of statins in cardiovascular disease found no statistically significant differences between statin and control groups with respect to melanoma incidence (Bonovas et al., 2010; Bonovas et al., 2006). Despite these observations, the usage of statins for melanoma chemoprevention continues, as the safety profile of

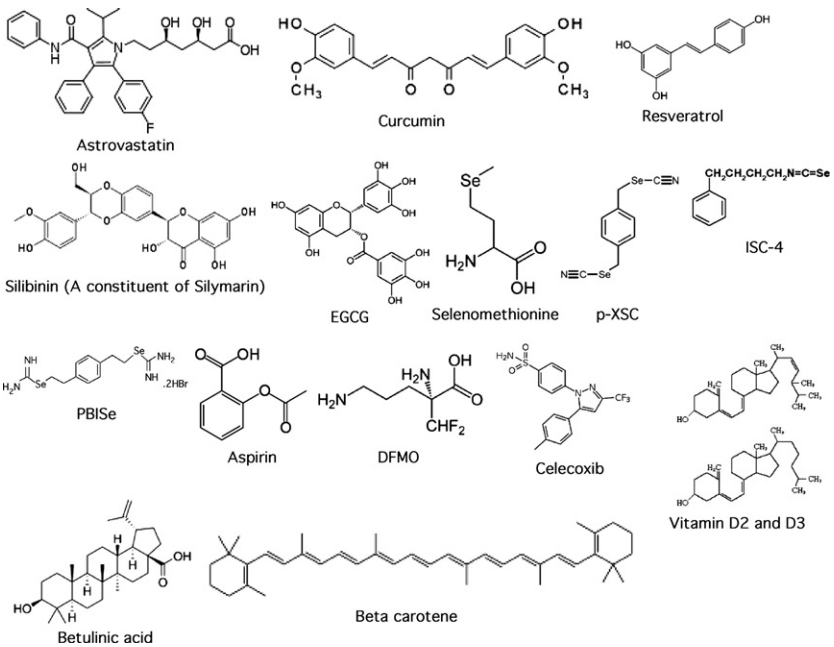

Figure 12.3 ***Structures of reported chemopreventive agents tested for melanoma chemoprevention.*** Structures of chemopreventive agents that have been tested using *in vitro* and *in vivo* models for preventing melanoma.

these compounds is very good (Demierre, 2005; Demierre et al., 2005). Moreover, some of the published meta-analysis reports failed to include the results of recent clinical trials, which showed positive association between statins' use and melanoma prevention (Bonovas et al., 2010; Kuoppala et al., 2008). Therefore, use of statins for preventing melanoma needs further evaluation in large multicentric trials. The Southwest Oncology Group (SWOG) has proposed a phase IIB chemoprevention study of statins versus placebo in a population of patients who have been treated for early-stage melanomas or the presence of clinically atypical nevi (Demierre & Sondak, 2005). This phase IIB trial will involve dermatologists and medical and surgical oncologists that will undertake prospective evaluation of biological markers in both blood and biopsied nevi (Demierre & Sondak, 2005a). Results of this clinical trial are expected to determine whether statins have a role in melanoma prevention.

3.2. Curcumins

Curcumin or diferuloylmethane, chemically known as 1,7-bis-[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, is a commonly used spice derived from *Curcuma longa* (turmeric) (Gupta et al., 2011; Kim et al., 2011) (Fig. 12.3). Although, the clinical efficacy of this yellow pigment is yet to be confirmed, *in vitro* observations using cultured cells and *in vivo* studies in xenografted and carcinogen-induced animal models suggested it may perform a chemopreventive role in melanomas (Baliga and Katiyar, 2006; Chen et al., 2011; Limtrakul et al., 2001; Limtrakul et al., 1997; Siwak et al., 2005). Curcumin and its derivatives seem to act by inhibiting key enzymes involved in melanoma tumor development (Limtrakul et al., 1997; Mimeault & Batra, 2011). For example, curcumin inhibits the xanthine oxidase, tyrosine kinase, cyclooxygenase (COX), and lipoxygenase (LOX) enzymes thereby exerting antioxidant effects (Gupta et al., 2011; Kim et al., 2011). Curcumin also inhibited cell survival by targeting NF-kB and XIAP in melanoma cells but not in melanocytes (Bush et al., 2001; Gupta et al., 2011; Kim et al., 2011; Marin et al., 2007). Topical application of curcumin inhibited UVB-induced NF-kB activation in cultured keratinocytes and TPA-induced tumor formation in mice (Huang et al., 1997; Kakar & Roy, 1994). Furthermore, curcumin treatment reduced lung metastasis of B16F-10 melanoma cells and increased animal life span (Menon et al., 1995; Ray et al., 2003). Mechanistically, curcumin treatment inhibited matrix metalloproteinases (MMP) to reduce melanoma cell invasion and metastasis (Banerji et al., 2004).

Curcumin protects from the UVA- and UVB-induced skin damage by triggering DNA repair mechanisms (Heng, 2010). Although UVB is primarily responsible for skin cancer initiation and progression, recent studies have also found the involvement of UVA in melanoma and non-melanoma skin cancer development (Autier et al., 2011). Despite its anti-cancer activity as well as safety and tolerability profiles, not many clinical trials have been conducted to study the efficacy of curcumin for inhibiting melanomas (Anand et al., 2008; Sa & Das, 2008). Some of the practical concerns associated with curcumin use for melanoma prevention include (a) its nature to stain; (b) poor absorption; and (c) rapid metabolism which limits its bioavailability (Anand, Sundaram et al., 2008). However, various semi-synthetic derivatives and nanoformulations have been developed that overcome these limitations (Anand, Thomas et al., 2008). A recent study also demonstrated augmentation of tumoricidal properties of curcumin when coupled with a cancer cell–specific antibody (Langone et al., 2011). This study coupled curcumin to a melanoma surface antigen recognizing Muc18 antibody, through a cleavable arm, for preventing B16F-10 melanoma tumor growth in mice (Langone et al., 2011). The Curcumin-Muc18 antibody complex was found ~230 fold more effective at inhibiting melanoma cell metastasis in mice than the unconjugated control (Langone et al., 2011). Although these preclinical trials are encouraging, clinical evaluation has not yet been undertaken.

3.3. Resveratrol

Chemically known as 3,5,4′-trihydroxy-trans-stilbene, resveratrol is a polyphenolic phytoalexin isolated from grapes, mulberries, and peanuts (Niles et al., 2003) (Fig. 12.3). Resveratrol is a good antioxidant (Aggarwal et al., 2004). Due to its anti-inflammatory and antiproliferative properties, resveratrol effectively inhibits initiation, progression, and metastasis of several cancers including those of the breast, prostate, and skin (Aggarwal et al., 2004). For example, topically applied resveratrol protects skin from UV-induced tumor growth by inhibiting COX-2 as well as the mTORC2 component rictor and hydrogen peroxide formation (Back et al., 2012; Bhat & Pezzuto, 2002). Resveratrol can also protect cells by preventing radiation-induced DNA damage (Aziz et al., 2005; Aziz et al., 2005). Studies have shown that this natural product scavenges free radicals and inhibits the activation of polyhydroxy aromatic hydrocarbon carcinogens (Aziz, Reagen-Shaw et al., 2005; Calamini et al., 2010; Holthoff et al., 2010).

Many *in vitro* and *in vivo* studies using animal models show that resveratrol (a) arrests cells in the G0/G1 phase of the cell cycle; (b) inhibits PI3K-Akt signaling; (c) downregulates NF-kB activation by blocking IKK; and (d) upregulates Egr-1, a known inhibitor of Cdk2 (Aggarwal et al., 2004; Calamini et al., 2010). In addition, resveratrol can also inhibit survivin, TGF-beta signaling, and sensitize cells to TRAIL (Aziz, Afaq et al., 2005; Kim, Back et al., 2011). Collectively, these combined effects mediated by resveratrol trigger apoptosis and inhibit cell proliferation in various tumor types.

Adding to its pluripotent anticancer effects, resveratrol has a good pharmacokinetic profile in animals leading to high absorption rates in the gut (Patel et al., 2011; Walle, 2011). Furthermore, its solubility makes it a suitable candidate for evaluation in clinical trials (Patel et al., 2011). The metabolites of resveratrol also retain the original chemopreventive activity, a key factor one should consider when using this agent in chemoprevention trials (Miksits et al., 2009). A Phase-1 interventional, open-label prevention trial has studied the side effects of oral resveratrol administration over 4 weeks to establish the mechanism through which it prevents cancer http://clinicaltrials.gov/ct2/show/NCT00098969 (April 12, 2012). This study measured the drug and carcinogen metabolizing enzymes, primarily cytochrome P450, in blood and urine collected from study participants who have taken resveratrol.

Although *in vitro* studies using resveratrol show potent anti-melanoma activity, an *in vivo* study found that resveratrol is rapidly metabolized in athymic nude mice and does not inhibit human melanoma xenograft growth (Niles et al., 2006). Administration of 110 or 263 μM resveratrol in the diet prior to subcutaneous injection of tumor cells had no tumor inhibitory effect; instead, mice treated with the highest resveratrol concentration had bigger tumors compared to control diet fed animals (Niles et al., 2006). Authors of this study hypothesized that rapid clearance as well as transformation of resveratrol when given in the diet might be responsible for this tumor-promoting effect (Niles et al., 2006). Further experiments designed to circumvent the rapid clearance of resveratrol when administered through oral gavage or in the diet have also failed to inhibit tumor growth, indicating that resveratrol on its own is not an effective chemopreventive agent for inhibiting melanoma development (Niles et al., 2006).

Derivatives of resveratrol with greater stability and efficacy have been created by chemical modifications and tested on cultured cells as well as in mouse models. Results of these studies identified that hydroxylated analogs

of resveratrol are more potent than resveratrol (Szekeres et al., 2010; Szekeres et al., 2011). For example, hexahydroxystilbene (M8) effectively inhibited COX-2 activity to inhibit the growth of various tumor cell lines at very low concentrations (Paulitschke et al., 2010; Szekeres et al., 2010). *In vivo*, intraperitoneal administration of M8 at 2.5 or 5 mg/kg/day for 4 weeks alone as well as in combination with 80 mg/kg DTIC (on days 4 and 6) inhibited the growth of palpable melanoma tumors in xenografted mice models (Paulitschke et al., 2010; Szekeres et al., 2011). In addition, M8 could also inhibit melanoma tumor metastasis as evidenced by decreased tumor development in the lymph nodes (Paulitschke et al., 2010; Szekeres et al., 2011). Although these preliminary findings are encouraging, further studies evaluating M8 in Phase-I and Phase-II trials in humans are needed. It is also unknown whether M8 could be combined with FDA-approved ^{V600E}B-Raf inhibitor Vemurafenib, and Akt inhibitor MK-2206 to cooperatively or synergistically inhibit melanoma development. Therefore, future studies should try these combinations for preventing melanomas.

3.4. Silymarin

Silymarin, a polyphenolic flavonoid isolated from *Silybum marianum* (milk thistle), is a potent antioxidant and anti-inflammatory agent (Afaq & Katiyar, 2011; Katiyar et al., 2011) (Fig. 12.3). Silymarin is a mixture of four isomeric compounds namely silybinin, silychristin, silydianin, and isosilybinin (Afaq & Katiyar, 2011; Katiyar et al., 2011). Studies using cultured cells and animal models demonstrated its chemopreventive ability against nonmelanoma skin cancers induced by chemical carcinogens and UV radiation (Li et al., 2004). Mechanistically, silymarin inhibits NF-kB, c-Jun N-terminal kinase, and COX-2 activities (Vaid & Katiyar, 2010). In addition, it also suppresses the production of reactive oxygen species thereby preventing DNA damage. Silymarin also inhibits cell proliferation by inducing a G0/G1 block, and suppresses invasion by inactivating PI3K-Akt as well as MAPK pathways (Li et al., 2006; Vaid & Katiyar, 2010). Current studies have demonstrated that silymarin inhibits melanoma cell migration by reducing MMP-2 as well as MMP-9 protein levels (Vaid et al., 2011). Further studies identifying the mechanistic basis of UV-induced melanoma chemoprevention showed that silymarin inhibits immunosuppressive IL-10 production in the skin as well as in draining lymph nodes (Katiyar, 2005). In addition, silymarin also acts on the immune system stimulating IL-12 to increase its levels, thereby protecting cells from UV-induced damage (Meeran et al., 2006). For example,

topical application of silymarin prevented UV-induced immune suppression only in wild-type mice but not in IL-12 knockout mice (Meeran et al., 2006).

3.5. Epigallocatechin-3-gallate

Epigallocatechin-3-gallate (EGCG), a major constituent of green tea, has been shown to protect from UV-induced skin cancers by inhibiting DNA damage and oxidative stress (Barthelman et al., 1998; Katiyar et al., 2007; Mittal et al., 2003) (Fig. 12.3). Experimentally, topical application of EGCG inhibited the reduction of antioxidants such as glutathione peroxidase and catalase in the epidermis, thereby protecting cells from oxidative stress (Katiyar et al., 2007; Mittal et al., 2003; Nihal et al., 2005). Studies have demonstrated that topical application or oral administration of EGCG reduced cutaneous edema and erythema and also decreased tumor incidence, multiplicity, and size (Lu et al., 2002; Mittal et al., 2003).

In cultured cells, treatment of metastatic A375M and Hs-294T melanoma cells with EGCG inhibited oncogenic BCL2 and upregulated Bax as well as caspase-3, 7, and 9 expression in a dose-dependent manner (Nihal et al., 2005). In addition, EGCG also reduced the expression of the proliferation regulator cyclin-D1 and induced cell cycle inhibitors p16, p21, and p27 (Nihal et al., 2005). Furthermore, in murine models of melanoma, EGCG reduced cell migration, induced apoptosis and cell cycle arrest thereby inhibiting melanoma tumor growth and the metastatic potential of the cells (Taniguchi et al., 1992). Additional studies showed effective anti-angiogenic properties of EGCG, as this compound reduced the production of VEGF (Liu et al., 2001; Konta et al., 2011). Since EGCG is less expensive and has negligible toxicity, it is an attractive candidate chemopreventive agent; however, no clinical trials using EGCG have been reported, warranting further study.

3.6. Selenium-Containing Agents for Preventing Melanoma

Anticancer activity of selenium has been suggested for preventing cancers of prostate, breast, and lung (Brozmanova et al., 2010). Many *in vitro* and *in vivo* studies also tested various selenium-containing compounds for inhibiting proliferation and inducing apoptosis as well as cell cycle arrest (Chung et al., 2011; Nguyen et al., 2011). However, a multicenter, double-blind, randomized, placebo-controlled trial of 1312 patients (mean age 63 years) with a history of basal cell carcinoma (BCC) or squamous cell carcinoma

(SCC) and a mean follow-up of 6.4 years showed that 200 μg of selenium in the form of brewer's yeast tablets did not have a statistically significant effect on BCC or SCC development (Clark et al., 1996). But, results from secondary end-point analyses showed that supplemental selenium might reduce the incidence (77 cancers in the selenium group versus 119 in controls) and mortality rates from carcinomas (29 deaths in the selenium treatment group versus 57 deaths in controls) (Clark et al., 1996). However, authors of this study stated that these results need further confirmation in additional clinical trials.

Selenomethionine was tested for its efficacy in a large multicenter chemoprevention trial, known as SELECT (selenium and vitamin E cancer-prevention trial), for preventing prostate cancer (Allen et al., 2008; Duffield-Lillico et al., 2004; Lippman et al., 2009) (Fig. 12.3). Results of SELECT raised further concerns regarding the clinical utility of selenium chemoprevention (Allen et al., 2008; Duffield-Lillico et al., 2004; Lippman et al., 2009). SELECT was the largest clinical trial ever conducted for prostate cancer prevention (Allen et al., 2008; Duffield-Lillico et al., 2004; Lippman et al., 2009). The trial, sponsored by NCI ($114 million) and NCCAM ($4.5 million) from 1999 to 2008, was initiated based on the results of the NPC trial showing 52–60% fewer new cases of prostate cancer following selenized yeast treatment compared to placebo (Clark et al., 1996; Duffield-Lillico et al., 2002). SELECT was a double-blinded, placebo-controlled study examining the role of nutritional supplementation of selenomethionine and/or vitamin E for preventing prostate cancer (Lippman et al., 2009). Based on the experts' opinion and available compelling evidence showing the efficacy of selenium-containing yeast in preclinical data, the SELECT study group decided to use 200 μg selenomethionine for the trial. Participant men between 50 and 55 years of age with no history of prostate cancer, and in good health, took pills constituting one of four possible combinations: two placebos; 200 μg selenomethionine and a placebo; vitamin E and a placebo or selenomethionine and vitamin E daily for 7–12 years with follow-up visits every 6 months. It was predicted to decrease prostate cancer by $\geq$25% (Lippman et al., 2009). Whereas selenized yeast reduced the incidence of prostate cancer in the NPC trial, the same trend was not observed in SELECT using selenomethionine. On September 15, 2008, the data and safety monitoring committee announced that all SELECT participants must discontinue supplements because, although statistically insignificant, more prostate cancer cases occurred in men taking only vitamin E and an increase in diabetes was noticed in the selenium groups.

Despite these negative results for inhibiting BCC and SCC with selenized yeast, use of selenium for preventing human skin cancers continues to be investigated. Possible reasons for considering selenium for melanoma prevention include (a) very low selenium levels noticed in the melanoma patient's serum; (b) an inverse correlation between the selenium concentration and melanoma incidence rates in population-based studies; (c) encouraging *in vitro* as well as *in vivo* studies demonstrating the efficacy of selenium for preventing melanomas; (d) feasibility of substituting selenium for sulfur for improving the efficacy of various chemopreventive agents; and (e) availability of a wide variety of selenium-containing compounds for better agent selection (Dennert et al., 2011, 2012). In fact, several selenium-containing agents have been prepared and tested *in vitro* as well as *in vivo* for safety and efficacy (Madhunapantula et al., 2008; Sharma et al., 2009). Results of these studies will be discussed in the following sections.

Isolated soy proteins (ISP) generated from high-Se as well as low-Se containing soybeans have been tested for efficacy to inhibit pulmonary metastasis of mouse melanoma cells (Li et al., 2004). Analysis of experimental data revealed an inverse correlation between selenium content in the mice and metastasis development (Li, Graef et al., 2004). Experimentally, ISP differing in selenium content has been given to mice 2 weeks before and after administration of B16BL-6 mouse melanoma cells and metastasis development in lungs quantified (Li, Graef et al., 2004). The results showed a significant decrease in tumor number and tumor size in the 10% high-Se ISP diet that contained 3.6 μg/g Se compared to 10% low-Se ISP diet having 0.13 μg/g Se (Li, Graef et al., 2004). Furthermore, addition of selenomethionine to the 10% low-Se ISP diet to levels equivalent to 10% high-Se ISP diet inhibited metastasis development similar to 10% high-Se ISP diet indicating that the active ingredient responsible for metastasis development inhibition in the high-Se ISP could be selenomethionine (Li, Graef et al., 2004).

Several other studies also have shown the ability of selenomethionine to inhibit metastasis development in animal models (Yan et al., 1999) (Fig. 12.3). For example, diet containing selenomethionine, one of the major constituents of selenized yeast, has been shown to inhibit pulmonary metastasis in a mouse model (Yan, 1999). Experimentally, mice were given a diet containing 2.5 or 5 ppm selenium as selenomethionine (experimental group) or as selenite (control group) 2 weeks before and after the intravenous injection of B16BL-6 murine melanoma cells, and the effect on number and size of the tumors developing in lungs was measured (Yan et al.,

1999). Authors of this study found that selenium in the form of selenomethionine or selenite could reduce lung metastasis; hence, selenomethionine was concluded to be the physiologically an active form of selenium (Yan et al., 1999).

In addition to selenomethionine, another study evaluating the effect of p-XSC on lung metastasis development also found decreased metastatic tumor nodules when the mice were fed with this agent (Tanaka et al., 2000) (Fig. 12.3). Mice were fed with experimental diets containing 4, 8, and 15 mg/kg p-XSC (corresponding to 2, 4, and 7.5 mg/kg selenium) before and after inoculation of B16BL-6 cells intravenously (Tanaka et al., 2000). Compared to controls, p-XSC fed mice were found to contain low numbers of lung metastasis (Tanaka et al., 2000). Mechanistic studies found that p-XSC could induce apoptosis in melanoma cells without affecting neighboring epithelial cells thereby reducing tumor development in lungs (Tanaka et al., 2000). Further studies have demonstrated that p-XSC can also inhibit tumor angiogenesis as well as the proliferation of melanoma cells (Tanaka et al., 2000). Hence, p-XSC could be a proliferative potential candidate for clinical evaluation.

Although selenomethionine, high-Se ISP, and p-XSC treatments reduced metastasis development, efficacy against melanoma tumor development and progression were not studied. Therefore, it is unknown whether selenium could inhibit very early events in melanoma development, and, if so, could selenium be used to prevent melanocytic lesion development in its very early stages.

Recent studies have synthesized selenium containing isoselenocyanates as well as isoselenoureas by substituting sulfur of the parent isothiocyanates and *S*,*S*′-(1,4-phenylenebis[1,2-ethanediyl)bis-isothiourea (PBIT) with selenium (Desai et al., 2010; Madhunapantula et al., 2008; Nguyen et al., 2011; Sharma et al., 2008, 2009) (Fig. 12.3). Results of *in vitro* and *in vivo* studies using laboratory-generated skin reconstructs and xenografted melanoma tumor models found greater tumor inhibition with selenium-containing derivatives (Chung et al., 2011; Nguyen et al., 2011). For example, topical application of isoselenocyanate-4 (ISC-4) and *S*,*S*′-(1,4-phenylenebis[1,2-ethanediyl)bis-isoselenourea (PBISe) significantly delayed xenografted melanoma tumors' growth (Chung et al., 2011; Nguyen et al., 2011). Two weeks after topical treatment, a 50–70% decrease in tumor volume was observed (Chung et al., 2011; Nguyen et al., 2011). Furthermore, topical administration of these compounds was safe with no major differences in vital organ histology or in blood parameters indicative

of major organ functions of treated mice (Chung et al., 2011; Nguyen et al., 2011). Therefore, selenium incorporated into the backbone of existing agents might be useful for melanoma prevention. Although both ISC-4 and PBISe inhibited growth of melanocytic nevi in laboratory-generated skin reconstructs as well as in subcutaneous xenografted melanoma tumors, efficacy and safety of these agents in humans yet to be established.

The mechanism through which the selenium-containing compounds function can vary to affect efficacy. Current studies have shown that ISC-4 could inhibit carcinogen-induced DNA adducts formation as well as modulate both phase-I and phase-II enzymes to prevent lung cancer development (Crampsie et al., 2011) (Fig. 12.3). In melanomas, ISC-4 reduced Akt3 signaling activity thereby inhibited melanoma cells proliferation and induced apoptosis (Sharma et al., 2009). In a separate study, it has been demonstrated that ISC-4 activates prostate apoptosis response protein-4 (Par-4) expression thereby inhibiting prostate tumor development in mice (Sharma et al., 2011).

PBISe is another selenium-containing compound found effective at inhibiting melanomas (Chung et al., 2011; Desai et al., 2010; Madhunapantula et al., 2008) (Fig. 12.3). Compared to its sulfur-containing analog PBIT, this compound effectively inhibited cell proliferation as well as survival of melanoma cells growing in culture (Chung et al., 2011; Desai et al., 2010; Madhunapantula et al., 2008). PBISe also retarded the growth of melanocytic nevi developing in skin reconstructs (Chung et al., 2011). Furthermore, mice receiving PBISe intraperitoneally or through topical application showed very slow tumor growth compared to PBIT or vehicle controls (Chung et al., 2011; Desai et al., 2010; Madhunapantula et al., 2008). Mechanistically, PBISe inhibited iNOS and Akt3 pathways, while inducing pErk1/2 expression (Chung et al., 2011). Elevated expression and activity of iNOS as well as Akt3 have been reported in melanoma (Ekmekcioglu et al., 2006; Stahl et al., 2004). Targeted inhibition of iNOS and Akt3 had been shown to reduce cell proliferation and induce apoptosis (Stahl et al., 2004; Sikora et al., 2010). In addition, PBISe induced the phosphorylation of endogenous Erk1/2 to levels that trigger senescence, by upregulating proliferation inhibitors p27 in melanoma cells (Cheung et al., 2008). Therefore, PBISe could be a potent melanoma chemopreventive agent.

3.7. Nonsteroidal Anti-Inflammatory Drugs

Use of nonsteroidal anti-inflammatory drugs (NSAIDs) for preventing cancers has been reported by several investigators as these agents have been

found to have better safety and health beneficial effects compared to many other chemopreventive agents (Friedman et al., 2002). In addition, many *in vitro* studies using cultured cells as well as mouse models showed efficacy of NSAIDs for preventing melanomas (Jeter et al., 2011). Interestingly the primary target of NSAIDs, COX2, which is expressed at a very high level in >93% patient tumors as well as in the majority of melanoma cell lines (Denkert et al., 2001). Many studies have shown the protective effects of NSAIDs for inhibiting colorectal cancers when used for extended periods of time (>5 years) with frequent administration. Similarly, several *in vitro* and observational studies testing the long-term use of NSAIDs and statins found reduced cutaneous melanoma development (Joosse et al., 2009). However, some conflicting reports have hampered further development of NSAIDs for cutaneous melanoma prevention (Asgari et al., 2008; Bard & Kirsner, 2011; Jeter et al., 2011). For example, a large cohort study measuring the association between NSAIDs' use and melanoma risk found no association indicating that the NSAIDs may not be good candidate drugs for melanoma chemoprevention (Asgari et al., 2008). In this study, 63,809 men and women from VITAL (vitamins and lifestyle) cohort study were linked to NCI Surveillance, Epidemiology and End Results (SEER) cancer registry to determine whether NSAIDs' used in the past 10 years had any association with melanoma risk (Asgari et al., 2008). This study also suggested the possibility that use of NSAIDs had no impact on tumor invasion, thickness, and metastasis (Asgari et al., 2008). However, a recent case–control study measuring the prevalence of cutaneous melanoma among populations using lipid lowering agents and NSAIDs reported that long-term use of at least one NSAID for >5 years decreased the likelihood of developing cutaneous melanoma by half compared with those who had taken NSAID for <2 years or who had not taken these anti-inflammatory agents (Curiel-Lewandrowski et al., 2011).

Acetyl salicylic acid (ASA, also known as aspirin) has been reported to half the risk of developing cutaneous melanoma compared to nonusers or those who have used ASA for <2 years (Curiel-Lewandrowski et al., 2011) (Fig. 12.3). Likewise, long-term use of low-dose (75 mg daily) aspirin also reduced the risk of developing many other cancers (Rothwell, Wilson et al., 2012). For example, results of the analysis of five large randomized trials of daily aspirin (≥75 mg daily) versus control for the prevention of cancers and the risk of metastases at presentation or on subsequent follow-up suggested that aspirin might help in the treatment of some cancers as well as for preventing distant metastasis (Rothwell, Wilson et al., 2012). In addition,

aspirin also reduced the death due to cancer in patients who developed adenocarcinoma without metastasis at time of diagnosis (Rothwell, Wilson et al., 2012).

Although it is known that low-dose aspirin reduces the long-term risk of death due to cancer, it is currently unclear about the short-term effect on cancer incidence (Rothwell, Price et al., 2012). To address this issue, a recent study evaluated the time-course effects of low-dose aspirin on cancer incidence. Results of this analysis showed reduced cancer deaths in the aspirin group beginning from 5 years onwards (Rothwell, Price et al., 2012). Furthermore, in some studies, it has been demonstrated that daily low-dose aspirin reduced the cancer incidence from 3 years onwards (Rothwell, Price et al., 2012). A different study also tested the efficacy of long-term use of low-dose (75–300 mg daily) aspirin on incidence and mortality due to colorectal cancers (Rothwell et al., 2010). Analysis of the pooled data showed that aspirin reduced the 20-year risk of colon cancer but not the rectal cancer risk in terms of incidence as well as mortality (Rothwell et al., 2010). The data in this study also suggested that increasing the dose of aspirin above 75 mg daily had no significant benefit in reducing cancer incidence (Rothwell et al., 2010).

Protective effects of ASA are primarily attributed to its influence on various signaling cascades regulating cell proliferation and survival (Curiel-Lewandrowski et al., 2011). For example, ASA has been reported to inhibit oncogenic NF-kB as well as BCL2, while upregulating the levels of tumor suppressor TP53, CDKN1A, and BAX (Park et al., 2010; Zhou et al., 2001). While some studies have shown an inverse association between NSAIDs' use and risk of developing cutaneous melanomas, others reported none (Asgari et al., 2008; Curiel-Lewandrowski et al., 2011). For example, in a prospective cohort study investigating the association between over-the-counter self-reported NSAIDs' use and melanoma risk, no association was found (Asgari et al., 2008). These conflicting results necessitate the need for further studies to confirm the clinical utility of NSAIDs for preventing melanomas.

3.8. Beta Carotene

Beta carotene is a potent antioxidant known to exhibit photoprotective effects and anticancer activity (Stahl & Sies, 2011) (Fig. 12.3). For example, an *in vitro* study using mouse melanoma models showed inhibition of angiogenesis as well as nuclear localization of transcription factors and induction of BAX-mediated apoptosis by beta carotene (Bodzioch et al.,

2005; Guruvayoorappan & Kuttan, 2007). A physicians' health study consisting of 21,884 male physicians showed that administration of 50 mg/kg oral beta carotene daily for ~12 years had no effect on the incidence of BCC and SCC (Frieling et al., 2000). Similarly, a separate community-based randomized trial with beta carotene in 1621 study participants from Nambour district, Southeast Queensland, Australia found that beta carotene alone and in combination with a sunscreen having a sun protection factor (SPF)-15 also had no beneficial effects for preventing the incidence of basal cell carcinoma (Green et al., 1999). Therefore, use of beta carotene for preventing progression and metastasis of melanoma is questionable. However, combination trials using beta carotene needs to be conducted before excluding this potent antioxidant from chemoprevention use.

3.9. Celecoxib

Celecoxib is a selective inhibitor of cyclooxygenases (Wilson, 2006a). Cyclooxygenase (COX, EC1.14.99.1) is an oxygenase responsible for the production of biological mediators such as prostaglandins, prostacyclin, and thromboxanes from arachidonic acid (AA) (Becker et al., 2009; Khan et al., 2011) (Fig. 12.3). COX is also called prostaglandin synthase (PHS) or prostaglandin endoperoxide synthase (EPS) (Fitzpatrick, 2004). Three COX isoforms—COX-1, COX-2, and COX-3 (a splice variant of COX-1)—have been identified in human tissues (Fitzpatrick, 2004). Although all COX isoforms are structurally similar (sharing >65% amino acid homology and having near-identical catalytic sites) and performing similar catalytic reactions, the tissue distribution and expression levels in response to various stimuli differ (Fitzpatrick, 2004). For example, COX-1 is a constitutive enzyme, whereas COX-2 is inducible in most instances (Fitzpatrick, 2004). In addition, COX-2 expression is elevated in the majority of tumors and is selectively inhibited by various pharmacological agents (Flower, 2003). Studies measuring the expression levels of COX-2 in melanomas found very high protein levels in early and late-phase melanoma patients compared to normal human melanocytes (Becker et al., 2009). Furthermore, levels of COX-2 were also upregulated when mice were exposed to UV light indicating a potentially important role of COX-2 expression in melanoma tumorigenesis (Rundhaug & Fischer, 2008). Similarly, COX-2 expression was elevated when human skins were exposed to UV radiation (Buckman et al., 1998). Additional studies also showed that targeted inhibition of COX-2 using siRNA or pharmacological agents could inhibit melanoma tumor growth and sensitize cells to radiation (Johnson et al., 2008).

A separate study evaluated the efficacy of celecoxib for preventing actinic keratosis in a double-blind, randomized, placebo-controlled trial (Elmets et al., 2010). Administration of 200 mg celecoxib twice daily to 240 high-risk men and women having 10–40 actinic keratoses and a history of previous skin cancer resulted in no response in terms of the incidence of actinic keratosis (Elmets et al., 2010). Therefore, the utility of celecoxib for treating nonmelanoma skin cancers was unclear.

In vitro studies using other COX-2 inhibitors showed that COX-2 is an effective chemopreventive target for reducing the metastatic potential of melanoma cells (Wilson, 2006b). Naturally occurring inhibitors such as berberine inhibited melanoma cell proliferation and metastasis by targeting COX-2 and ERK pathways (Kim et al., 2012). Another study tested the ability of celecoxib for preventing melanoma in 27 patients with surgically incurable recurrent melanoma (Wilson, 2006a). Data showed tumor regression in seven patients, among whom two patients had complete regressions, two experienced partial regressions, and three showed a mixed response (Wilson, 2006a). The median overall survival time from first incurable metastasis was 31.9 months (Wilson, 2006a). Analysis of median times to progressive disease and death from start of celecoxib was 4.3 months and 10.4 months, respectively (Wilson, 2006a). Although results of this study are encouraging, celecoxib failed to show similar efficacies in all patients despite the presence of high COX-2 expression, indicating that the level of inhibition might not be sufficient to prevent melanoma development in certain cases, warranting the development of more potent COX-2 inhibitors.

Since targeting COX-2 alone failed to lead to complete tumor inhibition *in vitro* as well as *in vivo*, further studies considered testing COX-2 inhibitors in combination trials (Wilgus et al., 2004). For example, a combination of COX-2 inhibitor celecoxib and 5-fluorouracil (5-FU) reduced the number of UVB-induced skin tumors 70% more effectively in mice compared to either of the single agents (Wilgus et al., 2004). Mechanistically, in addition to the inhibition of the cell cycle, celecoxib also facilitated the diffusion of 5-FU into tumor cells thereby increasing its efficacy to inhibit cell proliferation (Wilgus et al., 2004).

3.10. Alpha-Difluoromethylornithine

Alpha-difluoromethylornithine (DFMO), also known as eflornithine, is an irreversible inhibitor of ornithine decarboxylase and is involved in inhibiting

polyamine production (Sunkara & Rosenberger, 1987) (Fig. 12.3). Several cell culture–based and mice studies have shown the efficacy of DFMO for inhibiting pulmonary melanoma metastases (Kubota et al., 1987; Sunkara & Rosenberger, 1987). For example, a preclinical study evaluating the efficacy of DFMO in malignant mouse B16 amelanotic melanoma (B16a) showed a dose-dependent decrease in tumor growth as well as pulmonary metastasis development. Administration of 0.5, 1, and 2% DFMO in water, inhibited tumor growth by 0, 24.5, and 60%, while the same doses reduced metastasis by 55, 83, and 96% (Sunkara & Rosenberger, 1987). Since administration of DFMO did not inhibit experimental metastasis, authors of this study concluded that DFMO might be affecting invasion of melanoma cells (Sunkara & Rosenberger, 1987). A separate study tested the efficiency of DFMO in combination with Type I interferon in melanoma mouse models (Croghan et al., 1988; Sunkara et al., 1984). The data showed the anti-proliferative potential of DFMO, both alone and in combination in several tumor cell lines. For example, treatment of B16 melanoma cells with DFMO inhibited the growth with an IC_{50} of 31.1 μM. However, when used in combination, DFMO exhibited a marked synergism with Type I interferon. Mechanistically, DFMO enhanced the therapeutic efficacy of interferon treatment by Causing interferon receptor downregulation.

A previous Phase-II study using DFMO (2g/m2 po, q 8h) in 21 evaluable patients showed a complete response in 1 patient for 11 months (Meyskens et al., 1986). Seven other patients presented with stable disease for 8 weeks (Meyskens et al., 1986). However, due to toxicity and hearing loss observed in 5 patients, further use of DFMO was discouraged (Meyskens et al., 1986). Further studies are warranted to determine whether using a different DMFO schedule would prevent hearing loss (Meyskens et al., 1986). Future trials should also consider using DMFO in combination with other agents. A separate investigation has examined the antitumor and antimetastatic activities of DFMO by inducers of interferon, namely, tilorone and polyriboinosinic:polyribocytidilic acid complex [poly(l) X poly(C)] (Sunkara et al., 1984). Results of these combination trials indicated that interferon inducers could enhance the antitumor activity of DFMO against B16 melanoma in mice (Sunkara et al., 1984). DFMO, tilorone, or poly(l) X poly(C), when administered alone, showed 85, 39, and 39% inhibition of tumor growth, respectively (Sunkara et al., 1984). However, a combination of DFMO and tilorone or poly(l) X poly(C) resulted in 98 and 95% growth inhibition (Sunkara et al., 1984). Efficacy was linked to induction of interferon (Sunkara et al., 1984). Other studies with Lewis lung carcinoma

cells also showed similar DFMO-potentiating effects of interferons (Sunkara et al., 1984; Sunkara et al., 1989). A combination of DFMO and tilorone led to 78% inhibition of tumor growth and 99.5% inhibition of metastases, but the mechanisms remain to be fully elucidated (Sunkara et al., 1984, 1989). Enhancement of host immune response or interferon-mediated cytotoxicity would likely be the mechanism of action (Sunkara et al., 1984, 1989).

3.11. Sunscreens

Sunscreens are topically applied creams or gels to protect underlying skin cells from UV-induced damage (Burnett & Wang, 2011). Use of sunscreens is widely advocated as a preventive measure against sun-induced skin cancers (Drolet & Connor, 1992). However, to date, no epidemiologic study has reported decreased melanoma risk associated with sunscreen use (Weinstock, 1999). Furthermore, results from a collaborative European case–control study and animal studies raised concerns about the protection that sunscreens provide against UV radiation–associated cutaneous melanomas (Klug et al., 2010; Loden et al., 2011; Wolf et al., 1994). Moreover, meta-analysis of 18 studies investigating the association between melanoma risk and previous sunscreen use, suggested little or no beneficial correlation (Dennis et al., 2003; Huncharek & Kupelnick, 2002). Therefore, although sunscreens are known to act as physical barriers to protect skin from UV-induced damage, the role of these agents for preventing skin cancers requires further investigation. This is especially important since some studies suggest that skin cancer risk increased when sunscreens were used (Antoniou et al., 2008; Goldenhersh & Koslowsky, 2011; Gorham et al., 2007; Planta, 2011). Therefore, it is currently unknown whether sunscreens that have been designed to reduce exposure to UV radiation will reduce skin cancer incidence in humans. In addition, since host factors such as propensity to burn, variable numbers of benign melanocytic nevi, and atypical nevi may also increase the risk of developing cutaneous melanoma, clinical trials should consider these influencing factors while evaluating and testing the efficacy of sunscreens for preventing melanoma (Azizi et al., 2000; Holly et al., 1995a,b; Holly et al., 1995).

A very small randomized placebo-controlled study with 53 volunteers who had either clinical evidence of solar keratoses or nonmelanoma skin cancer was conducted using a sunscreen with an SPF of 29 (Naylor et al., 1995). The study showed, among 37 participants, a decrease in the rate of new solar keratoses in the sunscreen users compared to the placebo group

(Naylor et al., 1995). Another randomized controlled study evaluating the effect of regular sunscreen with SPF of 17 on solar keratoses in 431 patients demonstrated that individuals in the sunscreen group developed fewer new lesions and more remission of existing lesions than those in the base-cream placebo group (http://www.cancer.gov/cancertopics/pdq/prevention/skin/HealthProfessional/page4) (April 12, 2012). Furthermore, the development of new lesions and the remission of existing ones had been reported to correlate with the amount of sunscreen used. In contrast, a separate randomized study showed no statistically significant difference in incidence of BCCs with regular SPF-16 sunscreen use (Green et al., 1999). No difference was noticed in rates of melanoma on prescribed sunscreen application sites between the control and the experimental groups (Green et al., 1999). Although, results of this study indicate no protective effect of sunscreen on melanoma incidence, it has several important limitations (Green et al., 1999). For example, (a) melanoma was not the primary planned endpoint of the original trial, hence the selection of study subjects and endpoints might not be as effective when melanoma is considered as the primary outcome; (b) the confidence intervals of the outcome estimates are very wide, demonstrating substantial uncertainty regarding the magnitude of the effect; and (c) widespread use of the passive participant option during the follow-up phase of the study (Green et al., 1999).

A recent report is more positive regarding the importance of using sunscreens to prevent skin cancer. This study shows that use of sunscreen of SPF-15 reduced the incidence of squamous cell carcinoma in the sunscreen group compared to control groups not using it (van der Pols et al., 2006). Similarly, according to a recent report analyzing the long-term application of sunscreen on cutaneous melanoma in Nambour township, in Australia, reduced melanoma incidences were observed in daily sunscreen users (Green et al., 2011). This trial compared the incidence of melanoma between 1621 randomly assigned daily and discretionary sunscreen use groups and found an increase in the number of melanomas only in the discretionary use group but not in the daily users (Green et al., 2011). Ten years after trial cessation, the data showed 11 melanomas (3 of them invasive) in the daily sunscreen group compared to 22 melanomas, with half of them being invasive, in the discretionary user group indicating that regular use of sunscreens might help prevent melanoma (Green et al., 2011).

A separate case–control study with 418 melanoma cases and 438 healthy individuals also evaluated the influence of sunscreen use on the occurrence of cutaneous malignant melanoma (Autier et al., 1995). This study found

increased melanoma risk among psoralen sunscreen users compared to regular sunscreen users (Autier et al., 1995). The melanoma risk was 1.5 for regular sunscreen users whereas for psoralen sunscreen users it was 2.28 suggesting a negative influence of the psoralen sunscreen (Autier et al., 1995). This study supports the hypothesis that sunscreens do not protect against melanoma. This negative correlation with sunscreens use could be due to the prolonged exposure to unfiltered UV radiation, inducing melanoma. In support of this hypothesis, a separate study exposed C3H mice to UVB radiation twice a week for 3 weeks and used sunscreens containing 7.5% 2-ethylhexyl-*p*-methoxycinnamate, 8% octyl-*N*-dimethyl-*p*-aminobenzoate, 6% benzophenone-3, or the oil-in-water vehicle alone applied to the ears and tails of the mice 20 minutes before irradiation. UV-induced inflammation and histological alterations were measured (Wolf et al., 1994). Injection of melanoma cells into the external ears created melanomas in both control and experimental animals (Wolf et al., 1994). Although sunscreens protected from UV-induced ear damage, it failed to protect from melanoma indicating that the protection against sunburn does not necessarily imply protection against melanoma growth (Wolf et al., 1994). A comprehensive MEDLINE search analysis of reports published between 1966 and 2003 regarding the use of sunscreens and melanoma protection also showed no association (Dennis et al., 2003). Furthermore, this meta-analysis study suggested that the positive association reported in some prior studies could be due to failure to control for confounding factors such as the sensitivity to sun, age of the patient, frequency, and type of sunscreen use.

3.12. Betulinic Acid

Betulinic acid is a triterpene isolated from the bark of *Betula pubescens* (Pisha et al., 1995) (Fig. 12.3). Betulinic acid has been demonstrated to kill several cancer types including melanoma (Cichewicz & Kouzi, 2004; Pisha et al., 1995). Betulinic acid inhibited melanoma tumor development in mice without causing systemic toxicity (Pisha et al., 1995). Mechanistically, betulinic acid induced apoptosis in a p53- and CD95-independent manner in cancer cells to inhibit tumor development (Fulda et al., 1997). Other studies have suggested involvement of reactive oxygen species, inhibition of topoisomerase I, activation of Erk1/2 phosphorylation, suppression of tumor angiogenesis, and modulation of pro-growth transcriptional activators as well as aminopeptidase N activity for betulinic acid chemopreventive

activity (Mullauer et al., 2010). Due to encouraging *in vitro* and *in vivo* studies, safety, and cost-effectiveness, betulinic acid is currently being evaluated for the prevention of malignant melanomas (Surowiak et al., 2009; Struh et al., 2012) (http://clinicaltrials.gov/ct2/show/NCT00346502) (April 12, 2012). Betulinic acid was also found to be effective at preventing small and non–small cell lung, ovarian, cervical, and head and neck carcinomas (Mullauer et al., 2010).

While betulinic acid inhibits the growth of many cultured cancer cells, a separate study comparing the efficacy of betulinic acid for inhibiting MeWo melanoma cells (both drug sensitive and drug resistant) compared to normal melanocytes demonstrated greater killing efficacy of normal melanocytes, which is a concern (Surowiak et al., 2009). Another study has also shown increased sensitivity of keratinocytes to betulinic acid treatment compared to melanoma cells (Galgon et al., 2005). Therefore, additional experimentation is required to unravel the mechanistic basis for this effect on normal cells. Molecular mechanisms inducing resistance to betulinic acid have been investigated and found to be mediated by the PI3K-Akt pathway (Qiu et al., 2005). Since betulinic acid induces apoptosis by activating the Erk pathway and decreasing CDK4 expression, inhibitors reducing MEK1/2 activity such as U0126 have been reported to induce resistance to betulinic acid (Rieber & Rieber, 2006). Furthermore, resistance to betulinic acid could be due to induction of Akt activity and survivin expression (Qiu et al., 2005). Betulinic acid upregulates EGFR phosphorylation to promote Akt and survivin expression (Qiu et al., 2005). Targeted inhibition of EGFR using PD153035 decreased betulinic acid–induced EGFR phosphorylation and inhibited Akt activation to promote cancer cell destruction (Qiu et al., 2005). Compound combination studies have observed synergistic inhibition of cancer cell growth when betulinic acid is combined with PD153035, suggesting a new direction for future clinical trials (Qiu et al., 2005). Betulinic acid has also been reported to inhibit the migration of melanoma cells (Rieber & Rieber, 2006). For example, human metastatic C8161 melanoma cells, but not their non-metastatic variant C8161/neo6.3, were found to be more susceptible to betulinic acid treatment (Rieber & Rieber, 2006). In these cells, betulinic acid induced p53 expression thereby inducing apoptosis (Rieber & Strasberg-Rieber, 1998a, 1998b). A phase I/II study (NCT00346502) is currently evaluating the efficacy of 20% betulinic acid ointment for safety and efficacy for preventing dysplastic nevi from progressing into melanomas (http://clinicaltrials.gov/ct2/show/NCT00346502) (April 12, 2012).

3.13. Vitamin-D

In vitro and *in vivo* studies with vitamin-D as a chemopreventive agent for melanoma demonstrated reduced tumor growth (Eisman et al., 1987; Reichrath et al., 2007) (Fig. 12.3). Furthermore, a large case–control study using dietary vitamin-D found reduced melanoma risk (Millen et al., 2004). However, other studies have shown lack of melanoma inhibitory activity with vitamin-D, which raised concerns about using this natural product for preventing melanoma (Weinstock et al., 1992). A pilot study is underway to evaluate the effect of vitamin-D on melanocyte biomarkers (NCT01477463). The purpose of this study is to determine the signaling pathways and changes in gene expression in melanocytes of patients with a history of nonmelanoma skin cancer who are exposed to oral vitamin-D (http://clinicaltrials.gov/ct2/show/NCT01477463) (April 12, 2012). If vitamin-D inhibits a signaling pathway involved in the development of melanoma, such as that of the MAP kinase pathway involved in cell proliferation, then oral vitamin-D could be explored for melanoma chemoprevention.

4. CONCLUSION

Chemoprevention of melanoma if successful could be used to inhibit the transformation of nevi into invasive melanomas, which could reduce the incidence of this deadly disease. Even though several chemopreventive agents have been developed, currently, no single agent is effective for preventing melanomas, which is driving the search for more potent compounds or compound combinations having greater chemopreventive efficacy. Although encouraging data have been reported with selenium containing isoselenocyanates as well as isoselenoureas in laboratory-generated skin reconstructs as well as xenografted animal models, further studies are required to determine the safety and efficacy of these agents for human use. Similarly, studies are also warranted to determine the efficacy of naturally occurring, cost-effective compounds such as curcumins and EGCGs for melanoma prevention.

Future studies with the goal of effective melanoma chemoprevention should consider (a) using targeted nanotechnologies for effective delivery of chemopreventive agents; (b) determining the effectiveness of compound derivatives such as those for curcumin, for safety and efficacy; (c) developing better preclinical models for evaluating the chemopreventive efficacy of

various agents; and (d) evaluating various compound combinations primarily focusing on target-based preventive strategies.

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ABBREVIATIONS

ASA acetyl salicylic acid
BAX Bcl-2-associated X protein
BCC basal cell carcinoma
BCL-2 B-cell CLL (chronic lymphocytic leukemia)/lymphoma-2
CD95 cluster of differentiation-95
CDK2 cyclin-dependent kinase 2
COX cyclooxygenase
DFMO difluoromethylornithine
DTIC dacarbazine
EGCG epigallocatechin gallate
EGFR epidermal growth factor receptor
EGR-1 early growth response protein-1
EPS endoperoxide synthase
ERK extracellular signal-regulated kinase
5-FU 5-fluorouracil
HGF-SF hepatocyte growth factor-scatter factor
IKK IκB kinase
IL interleukin
iNOS inducible nitric oxide synthase
ISC-4 isoselenocyanate-4
ISP isolated soy protein
LOX lipoxygenase
MAPK mitogen-activated protein kinase
MMP matrix metalloproteinase
mTORC2 mammalian target of rapamycin complex-2
NCCAM National center for complementary and alternative medicine
NCI National Cancer Institute
NFκB nuclear factor kappa B
NPC nutritional prevention of cancer
NSAID nonsteroidal anti-inflammatory drug
PAR-4 prostate apoptosis response protein-4
PBISe *S*,*S*′-(1,4-phenylenebis[1,2-ethanediyl)bis-isoselenourea
PBIT *S*,*S*′-(1,4-phenylenebis[1,2-ethanediyl)bis-isothiourea

PI3K phosphoinositide 3-kinase
p-XSC 1,4-phenylenebis(methylene)selenocyanate
SCC squamous cell carcinoma
SEER surveillance epidemiology and end results
SELECT selenium and vitamin E cancer prevention trial
SPF sun protection factor
SV-40 simian vacuolating virus-40
SWOG southwest oncology group
TGF-β transforming growth factor beta
TP53 tumor protein p53
VEGF vascular endothelial growth factor

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CHAPTER THIRTEEN

Whole Genome and Exome Sequencing of Melanoma: A Step Toward Personalized Targeted Therapy

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Abstract

Melanoma has historically been refractive to traditional therapeutic approaches. As such, the development of novel drug strategies has been needed to improve rates of overall survival in patients with melanoma, particularly those with late stage or disseminated disease. Recent success with molecularly based targeted drugs, such as Vemurafenib in *BRAF*-mutant melanomas, has now made "personalized medicine" a reality within some oncology clinics. In this sense, tailored drugs can be administered to patients according to their tumor "mutation profiles." The success of these drug strategies, in part, can be attributed to the identification of the genetic mechanisms responsible for the development and progression of metastatic melanoma. Recently, the advances in sequencing technology have allowed for comprehensive mutation analysis of tumors and have led to the identification of a number of genes involved in the etiology of metastatic melanoma. As the methodology and costs associated with next-generation sequencing continue to improve, this technology will be rapidly adopted into routine clinical oncology practices and will significantly impact on personalized therapy. This review summarizes current and emerging molecular targets in metastatic melanoma, discusses the potential application of next-generation sequencing within the paradigm of personalized medicine, and describes the current limitations for the adoption of this technology within the clinic.

1. INTRODUCTION

Melanoma is a malignant skin cancer originating from the unregulated growth of melanocytes, cells responsible for pigmentation in the skin. In the United States, 76,250 new cases of melanoma have been projected for 2012 (Siegel et al., 2012). Of significance is the increasing incidence of melanoma that has been observed in the United States and worldwide,

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which has more than doubled over the last 20 years (Linos et al., 2009; Welfare, 2010). Furthermore, late stage or disseminated melanoma accounts for the majority of skin cancer related deaths; it is estimated that 9,180 Americans will succumb to the disease in 2012 (Siegel et al., 2012). The high mortality rate of metastatic melanoma has essentially been due to the lack of efficacy of traditional therapeutic approaches. Significant improvements to existing therapies or the development of novel drug strategies are required in order to increase survival for patients with metastatic melanoma.

1.1. Traditional Therapeutic Approaches

Melanoma has historically been refractive to chemotherapeutic treatments. Although a number of agents have been assessed in clinical trials (Yang & Chapman, 2009), dacarbazine (DTIC), until recently, has been the standard approved treatment option for patients with advanced (stage IV) melanoma. DTIC is a cytostatic agent with alkylating properties that inhibits DNA synthesis and promotes growth arrest. Intravenously administered, DTIC is a prodrug that requires processing within the liver to release the active compound 5-(3-methyl-1-triazeno)imadazole-4-carboxamide (MTIC). Although complete responses in patients are occasionally observed, clinical trials have demonstrated response rates in only 5–15% of the patients, with a median durability of 6–12 months (Chapman et al., 1999).

Due to the inherent resistance of metastatic melanoma to chemotherapy, alternative avenues of treatment were investigated and led to the approval of immunological drugs, interferon alpha (IFN-α) and high-dose interleukin-2 (IL-2), during the mid-1990s. The use of these drugs is typically associated with response rates of 10–20%, with approximately 5% of the patients exhibiting long-term responses, in some cases, remission of up to 5–10 years (Atkins et al., 1999; Kirkwood et al., 1996). However, due to the nature of these therapies eliciting strong immune reactions, severe adverse side effects are frequently observed. As such, treatment via these modalities is usually limited to those patients who are relatively healthy and have excellent organ capacity, but they still require intensive clinical observation during treatment. These drugs can be used in conjunction with standard chemotherapeutic strategies; however, this approach is associated with the risk of increased toxicity with minimal survival benefit (Stoter et al., 1991).

1.2. Modern Therapeutic Approaches

In the 20 years following approval of IFN-α and IL-2, only two new therapies have been approved for late stage melanoma. Both drugs, approved in 2011, have already demonstrated a significant impact on patient outcome. The first of these, Ipilimumab (Yervoy), is an anti-CTLA4 antibody approach designed to promote sustained T cell activation. Phase III clinical trials of Ipilimumab used as a single agent or in conjunction with DTIC, have improved rates of overall survival (Hodi et al., 2010; Robert et al., 2011); however, response is often associated with initial delays in tumor regression. Similar to the immunological approaches of IFN-α and IL-2, the use of Ipilimumab can promote severe grade III and IV side effects leading to premature termination of therapy, and on rare occasions, treatment-related mortalities (Hodi, et al., 2010).

A number of challenges regarding the clinical management of Ipilimumab remain, and hopefully, with the identification of positive biomarkers of drug response, improvements in the clinical utility of this drug will occur. Investigations into biomarkers are currently in their infancy; however, tumors with active immune microenvironments (Ji et al., 2011) and those expressing immune-related genes (Hamid et al., 2011) may indicate favorable responses in patients. Despite the current lack of robust biomarkers, Ipilimumab has quickly been established as the standard treatment for non-*BRAF*-mutated melanoma patients.

Another promising avenue of treatment is the use of molecularly based targeted therapy, that is, a novel drug approach that counteracts the effect of acquired mutations responsible for tumorigenesis. The first forays into targeted therapies in melanoma stemmed from the seminal finding of somatic oncogenic mutations in *BRAF*, a member of the serine/threonine family of protein kinases, occurring in 66% of melanomas (Davies et al., 2002). Mutation of *BRAF* has since been more accurately estimated to occur in ~50% of melanomas (Jakob et al., 2011), the majority of which are accounted for by a valine to glutamate substitution at coding position 600 (V600E, initially reported as V599E). Mutations such as V600E disrupt the inactive conformation of the kinase domain resulting in constitutive autophosphorylation and downstream signaling of the mitogen activated protein kinase (MAPK) pathway (Wan et al., 2004).

Although preclinical assessment of BRAF inhibitor strategies were promising (Hingorani et al., 2003; Sharma et al., 2005; Wellbrock et al., 2004), phase II and III clinical trials investigating a small molecule inhibitor of tyrosine kinases, Sorafenib, did not demonstrate a significant survival

advantage compared to standard chemotherapeutic strategies (Eisen et al., 2006; Hauschild et al., 2009). Further refinement in molecular drug design led to the development of highly selective inhibitors of *BRAF* V600E tumors (Tsai et al., 2008). One of these, Vemurafenib (also known as PLX4032 or Zelboraf), was the second drug to receive approval for use against metastatic melanoma in 2011.

Vemurafenib results in dramatic rates of initial tumor regression, demonstrating an increase in overall survival at 6 months compared to DTIC (Chapman et al., 2011). However, long-term response rates have been hindered by tumor acquired drug resistance observed in the majority of patients. Multiple mechanisms of tumor resistance to Vemurafenib have been identified (Emery et al., 2009; Jiang et al., 2011; Nazarian et al., 2010; Poulikakos et al., 2011; Shi et al., 2012; Villanueva et al., 2010; Wagle et al., 2011), and it is hoped that with continued research, long-term response rates and overall survival will be improved. A recent phase II trial of Vemurafenib in BRAF V600-mutant patients with previously treated melanoma demonstrated a median overall survival of 16 months, providing evidence that longer-term responses in patients can be achieved (Sosman et al., 2012).

Interestingly, the use of Vemurafenib frequently results in the development of skin lesions, such as squamous cell carcinomas, in patients undergoing therapy (Su et al., 2012). Although not life threatening when managed by frequent clinical observation and surgical removal, it is a concerning phenomenon pointing toward off-target side effects. Paradoxically, the use of BRAF inhibitors in non-*BRAF*-mutant tumors results in the activation of the MAPK pathway (Halaban et al., 2010; Hatzivassiliou et al., 2010), thus promoting cell proliferation and tumor progression. This finding highlights the critical importance of drug selection based on the presence of a *BRAF* V600E mutation within a patient's tumor, an example of personalized medicine.

The successful development and recent approval of two drugs for metastatic melanoma, in particular the molecularly based targeted approach of Vemurafenib, can be attributed to the extensive effort in understanding the genetic etiology of melanoma. This, in part, has largely been driven by recent advances in technology, including the advent of next-generation sequencing platforms.

1.3. Identifying the Genetic Mechanisms Underlying Melanoma

Although predisposition to a subset of melanomas has a hereditary component, the majority arise through the gradual accumulation of genetic

abnormalities caused by carcinogenic exposure to solar ultraviolet radiation (UVR). It is the acquisition of somatic mutations in critical genes controlling a range of important cellular processes that results in the proliferation and dissemination of melanoma throughout the body. Characterization of the multitude of genetic alterations promoting the development and progression of melanoma has led to the identification of several frequently mutated genes, of which, a proportion is amenable to therapeutic intervention.

The discovery of mutated genes driving the development of melanoma has dramatically changed over time as a result of advances in technology. More recently, next-generation sequencing has allowed unparalleled, unbiased analysis of the cancer genome. The first catalog of somatic mutation of a melanoma genome involved the sequencing of a commercially available metastatic melanoma cell line, COLO-829, and its matched lymphoblastoid cell line (Pleasance et al., 2010a). A total of 292 somatic protein altering mutations were found, of which 187 were nonsynonymous—a mutation rate considerably higher than that of other solid tumors such as breast cancer (Shah et al., 2009) or glioblastoma multiforme (Parsons et al., 2008).

Interestingly, analysis of the somatic base substitutions in COLO-829 revealed a mutation profile consistent with a UVR-based carcinogenic signature (Pfeifer et al., 2005). The majority of mutations detected were C>T (G>A) transitions, with ~70% being CC>TT/GG>AA; these are predicted to be caused by UVR-induced DNA damage resulting in the formation of covalent links between two adjacent pyrimidines (Daya-Grosjean & Sarasin, 2005). A carcinogenic signature of G>T/C>A transversions has since been identified in a small-cell lung cancer associated with excessive tobacco exposure (Pleasance et al., 2010b).

Mutations are thought to stochastically accumulate within the genome, a large majority of which is not likely to confer a growth advantage to the cell. This type of mutation, known as a "passenger" mutation, may be present prior to, or gained during, clonal expansion of the tumor. In contrast, a small handful of deleterious pro-oncogenic mutations will be responsible for "driving" the process of tumorigenesis. One of the main challenges faced in analyzing large amounts of data produced from genome-wide studies is determining "passenger" mutations from "driver" mutations (Parmigiani et al., 2009).

To assess the significance of mutations with respect to driver versus passenger events, one approach is to use the nonsynonymous to synonymous (N:S) mutation ratio (Greenman et al., 2006; Greenman et al., 2007). This

statistic is based on the assumption that nonsynonymous mutations are biologically selected for since these mutations can affect the structure of proteins. As such, higher N:S ratios indicate positive selection overall compared to what is expected by chance. The N:S ratio of the COLO-829 genome was 1.78, not higher than the N:S ratio of 2.5:1 predicted for nonselected passenger mutations; this indicated that the majority of mutations are likely to be passenger mutations not relevant for pathogenesis of melanoma. This observation, in conjunction with considerably higher mutation rates in melanoma compared to other malignancies, highlights a potential difficulty in identifying causal genes involved in this disease. One approach to overcome this problem is by analyzing large numbers of tumors to identify frequently mutated genes.

Integrative analysis of RNA-seq and high-resolution chromosomal copy number data was an early approach taken to comprehensively assess the mutation rate in a large set of melanomas (Berger et al., 2010). Although a number of interesting mutations were identified, this study was limited by the detection of mutations in only the most abundant transcripts expressed in melanoma and the lack of matched normal samples for comparison. An improvement to this approach involved the application of whole-exome sequencing to cancer.

The first melanoma related exome report, released in mid-2011, analyzed 12 metastases and their matched normal samples (Wei et al., 2011b). Although the N:S ratio was 2.0:1, suggesting the majority of mutations were passenger events, a number of interesting genes were identified when the lists of mutations were compared between samples. This highlighted a recurrent mutation of *TRRAP* in 4% of the melanomas, as well as ~25% of the melanomas exhibiting *GRIN2A* mutations. Despite the high burden of mutation in melanoma, this study provided a proof of principle that genes relevant to the pathogenesis of the disease could be detected with small sample sets. Since this initial report, other whole-exome sequencing studies in melanoma have been published and have identified a number of new genes involved in the etiology of this disease (Harbour et al., 2010; Nikolaev et al. 2012; Stark et al., 2012).

2. MELANOMA GENETICS

Since the identification of *BRAF* mutations in melanoma, studies have identified a number of oncogenes and tumor suppressor genes involved in

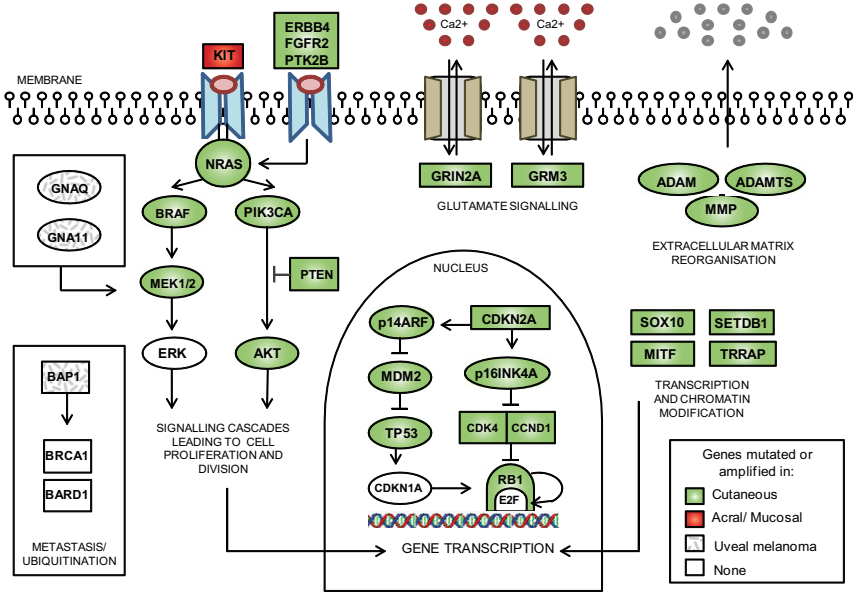

Figure 13.1 Pathways frequently deregulated in metastatic melanoma. Significant advances in understanding the genetics of metastatic melanoma have recently been achieved through the use of next-generation sequencing strategies. For color version of this figure, the reader is referred to the online version of this book.

a variety of key pathways, including cell signaling, division, and apoptosis. As the introduction of new technologies is making powerful genome-wide scale studies achievable, it is becoming apparent that determining commonly affected pathways, rather than single genes in isolation, will be important in understanding tumorigenesis (Vogelstein & Kinzler, 2004). This section reviews the well-characterized classical pathways of cutaneous melanoma development in addition to novel emerging pathways revealed by recent sequencing efforts and is summarized in Fig. 13.1.

2.1. Classical Pathways to Melanoma Development

The MAPK pathway regulates cell growth and survival through a series of signaling cascades in response to external stimuli (reviewed extensively in Fecher et al., 2008). Under normal physiological conditions, extracellular signals initiate the binding of receptor tyrosine kinases (RTK) to RAS, a membrane-bound GTPase at the cell surface membrane. This process leads to a series of downstream phosphorylation cascades causing stepwise activation of BRAF, MEK1/2, and ERK1/2 and ultimately leads to regulation

of cell proliferation, angiogenesis, invasiveness, and metastasis. Although ERK activity is tightly regulated in melanocytes, high constitutive activity of the MAPK pathway is frequently observed in melanoma, largely due to the acquisition of oncogenic mutations in components of this pathway (Cohen et al., 2002; Smalley, 2003).

MAPK activation in melanoma is predominantly driven by mutation of *BRAF* (~50% of the melanomas); however, some tumors exhibit mutations in *RAS*. *RAS* mutations have been observed in 10–20% of the melanomas (Herlyn & Satyamoorthy, 1996), the most frequently mutated member of this family being *NRAS*. Notably, mutations of *BRAF* and *NRAS* tend to be mutually exclusive (except for a few rare cases) indicating redundancy in their biological function. As mentioned previously, BRAF V600-mutant tumors are amenable to therapeutic intervention with modern BRAF inhibitors, such as Vemurafenib.

Recently, exome sequencing has revealed mutations in *MAP2K1* (MEK1) and *MAP2K2* (MEK2), which when sequenced in a larger cohort of samples were mutated in approximately 6% and 2% of the melanomas, respectively (Nikolaev et al. 2012). Interestingly, MAP2K1/2 mutations not only cause constitutive activation of the MAPK pathway, but can also be acquired in drug resistant tumors following use of BRAF inhibitors (Emery et al., 2009; Wagle et al., 2011).

Apart from the MAPK pathway, NRAS also signals through phosphatidylinisitol-3-kinase (PI3K) to activate AKT (Cully et al., 2006; Wu et al., 2003). AKT interacts with a number of other signaling networks that control a variety of cellular functions including cell survival, proliferation, apoptosis, and tumor cell chemoresistance. PTEN (phosphatase and tensin homolog, deleted from chromosome 10) negatively regulates this pathway by preventing downstream AKT signaling and controls cell cycle progression.

Activation of the PI3K pathway in melanoma occurs primarily through *NRAS* mutation (~20%); however, oncogenic mutations can also occur in *PIK3CA* and *AKT*, albeit at low frequencies (Davies et al., 2008; Omholt et al., 2006). In contrast, *PTEN* mutation results in deregulation of the PI3K pathway through the loss of negative regulation of AKT. *PTEN* mutation has been observed at high frequency in melanoma and was originally identified by its frequent deletion in a number of other cancers. A variety of mutations including missense and splice site mutations, deletions, and insertions in *PTEN* have since been observed in up to 30–50% of melanomas (Guldberg et al., 1997; Tsao et al., 1998). As mutation of *NRAS*

results in the deregulation of both the MAPK and PI3K pathways, mutation of *PTEN* is generally associated with *BRAF*-mutant tumors.

The Rb pathway, responsible for controlling cell cycle division and progression, is another frequently deregulated pathway in melanoma (Sharpless & Chin, 2003). A key regulator of this pathway is *CDKN2A* (cyclin-dependent kinase inhibitor 2A), a tumor suppressor gene identified in a range of tumors including melanoma (Kamb et al., 1994). *CDKN2A* encodes two different proteins, p16INK4A and p14ARF through alternative transcription start sites and use of different reading frames. Similar to *PTEN*, deletion of a region of chromosome 9 (where *CDKN2A* is located) was observed in a number of melanomas, indicating the presence of a putative tumor suppressor gene (Fountain et al., 1992). Deletions of *CDKN2A* have since been observed in up to 50% of melanomas (Flores et al., 1996).

The *CDKN2A* product p16INK4A negatively regulates cell division by inhibiting kinases CDK4 and CDK6 bound to CCND1. The CCND1-CDK4/6 complex, when not inhibited, phosphorylates pRb (*RB1*), an active repressor of E2F-mediated gene transcription, allowing expression of a variety of genes that promote cell division. Besides inactivation of p16INK4A, deregulation of this pathway can occur through mutation of *CDK4*, *CDK6*, and *RB1*, or alternatively, by amplification of *CCND1* or *CDK4* (Bartkova et al., 1996; Muthusamy et al., 2006; Sauter et al., 2002; Tang et al., 1999; Wolfel et al., 1995).

CDKN2A, through an alternative reading frame, encodes another tumor suppressor called p14ARF. p14ARF is responsible for the inhibition of MDM2 which in turn regulates the activity of p53 (*TP53*), a well-known tumor suppressor involved in DNA repair, apoptosis, and cell cycle regulation. One role of p53 is to activate p21 (*CDKN1A*) which, like p16INK4A, prevents the phosphorylation of pRb by binding to CDK2/CCNE1 complexes. Besides inactivation of p14ARF, deregulation of the p53 pathway occurs through mutation or deletion of *TP53* in approximately 20% of melanomas, or amplification of *MDM2* (Florenes et al., 1994; Muthusamy, et al., 2006; Weiss et al., 1993).

2.2. Classic Drug Targets Amenable to Traditional Pharmaceutical Intervention

2.2.1. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are cell surface receptors that respond to external stimuli and are responsible for the control of a variety of cellular

processes. These kinases have been extensively studied due to their frequent involvement in tumorigenesis and ability to be targeted for pharmacologic inhibition (Futreal et al., 2004; Sawyers, 2004). In melanoma, the most well characterized RTK with therapeutic potential is KIT; upon activation KIT signals via *NRAS* and can thus activate both the MAPK and PI3K pathways.

The first forays into the investigation of KIT as a therapeutic target stemmed from early investigations into the efficacy of Imatinib in other cancers; this drug is an inhibitor of tyrosine kinases and prevents substrate phosphorylation through competitive inhibition of the ATP binding domain. Imatinib has been approved for use in *BCR-ABL* mutant chronic myeloid leukemia, and gastro-intestinal stromal tumors (GIST), a cancer that exhibits oncogenic mutations of *KIT* in approximately 80% of patients (Heinrich et al., 2000; Hirota et al., 1998). The positive response of Imatinib in GIST, combined with an early observation of *KIT* expression in melanoma, led to clinical trials of Imatinib in metastatic melanoma. Although overall results of Imatinib lacked efficacy in the treatment of melanoma (Hofmann et al., 2009; Kim et al., 2008; Wyman et al., 2006), closer analysis of a single patient who responded favorably identified them to harbor a mutation in *KIT*, suggesting putative efficacy of Imatinib in a subset of melanoma patients.

Interestingly, sequencing analysis has revealed a distinct lack of *KIT* mutation in intermittently sun-exposed cutaneous melanomas but an increased representation of mutation in acral, mucosal, and chronically sun-exposed melanomas (Curtin et al., 2006). As the latter subtypes of melanoma are rare, the poor efficacy in early Imatinib trials is most likely explained by the underrepresentation of *KIT*-mutated tumors within the studies. Subsequent case reports have since demonstrated major responses to Imatinib in patients with *KIT*-mutated acral and mucosal melanoma (Hodi et al., 2008; Lutzky et al., 2008; Satzger et al., 2010; Yamaguchi et al., 2011), and more recently, a number of phase II clinical trials have proved encouraging (Carvajal et al., 2011; Guo et al., 2011). Mutations in KIT can occur throughout the gene but are frequently localized at critical residues in functional domains of the kinase. The majority of responders in the phase II clinical trials had mutations predominantly in exons 11 or 13. This suggests that further selection criteria for drug eligibility of patients to be given KIT inhibitors may be beneficial.

Trials into other KIT inhibitors are also currently in progress, including the recent completion of a Sunitinib trial for *KIT*-mutated melanoma

(Minor et al., 2012). It will be interesting to compare the activity of these drugs, particularly in regard to their efficacy in targeting the variety of mutation events observed in *KIT*. Regardless, the use of Imatinib in KIT-mutated melanomas is an excellent example of personalized medicine utilizing an "off the shelf" drug approach. The availability of existing therapeutics that show efficacy in malignancies with comparable mutation profiles will allow rapid trials and case studies for the treatment of new molecular subtypes of melanoma.

Early studies identifying frequent RTK mutation in cancer (Futreal et al., 2005; Stephens et al., 2005), including KIT mutations in melanoma (Willmore-Payne et al., 2005), suggested the possibility of other deregulated RTKs in melanoma development. To investigate this premise, Prickett et al. (2009) performed a comprehensive analysis of the tyrosine kinase family in melanoma. A total of 99 nonsynonymous mutations was found in 19 protein tyrosine kinases, with the highest frequency occurring in *ERBB4* (19%), *FLT1* (10%), and *PTK2B* (10%). Focusing on *ERBB4*, *in vitro* functional analysis revealed that mutation led to an increase in cell growth and receptor activation via autophosphorylation. Importantly, cells transfected with mutant *ERBB4* had increased sensitivity to the drug Lapatinib, an FDA approved ERBB pharmacological inhibitor. These results, if confirmed through additional *in vivo* experiments, suggest that *ERBB4* could be a *bona fide* target for existing ERBB inhibitors in this subset of patients.

2.2.2. G protein Coupled Receptors

The first study using exome sequencing analysis in metastatic melanoma provided a glimpse into the melanoma genome and identified a number of novel recurrently mutated genes (Wei et al., 2011b). Aside from *BRAF*, the most frequently mutated gene found in this discovery screen was *GRIN2A*, which was mutated in ~25% of the melanomas. Mutations occurred throughout the entire length of the gene and were most likely inactivating, suggesting that *GRIN2A* acts as a tumor suppressor.

GRIN2A, an N-methyl-D-aspartate (NMDA) receptor, belongs to a class of ionotropic glutamate-gated ion channels. Binding of glutamate to *GRIN2A* allows calcium and potassium to traverse the cell membrane; however, the biological effect of *GRIN2A* mutation and its role in melanoma has yet to be determined. Targeted exon capture paired with next-generation sequencing of the G protein coupled receptor (GPCR) family in melanoma identified mutations in members of a second class of glutamate receptors, the metabotropic glutamate receptors (Prickett et al., 2011). This

included mutation of *GRM3* and *GRM8* in ~16% and ~9% of melanomas, respectively. Biochemical analysis of mutant *GRM3* showed that it caused an increase in anchorage-independent growth and cell migration *in vitro* and *in vivo*.

Additional evidence for the role of this emerging pathway in melanoma is demonstrated by mutation of *PLCB4*, a downstream effector of GRM signaling (Wei et al., 2011b). Other members of the GRM family have also been implicated in melanomagenesis; this includes the correlation of *GRM1* expression to hyperproliferation of mouse melanocytes and increased expression of *GRM1* in human melanoma biopsies compared to melanocytes (Pollock et al., 2003). Lastly, mutant *GRM3* was shown to increase the activation of MEK, suggesting crosstalk between the MAPK and glutamate pathways. Exposure of cells carrying mutant *GRM3* to AZD6244, a small selective molecular inhibitor of MEK, resulted in greater growth inhibition and drug sensitivity compared to cells with wild-type *GRM3*, suggesting this may be a viable drug strategy in patients with mutations of the glutamate pathway.

2.2.3. Guanine Nucleotide Binding Proteins and Uveal Melanoma

Melanomas of the skin (cutaneous melanoma) account for approximately 90% of all the diagnosed melanomas; the remaining melanomas arise within the eye (uveal ~5%), or from mucosal membranes of the body (mucosal ~2%) (Chang et al., 1998). Notably, uveal tumors rarely have mutations in *BRAF* or *NRAS*, this is despite the presence of constitutive activity of the MAPK pathway (Zuidervaart et al., 2005). Recently, frequent mutations in guanine nucleotide binding proteins, downstream effectors of GPCRs, have been identified in uveal melanomas.

The discovery of hypermorphic mutations in *GNAQ* and *GNA11* in dermal hyperpigmented mice from mutagenesis screens led to sequencing of these genes in melanoma (Van Raamsdonk et al., 2004). This study found somatic mutations of *GNAQ* in 83% of blue naevi and 46% of uveal melanomas (Van Raamsdonk et al., 2009). Mutation of *GNAQ* nearly exclusively occurs in a single coding position (Q209), locking the GTPase in a manner that leads to constitutive activity, and downstream signaling of the MAPK pathway.

A subsequent study that sequenced the highly homologous gene family member *GNA11*, led to the identification of mutations in 7% of blue naevi, 32% of primary uveal melanomas, and 57% of uveal melanoma metastases (Van Raamsdonk et al., 2010). Interestingly, mutation of *GNA11* occurs

mutually exclusively to *GNAQ* mutation, and together they account for ~85% of uveal melanomas. As both genes lead to activation of the MAPK pathway, it was proposed that drugs targeting this pathway, such as MEK inhibitors (e.g., AZD6244), may represent an effective therapeutic avenue. Currently, clinical trials using this approach in uveal melanoma are underway; however, a recent study revealed only mild sensitivity to AZD6244 of *GNAQ* mutant uveal melanoma cell lines *in vitro* (Mitsiades et al., 2011).

Uveal melanomas appear to be a distinct molecular subtype of melanoma and mutations identified in these tumors rarely occur in cutaneous melanomas. Further evidence to support this comes from exome sequencing of two class 2 uveal melanoma tumors, that is, those with high metastatic risk, and their matched normal counterparts (Harbour et al., 2010). This revealed inactivating mutations in *BAP1* (encoding BRCA1 associated protein 1), which, when screened in a larger set of tumors, revealed mutations in 26 of 31 (84%) class 2 uveal melanomas. BAP1, a nuclear ubiquitin carboxyterminal hydrolase, has binding domains for the tumor suppressors BRCA1 and BARD1, and can complex with the histone modifier HCFC1. Although targeting of this gene in a therapeutic sense is challenging, inhibition of RING1 deubiquitinating activity may be a viable approach (Harbour et al., 2010).

2.2.4. Kinases

As mentioned above, mutations within CDK4 and PIK3CA occur at low frequency in melanoma (Omholt et al., 2006; Wolfel et al., 1995); however, these may be susceptible to therapeutic intervention. *CDK4*, or cyclin-dependent kinase 4, has recently been screened in a large panel of samples to determine an accurate estimate of mutation rate (Dutton-Regester et al., 2012). Mutation of *CDK4*, like *BRAF*, occurs at a mutation hotspot at coding position arginine 24, and was mutated in 8 of 252 (~3%) melanomas. CDK4 inhibitors, such as UCN-01, have been assessed in phase I and phase II clinical trials but overall did not demonstrate significant clinical efficacy; however, these trials consisted of small cohorts of patients and did not assess the mutation status of *CDK4* (Li et al., 2010; Sausville et al., 2001). As such, it may be possible that CDK4 inhibitors may be more efficacious in *CDK4* mutant or amplified melanomas. Alternatively, numerous drugs targeting the PI3K pathway are under current investigation; mutation of PIK3CA may be susceptible to intervention by these strategies (Aziz et al., 2010; Yuan et al., 2011).

2.3. Emerging Therapeutic Targets

2.3.1. Extracellular Matrix Regulation

A number of recent studies have identified frequent mutations in gene families involved in the regulation of the extracellular matrix and may affect cell motility, invasion, or metastasis. Together, these studies have resulted in the emergence of a novel pathway to melanoma development.

Matrix metalloproteinases (MMPs) belong to a family of 23 proteolytic enzymes that degrade the extracellular matrix and basement membranes surrounding cells. The role of MMPs in cell invasion, including that of melanoma, has long been identified (Hofmann et al., 2005). However, investigations into somatic mutations within this family of proteins have only recently been performed (Palavalli et al., 2009). Mutations were found in 8 MMP genes in 23% of the melanomas, of these, *MMP8* and *MMP27* were most frequently mutated. Interestingly, mutant MMP8 showed a decrease in proteolytic activity but resulted in an increase in tumor growth both *in vitro* and *in vivo*. These findings suggest that wild-type MMP has the ability to inhibit melanoma progression and thus has a putative tumor suppressor role.

Another related family, disintegrin-metalloproteinases with thrombospondin domains (ADAMTS), is part of a larger superfamily of zinc-based proteinases called metzincins, to which the MMPs belong. The role of ADAMTS proteins in cancer has not been well established; however, *ADAMTS15* was shown to be genetically inactivated in colorectal cancer (Viloria et al., 2009). This prompted mutational analysis of the ADAMTS family in melanoma (Wei et al., 2010), a study which identified a large fraction of tumors (~37%) harboring mutations in 11 of the 19 genes comprising the family. Mutant *ADAMTS18*, the most frequently mutated member at ~18%, was shown to be critical for cell migration *in vitro* and caused increased metastases *in vivo*, suggesting an oncogenic role in the proliferative and migratory capability of metastatic melanomas.

Mutational analysis of a third family of the metzincins, the disintegrin and metalloproteinase (ADAM) family, also revealed high rates of mutation in melanoma (Wei et al., 2011a). ADAMs are a group of membrane-bound glycoproteins that have a variety of biological roles including cell adhesion, migration, and proteolysis. Sequencing of the 19 ADAM genes revealed 8 genes collectively being mutated in 34% of the melanomas, the most frequently occurring in *ADAM7* (~12%) and *ADAM29* (~15%). Functional analysis demonstrated that mutant *ADAM7/29* affected the adhesion

capacity to a variety of extracellular matrix proteins and increased cell migration.

Although early clinical trials investigating first generation pan-inhibitors of proteolytic activity of MMPs yielded disappointing results (Overall & Lopez-Otin, 2002), numerous investigations using novel approaches targeting secretase activity are currently underway (Tolcher et al., 2010).

2.3.2. Transcriptional and Chromatin Modification

Micropthalmia-associated transcription factor (*MITF*) is a key regulator of melanocyte development controlling a variety of processes such as pigmentation, apoptosis, and cell cycle progression. In an early study using high density SNP arrays to investigate chromosomal copy number change in the NCI60 panel of cell lines, amplifications at a locus on chromosome 3p were identified that defined the melanoma subcluster (Garraway et al., 2005). Within this region, *MITF* was the only gene that showed strong correlation between amplification and high transcript expression. Subsequent analysis revealed that between 10 and 20% of the melanomas exhibited amplification of *MITF* and that its deregulation, in combination with *BRAF* V600E mutation, was capable of transforming melanocytes. As such, somatic alteration of *MITF* by amplification was suggested to define a specific oncogenic subclass based on "lineage survival" or "lineage addiction."

Further analysis of *MITF* revealed that in addition to amplification, somatic mutation also occurs in ~8% of cutaneous melanomas (Cronin et al., 2009). Additionally, a gene upstream of MITF, *SOX10*, was found to have putative inactivating mutations in a small proportion of melanomas; these mutations occurred in a mutually exclusive pattern to those in MITF, possibly indicating functional redundancy. Both of the aforementioned studies documented an association between *MITF* and *BRAF* mutation and mutual exclusivity to *NRAS* mutation. *MITF* has been shown to act through the TP53 and RB1 pathways (Carreira et al., 2005) and recently was characterized for direct interactions of genes involved in DNA replication, repair, and mitosis (Strub et al., 2011). Due to the complexity of *MITF* interactions, additional studies will be required to determine if this critical melanocyte gene can be targeted therapeutically.

Studies have also revealed several other genes implicated in melanoma development that are involved in transcriptional control and chromatin modification. Exome sequencing revealed a recurrent mutation in a novel

gene, *TRRAP,* with a role in transcription and DNA repair and complexes with histone acetyltransferases (Wei et al., 2011b). Mutations in *TRRAP* clustered locally, similar to *BRAF*, *PIK3CA*, and *RAS*, suggesting that *TRRAP* may be a new oncogene involved in metastatic melanoma. *TRRAP* mutation occurred in ~4% of the melanomas and mutant *TRRAP* was shown to be essential for cell survival and transformation.

In further regard to chromosomal copy number alterations, functional screening using a zebrafish model revealed that *SETDB1*, which maps to a region of recurrent amplification of human chromosome 1, could cooperate with *BRAF* (V600E) to promote melanoma development (Ceol et al., 2011). *SETDB1* is a histone methyltransferase and contributes to cellular functions involving histone methylation, gene silencing, and transcriptional repression. Alternatively, homozygous deletions in a histone deacetylase, *HDAC4*, have also been documented in metastatic melanoma, although the consequences of its deletion have not been determined (Stark & Hayward, 2007). With increasing exome sequencing reports identifying mutations in histone and chromatin modification genes in cancer (Morin et al., 2011; Varela et al., 2011) it will be interesting to see how mutations of this class contribute to the development of melanoma and whether some are amenable to histone deacetylase inhibition.

3. PERSONALIZED THERAPEUTICS

Recent advances in technology have resulted in an inverse relationship between the associated costs and output capabilities of next-generation sequencing platforms. With this increased capability for the generation of data, it is expected that an avalanche of new genes and mutation events contributing to melanomagenesis will be discovered. This section describes the applicability of sequencing technology and mutation detection within a clinical setting, with particular emphasis on the use of this data in personalized therapeutics.

3.1. Molecularly Based Targeted Strategies

Recent advances in molecularly based targeted drug strategies have begun to show a significant impact on overall survival for patients with metastatic melanoma. Notably, the approval of Vemurafenib in August 2011 was a significant milestone for the melanoma research community and the field of personalized therapeutics. Furthermore, a number of promising molecular

based drugs for use in melanoma are currently under investigation, or are on the horizon; this includes the use of Imatinib or Lapatinib in *KIT* or *ERBB4* mutant melanomas, respectively (Guo et al., 2011; Prickett et al., 2009). However, the successful application of molecularly based targeted drugs within the clinic strongly relies on the correct stratification of patients based on their tumor mutation profiles, essentially guiding drug efficacy and/or resistance (Fig. 13.2).

Oncogenic mutation screens, such as the OncoCarta® and MelaCarta® mutation panels can be used for the identification of clinically relevant mutation profiles within tumors (Dutton-Regester et al., 2012; Thomas et al., 2007). These oncogenic mutation panels have a number of advantages compared to alternative methodologies and benefit from minimal sample requirements, cost effectiveness, and high throughput analysis. The latter is of significance for the successful clinical application of mutation detection; delays in implementing treatment can be a critical factor determining patient

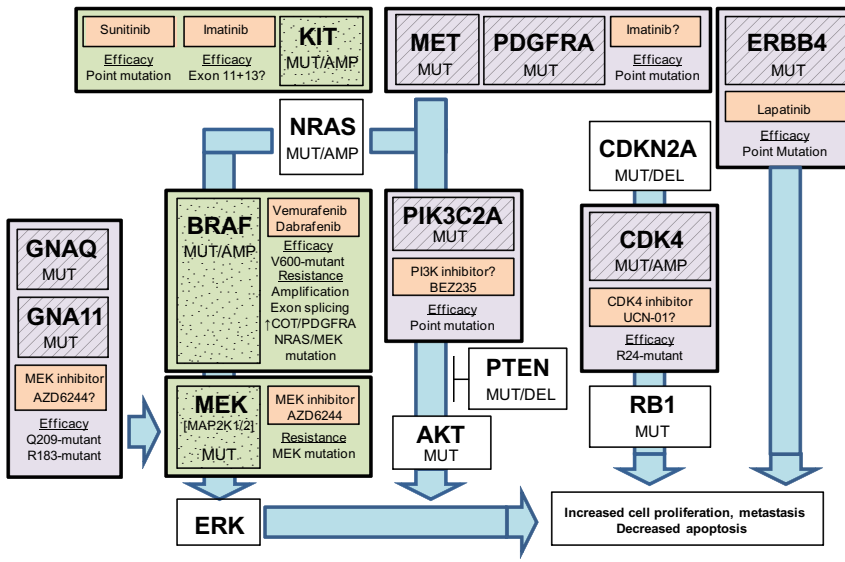

Figure 13.2 The application of genetic data with molecularly based targeted drugs in metastatic melanoma. Mutation profiling of the patient's tumor can be used to determine the appropriate therapeutic approach. For example, if a patient presents with a BRAF V600E mutation, then use of a BRAF inhibitor such as Vemurafenib would be an effective strategy. Green/dotted boxes indicate robust drug targets that have been extensively investigated. Purple/diagonal line boxes indicate emerging targets of significance that need further investigation. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

survival, in particular, for those who have aggressive late stage or disseminated disease.

One consideration that has yet to be comprehensively explored is inter- and intraheterogeneity of tumor specimens within a patient. This includes differences between the mutational evolution of primary to metastatic tumor sites, variability between multiple metastatic deposits throughout the body, or the spectrum of mutations, or subclones present within a given tumor. This was recently addressed through a comprehensive genomic analysis of multiple deposits and tumor sections from biopsies of several patients with renal carcinoma (Gerlinger et al., 2012). Significant tumor heterogeneity was observed, with 63–69% of all the somatic mutations not being present in every tumor, and frequent mutant:wild-type allelic imbalances seen between tumors.

Two main clinical implications arise with the observation of tumor heterogeneity. First, singular biopsy analysis, as is routinely performed within the clinic, may be insufficient for estimating the entire spectrum of mutations within a tumor. Secondly, tumor heterogeneity may result in inappropriate choices of treatment strategies using molecularly based drugs. Thus, the unique capabilities of mutation-screening panels would allow easy and cost effective analysis of multiple, spatially separated biopsies within a single tumor, and/or, the testing of multiple metastatic deposits in a patient. However, it must be noted that in regards to trials investigating Vemurafenib, inter- and intra-heterogeneity of BRAF mutation does not currently appear to be an issue due to the observation of widespread regression of multiple metastatic lesions in patients upon treatment, and the retainment of BRAF mutation in tumors that acquire resistance.

Despite the advantages, mutation-screening panels such as the melanoma specific mutation panel (Dutton-Regester et al., 2012) are limited by their ability to only assess "oncogenic" or specific nucleotide mutation events. Tumorigenesis is a complex interaction of genetic abnormalities contributing to the neoplastic process involving activating oncogenic mutations in combination with inactivating tumor suppressor mutations. The latter, due to the propensity of mutations to occur throughout the entire length of the gene, essentially relies on the use of sequencing technology for the successful identification of all mutations. As such, the use of next-generation sequencing will likely be a desirable platform to comprehensively assess mutation profiles. Indeed, specialist oncology clinics have already begun implementing routine next-generation sequencing to ascertain

therapeutically relevant mutations within individual patient tumors in order to personalize treatments (Roychowdhury et al., 2011).

Although the clinical promise of next-generation sequencing seems achievable with existing technology, a number of technical limitations have yet to be solved before it is likely to be widely adopted by oncology clinics (reviewed in more detail by Dancey et al., (2012) and Desai & Jere, (2012)). Of utmost importance to the implementation of any methodology within a clinical setting, and not just concerning the concept of next-generation sequencing, is accuracy.

The clinical laboratory improvement amendments (CLIA) certification (or its equivalent) is a regulatory standard to which all clinical laboratory testing must be adhered. Within these guidelines, strict adherence to set protocols is required to uphold consistent accuracy, reliability, and timeliness of test results. This is highly significant in a clinical diagnostic cancer setting, as patient survival and prognosis is intimately associated with the rapid implementation of efficacious treatment regimens. Thus, any technology used within this arena will require high accuracy with low rates of false positive and false negative calls. This is problematic for current next-generation sequencing platforms where high throughput, which is desirable in a research setting, offsets the rate of accuracy. Although excessive coverage increases the rate of accuracy, this in itself poses a number of issues, particularly the additional cost and associated bioinformatic processing time. Platform-specific biases must be also considered and is why, if possible, combinations of technologies can significantly improve data quality and output.

Reliability is another critical issue of concern for the implementation of next-generation sequencing platforms. As stated above, strict adherence to protocols is required in order to maintain accuracy and consistency. Due to the rapidly progressive nature of sequencing technology, upgrades to machines or improvements to sequencing chemistry are consistently being released to increase data output and reduce sequencing costs, sometimes at biannual frequency. This is problematic in a CLIA setting due to the investment of time and expense required for the establishment of standardized workflows and procedures. Other technical considerations include the adoption of automated library preparation to reduce labor-intensive procedures and to improve reliability, the standardization of bioinformatic analysis methods, and the current need for independent platform validation of identified mutations.

These concerns aside, another debate currently exists into what sequencing depth, or coverage, should be required for use in a clinical setting, specifically, whether to analyze patient samples with whole-genome, exome, or targeted gene sequencing strategies. Regardless, it must be noted that in relation to acquired drug resistance using existing molecular based targeted therapies, genetic testing alone will be insufficient to comprehensively determine all mechanisms of resistance. For example, Vemurafenib resistance in *BRAF*-mutant melanomas includes acquired mutations in *MEK* and *NRAS*, differential splicing and amplification of *BRAF* and upregulation of tyrosine kinases such as PDGFRA and COT1 (Emery et al., 2009; Jiang et al., 2011; Nazarian et al., 2010; Poulikakos et al., 2011; Villanueva et al., 2010; Wagle et al., 2011). Determining these mechanisms will require analysis using multiple platforms, and for some, nonsequence based analytical approaches.

Although it is hard to make conclusive predictions due to the regular and rapid advances in the sequencing industry, it is unlikely that widespread adoption of next-generation sequencing within the clinic will occur for at least another 5 years. However, during these interim years, analysis will most likely concentrate on the identification of mutations with known clinical significance to existing molecularly based targeted drug strategies. Although it would be ideal to utilize next-generation sequencing throughout all stages of clinical presentation of melanoma, routine use of this technology for the majority of oncology clinics will most likely be restricted to metastatic disease, due to the cost, bioinformatic needs, and labor (Fig. 13.3).

3.2. Immunological Approaches

Alongside the recent success of molecularly based targeted drugs such as Vemurafenib (Chapman et al., 2011), and CTLA4 inhibition with Ipilimumab (Hodi et al., 2010), a number of alternative strategies are currently being investigated. One approach that is showing promising results in patients with metastatic melanoma is adoptive cell therapy (ACT) with use of tumor infiltrating lymphocytes (TIL) (Rosenberg et al., 2008). This strategy involves autologous TIL isolation and cultivation *in vitro* with IL-2, selection of tumor-reactive cultures in matched tumor cell lines, then systemic reintroduction of cultured reactive TILs (Dudley et al., 2003). Patients will also typically undergo lymphodepletion regimens during cell preparation as this method results in long-lasting responses (Dudley et al., 2005). Using this therapeutic approach, complete responses in 20 of 93

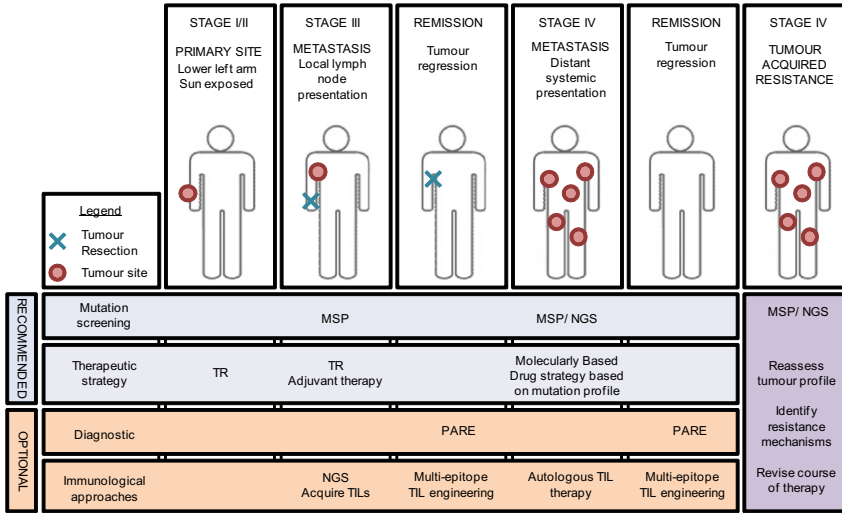

Figure 13.3 Flow diagram depicting the use of mutation profiling in a personalized therapeutic setting. Above describes the presentation of a progressive disease scenario of a patient with metastatic melanoma. Below details current approaches for integrating personalized medicine into a clinical setting following the course of treatment, including tumor-acquired resistance. MSP: melanoma specific mutation panel, NGS: next-generation sequencing strategies (whole genome, exome, or targeted), TR: tumor resection, PARE: personalized analysis with rearranged ends, and TIL: tumor infiltrating lymphocyte. For color version of this figure, the reader is referred to the online version of this book.

(22%) metastatic melanoma patients were observed, with 19 patients still alive 3 years posttreatment (Rosenberg et al., 2011).

Despite these impressive results, a number of limitations to the method have been outlined and it is recognized that TIL therapy will only be available for ~50% of all melanoma patients (Rosenberg et al., 2011). This is largely due to the requirement of clinical resection of tumor nodules of at least 2 cm in diameter in order to obtain sufficient TILs, and the subsequent isolation of sufficient tumor-reactive lymphocytes. Recently, the efficacy of ACT acquiring TILs using ultrasound-guided needle biopsy in 11 patients was performed; although this was a small cohort, 4 patients demonstrated objective clinical responses, highlighting the efficacy of this less invasive, less expensive approach (Ullenhag et al., 2011). However, the true clinical benefit of this technique needs to be determined through testing in a larger set of patients and, as mentioned earlier, the significance of intertumor heterogeneity may need to be determined for this specific application (Gerlinger et al., 2012).

A promising alternative that circumvents the need for cultivation of TILs from a dissected tumor mass is the genetic engineering of peripheral blood lymphocytes (Morgan et al., 2006). This strategy can also overcome the difficulty of identifying tumor reactive TILs and involves the manipulation of blood lymphocytes to react to specific antigens presented within the tumor. A seminal paper released by Morgan et al. (2006), reported the engineering of peripheral blood lymphocytes to recognize MAGE-1, a melanoma differentiation antigen, in order to replicate immunogenicity with autologous TILs. Although the response rate with modified blood lymphocytes (2 of 15 patients, or 13%) did not achieve the same level of efficacy as the autologous TIL approach (~50%), further research efforts into improvements of the technique may reduce the disparity between these methods.

The promising results of ACT with TILs may benefit from concurrent use of next-generation sequencing technologies. It has been shown that point mutations can generate novel epitopes, such as *NRAS* Q61R in melanoma (Linard et al., 2002), and elicit strong immunogenic responses in cancer patients. As such, it may be possible to harness next-generation sequencing to comprehensively identify mutations in tumors in order to develop highly specific, individualized, and engineered TILs from a cocktail of mutation-derived epitopes (reviewed in more detail by Nelson, (2011)).

A number of reports have begun to determine the efficacy and details of such an approach; this includes a recent comprehensive investigation of T cell antigen specificity in human melanoma (Sick Andersen et al., 2012). In this approach, a list of all known melanoma-associated antigens were compiled and tested for immunogenicity against 63 TIL cultures from 19 patients. A total of 175 tumor-associated antigens that included mutated and overexpressed antigens, as well as those involved in differentiation and cancer-testis/onco-fetal origin, resulted in 90 responses against 18 epitopes. Notably, the majority of the responses was derived from differentiation antigens and not from mutant epitopes; however, the authors failed to assess the mutation status of the tumors from which the TILs were isolated and this may explain the lack of response with this class of antigen.

Building on this finding, Castle et al. (2012) assessed the mutanome of B16F10 murine melanoma cells for its ability to generate an immunogenic response, specifically, in the context of establishing a multi-epitope tumor vaccine. Next-generation sequencing revealed a total of 962 non-synonymous point mutations, of which, 563 were within genes that were highly expressed. Immunization of mice with long peptides containing 50 of

the validated mutations resulted in one-third eliciting *in vivo* immunogenic responses (16 of 50); furthermore, 60% of the responders showed preferential sensitivity of mutant epitope compared to wild-type sequence. In addition, mutant peptide immunization *in vivo* conferred tumor control, indicating the efficacy of single amino acid alterations as epitopes in a therapeutic vaccine setting.

These results provide a proof of principle for the potential application of personalized, molecularly engineered TILs that are specific to an individual's tumor mutation profile. However, the application of this strategy has some important considerations and limitations. First, the sequencing technology required to identify mutant epitopes eliciting an immune response is currently time-consuming and laborious; however, the improvement of sequencing technologies and bioinformatic analyses should reduce the impact of this process. One interesting possibility is the curation of a database containing documented immunogenic mutant epitopes, observed experimentally or within the clinic, for the rapid identification of targets for vaccine design. This is exemplified in the aforementioned study where a previously identified epitope in ACTN4 (Echchakir et al., 2001) was replicated in the mutanome study of B16F10 (Castle et al., 2012). Another potential limitation is the loss of expression or clonal deletion of mutant epitopes within the tumor, including evolutionary selection pressures at play during the course of therapy. Multi-epitope therapeutic vaccine design has the potential to overcome this problem; however, this approach requires further investigation.

One potential advantage for personalized TIL therapy is that discerning the difference between driver and passenger nonsynonymous mutations should not be necessary as both can elicit immunogenic reactions. This was demonstrated in the B16F10 mutanome study where one of the strongest reactions specific to a mutant epitope was a K739N mutation in KIF18B; this mutation does not localize to any functional or conserved domain and most likely represents a passenger mutation (Castle et al., 2012). If so, this shows promise for immunological therapies as it expands the potential pool of mutant epitopes available; even more so for melanoma where the intrinsic rates of mutation are considerably higher than other cancers due to carcinogenic exposure to solar UVR.

Expanding the suite of treatments available for metastatic melanoma will act positively on rates of overall survival and help overcome issues of therapeutic resistance or tumor remission; any therapy that shows an improvement in overall survival or clinical activity will warrant further investigation.

As TIL therapy has already demonstrated robust responses in patients who have undergone multiple refractive therapeutic treatments, including dacarbazine and Ipilimumab (Rosenberg et al., 2011), first line treatment regimens concurrent with TIL preparation may be an effective strategy for improving overall survival (Fig. 13.3). In this case, if the first line of treatment fails, TIL therapy can be administered rapidly thereafter as strong immunogenic personalized TILs will by then have been established; however, this would be at considerable expense but may represent an effective short-term strategy until robust drug combinations are implemented. The combination of individualized TIL therapy through the identification of tumor-specific epitopes using next-generation sequencing is an exciting prospect for future treatment of patients with metastatic melanoma; however, this approach requires further research.

3.3. Diagnostic Applications

Next-generation sequencing technology has demonstrated value in personalized biomarker identification for the clinical management of patients (Leary et al., 2010). In this study, Leary and colleagues utilized massively parallel sequencing to identify chromosomal translocation events in a method called "personalized analysis of rearranged ends" or PARE. In this process, fusion events in solid cancers were initially identified using PARE, before sensitive digital polymerase chain reaction (PCR) assays were designed to detect these rearrangements from circulating DNA in patient plasma samples. This approach was highly sensitive, and able to detect rearrangements at a frequency of 0.001% in sample material also containing normal DNA.

The application of PARE in a series of plasma samples taken throughout the course of a patient's therapy highlighted the potential benefits of this approach in a clinical setting. Levels of the identified rearrangement detected in circulating DNA from plasma showed a significant decrease after primary resection, an increase after metastatic dissemination, and a decrease after the commencement of chemotherapy; effectively, levels of the detected rearrangement in plasma closely followed the tumor burden within the patient. As such, PARE could provide an effective and highly sensitive method to determine disease progression following treatment. In the assessment of tumor acquired drug resistance, PARE may detect patient relapse more rapidly than conventional approaches such as computed tomography (CT) scans; however, this has yet to be determined.

Reciprocal to the potential of personalized TIL therapy, the need for discerning driver and passenger translocations is largely negligible; the only requirement is retained tumor expression of the fusion gene throughout the course of treatment. It is interesting to speculate whether point mutations identified through next-generation sequencing may act as superior biomarkers to PARE. Although single-base mutations can appear as artifacts through the introduction of errors via PCR, simultaneous analysis of multiple mutation events within the tumor may increase accuracy while circumventing the issue of clonal selection or heterogeneity within the tumor (Fig. 13.3). Further research into the application of next-generation sequencing in biomarker identification may have considerable significance for managing patient therapy in the clinic.

3.4. Melanomas Arising in Different Tissues

As mentioned previously, melanomas of uveal origin have distinct mutation profiles compared to cutaneous melanomas (Harbour et al., 2010). Furthermore, evidence from exome sequencing has indicated stark differences between the overall rate of mutation between uveal and cutaneous melanomas, with an average of 27 and 250 nonsynonymous mutations in coding regions, respectively (Harbour et al., 2010; Nikolaev et al. 2012; Stark et al., 2012; Wei et al., 2011b) (Fig. 13.4).

A small proportion of melanomas (~5%), although of cutaneous origin, occur in typically non-UV-exposed regions of the body such as the palms or soles and are classified as a distinct subtype of melanoma (acral). Recently, the first glimpse into the genetic architecture of acral melanoma was reported through the whole-genome sequencing of a chemo-naïve primary acral melanoma and its matched lymph node metastasis (Turajlic et al., 2012). Not surprisingly, the total number of nonsynonymous mutations detected in these tumors was 40, about 10-fold less than that of the observed mutation rates of sun-exposed cutaneous melanomas. This rate of mutation is also consistent with the observed rates in noncarcinogen exposed solid tumors such as breast (Shah et al., 2009) and prostate cancer (Berger et al., 2011).

The lower rates of mutation in uveal and acral melanomas may have a significant impact on the application of personalized therapeutics. First, the low mutation rate may be of benefit in the identification of driver mutations as there will be an inherently lower proportion of passenger mutations. However, in the same context, the lower number of mutations may also limit or reduce the number of therapeutic targets applicable for intervention

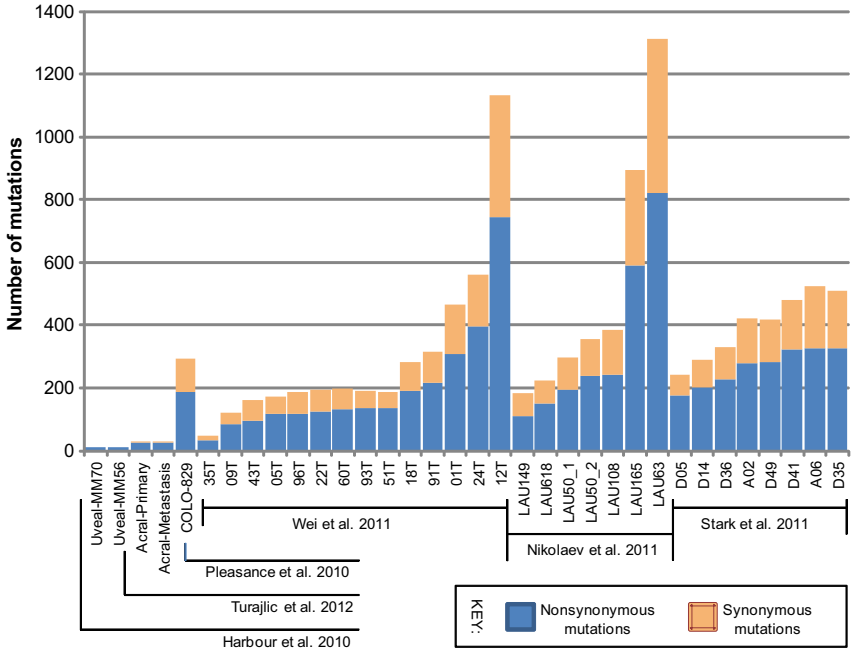

Figure 13.4 Number of mutations in coding regions identified from whole genome and exome sequencing studies of metastatic melanoma tumors and cell lines. Uveal and acral melanomas (labeled accordingly) have a low mutation rate compared to cutaneous melanomas. This is likely to have significance for personalized therapeutic strategies in regard to driver mutation events, diagnostic applications, and immunological approaches such as engineered autologous tumor infiltrating lymphocyte therapy. For color version of this figure, the reader is referred to the online version of this book.

within the tumor. In addition, application of engineered TIL therapy and personalized diagnostics, as described in the previous sections, will likely be greatly reduced for these molecular subtypes. Further investigation using exome sequencing strategies in a larger number of tumors are required before the clinical significance of different mutation rates in these rare subtypes of melanomas is understood.

3.5. Identification of Therapeutic Targets Not Directly Amenable to Therapeutic Intervention

The rate of mutation in cutaneous melanoma is high; although the majority of these mutations represent passenger events, it is still undetermined how many driver mutations are required for melanomagenesis. A proportion of nonsynonymous mutations in genes, such as *BRAF* V600E, are amenable to

therapeutic intervention through the design of mutation specific inhibitor strategies, or via high throughput chemical drug screens. However, it has been suggested that in regards to the "druggability" of proteins within the human genome, only ~10% of the genes can be targeted effectively with traditional pharmaceutical drug design (Hopkins & Groom, 2002; Southan et al., 2011). As such, a significant number of mutations identified from large scale cancer genomic studies, even if responsible for driving tumorigenesis, will not be able to be directly targeted therapeutically. This raises an important issue for the application of personalized molecularly based medicine in a clinical setting, particularly for the subset of patients whose mutation profile does not present with druggable targets.

One strategy to address the abovementioned problems is the use of pathway analysis and requires an understanding of the functional role that mutations play within signaling networks. In this sense, it is theoretically possible to achieve therapeutic success by targeting genes upstream or downstream of the mutant gene in question. An example of this approach has recently been suggested with the inhibition of ERK1/ERK2, proteins downstream of BRAF and MEK in the MAPK pathway, with the use of shRNA (Qin et al., 2012). Although for 50% of the melanoma patients, the MAPK pathway can be targeted through use of BRAF inhibitors, due primarily to the presence of *BRAF* V600E mutations, patients with *NRAS* mutations, or who are *BRAF/NRAS* WT, are currently refractive to this therapeutic approach despite constitutive activation of the MAPK pathway. In the study by Qin et al. (2012) *in vitro* inhibition of *ERK1/ERK2* in *BRAF*-mutant A375 melanoma cells was more effective at promoting apoptosis than BRAF inhibitors, such as PLX4032. Although the effect of *ERK1/ERK2* inhibition on *BRAF* WT melanomas was not assessed, this approach may be an effective strategy in this subset of melanoma patients.

Despite the ability to target downstream or upstream members of biologically important pathways in an experimental *in vitro* setting, a number of issues are raised when this concept is considered in a clinical setting. One approach that has gathered significant interest since their identification is the use of siRNA knockdown strategies to inhibit overactive protein activity, or signaling networks. Although siRNAs are effective within *in vitro* cell culture experiments, delivery of the siRNA becomes difficult *in vivo* as current approaches are ineffective. However, significant research in improving the delivery is currently underway and is beginning to demonstrate clinically actionable results in melanoma (Davis et al., 2010; Lee et al., 2012). It will be interesting to see how the application of pathway analysis and inhibitor-

based strategies will affect treatment options in the future; however, an extensive understanding of the biology of the targeted pathways will be required before success with these approaches is achieved.

4. CONCLUSION

Melanoma is an aggressive cancer that accounts for nearly all skin cancer related mortality; this is largely due to late stage or disseminated melanoma, which has been refractive to traditional chemotherapeutic strategies. However, recent success with molecularly based targeted drugs in metastatic melanoma, such as Vemurafenib in patients with *BRAF* V600E mutations, has begun to demonstrate an improvement in overall survival and supports the use of "personalized medicine" within the clinic. Thus, understanding the genetic mechanisms of metastatic melanoma development will ultimately lead to the establishment of novel drug strategies, while also improving existing therapeutic approaches in treating this disease.

As the mutation events contributing to melanomagenesis are increasingly identified and their subsequent significance to current or novel drug strategies determined, the utilization of this information will progressively move from a research setting toward routine clinical applications. For instance, the development of a melanoma specific mutation panel is one such example of how genetic information could be used to guide efficacious treatment strategies with molecularly based targeted drugs. However, a number of ethical issues and technical considerations will need to be discussed before the use of mutation-screening panels or next-generation sequencing platforms can be implemented into routine use within oncology clinics. This aside, a number of specialist clinics are currently embracing this technology and no doubt, will contribute to the development of the standardized practices required for the widespread adoption of this technology.

The introduction of next-generation sequencing to cancer genetics has significantly progressed our understanding of the genetic mechanisms of melanoma development and will hopefully lead to improved therapeutic outcomes for patients with this disease.

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ABBREVIATIONS

ACT adoptive cell therapy
ADAM a disintegrin and metalloproteinase
ADAMTS a disintegrin and metalloproteinase with thrombospondin domains
CLIA clinical laboratory improvement amendments
DTIC dacarbazine
GPCR G protein coupled receptor
GTPase guanosine triphosphatases
IFN-α interferon alpha
IL-2 interleukin-2
MMP matrix metalloproteases
MSP melanoma specific mutation panel
MTIC 5-(3-methyl-1-triazeno)imadazole-4-carboxamide
PARE personalized analysis of rearranged ends
RTK receptor tyrosine kinase
TIL tumor infiltrating lymphocyte
TR tumor resection
UVR ultraviolet radiation

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CHAPTER FOURTEEN

Targeted Therapy for Gastric Adenocarcinoma

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Abstract

Gastric cancer (GC) is one of the leading causes of cancer death worldwide. Despite significant improvement in understanding disease biology and recent improvements in surgical outcome, radiation techniques, and chemotherapy, the 5-year survival rates remain divsmal.

Several pathways related to cell proliferation, invasion, and metastasis have been identified and evaluated as candidates for targeted treatment but despite promising preclinical data, the majority of targeted agents failed to improve outcome in this disease. Recently, adding Trastuzumab—a HER-2 monoclonal antibody—to cisplatin-based chemotherapy in patients with HER-2 overexpressing gastric and gastroesophageal junction (GEJ) adenocarcinoma resulted in statistically significant improvement in response rate, progression-free survival, and overall survival in phase III trial.

We have reviewed the different pathways relevant to gastric cancer development with focus on the recent advances in targeting these pathways in order to improve outcomes in this disease.

1. INTRODUCTION

Gastric cancer (GC) is one the most common cancers and a leading cause of cancer-related mortality worldwide. Despite considerable improvement in diagnosis, surgical techniques, and multidisciplinary therapy, the clinical outcome for advanced GC remains poor with 5-year overall survival rates between 5 and 15%. In the metastatic setting, chemotherapy remains the cornerstone of palliation with dismal prognosis. The development of new treatment to be combined with cytotoxic treatment is an urgent priority. Treatment with combination of three chemotherapy agents might lead to modest improvement in survival compared to two-agent regimen but at the expense of toxicity (Ajani et al., 2007).

Targeted agents have emerged as a new treatment strategy to improve outcomes in colon, lung, and breast cancer among others. Molecules related

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to cell proliferation, invasion, and tumor metastasis have been studied in GC and agents targeting these molecules have been evaluated in preclinical setting and are rapidly moving to patient testing. The proposed agents will target vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) including human epidermal growth factor type 2 (HER2), insulin-like growth factor 1 receptor (IGF-1R), and P13k/Akt/mTor pathway, as well as other pathways including c-Met pathways, fibroblast growth factor receptor, etc.

The ToGA trial (Bang et al., 2010) comparing trastuzumab plus cisplatin-based chemotherapy with chemotherapy alone in patients with HER2-positive advanced gastric or gastrointestinal junction (GEJ) cancer demonstrated that adding trastuzumab leads to better overall survival. These results have opened the door to other molecular targeted agents in the treatment of GC.

During recent years, many molecular abnormalities underlying gastric carcinogenesis and progression have been identified. This improved our understanding of the biology of GC and stimulated the search for novel therapeutic approaches in this disease. This chapter discusses the molecular targets and the novel drugs currently in development in patients with GC.

2. MOLECULAR TARGETS IN GASTRIC CANCER

The development of GC involves multiple genetic and epigenetic alterations, chromosomal aberrations, gene mutations, and altered molecular pathways. Some of the molecular abnormalities and signaling pathways are amenable to pharmacological interventions (Fig. 14.1). Multiple agents targeting these pathways are now in clinical development and some are being tested in patients with GC (Table 14.1).

2.1. Cell Surface Receptor Inhibitors

2.1.1. Vascular Endothelial Growth Factors Inhibitions (Anti-Angiogenesis)

Angiogenesis is an important aspect of tumorgenesis and is critical for tumor growth and survival. VEGF plays a pivotal role in the control of angiogenesis, tumor growth, and metastasis in most human cancers (Carmeliet, 2003) including GC, which makes it an attractive target for treatment. VEGF-A is an essential mediator of physiologic and pathologic angiogenesis (Ferrara et al., 2003), and its activities are mediated by two tyrosine kinase

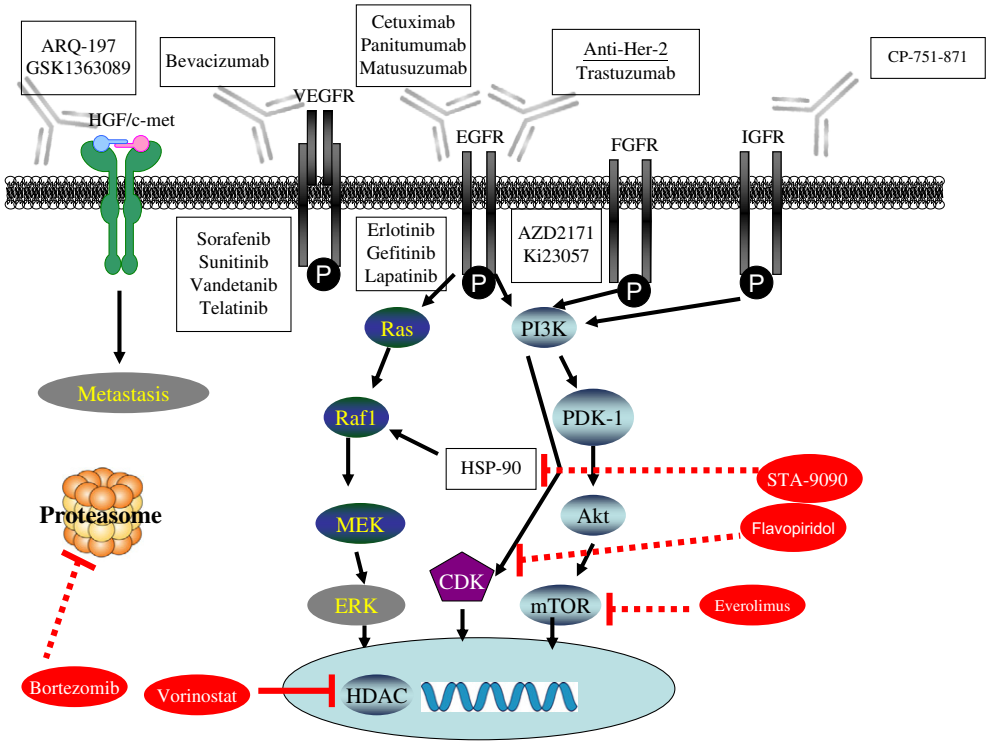

Figure 14.1 Diagram of the signal transduction pathways in gastric cancer and targeted agents. EGFR: epidermal growth factor receptor, VEGFR: vascular endothelial growth factor receptor, IGF-1R: insulin-like growth factor 1 receptor, HER-2: human epidermal growth factor type 2, FGFR: fibroblast growth factor receptor, mTOR: mammalian target of rapamycin, ERK: extracellular signal-regulated kinase, PI3-kinase: phosphatidylinositide-3-kinase, PDK: pyruvate dehydrogenase kinase, HGF: hepatocyte growth factor, HDAC: histone deacetylase, HSP-90: heat shock proteins. For color version of this figure, the reader is referred to the online version of this book.

Table 14.1 Targeted Agents and Clinical Trials for Gastric and Gastroesophageal Cancer

Drugs and Their Targets	Agents	Clinical Trials
Cell surface receptor inhibitors		
VGFR inhibitors		
Monoclonal antibody	Bevacizumab	Phase III
Receptor tyrosine kinase	Sunitinib	Phase II
	Sorafenib	Phase I/II
	Vandetanib	Phase I/II
	Telatinib	Phase II
EGFR inhibitors		
Monoclonal antibody	Cetuximab	Phase III
	Panitumumab	Phase III
	Matuzumab	Phase I/II
Receptor tyrosine kinase	Gefitinib	Phase II
	Erlotinib	Phase II
HER-2 inhibitors		
Monoclonal antibody	Trastuzumab	Phase III
Receptor tyrosine kinase	Lapatinib	Phase II
IGF-1R inhibitors		
Monoclonal antibody	CP-751-871	Phase I
c-Met inhibitors		
Receptor tyrosine kinase	GSK1363089	Phase II
	ARQ197	Phase I/II
FGFR inhibitors		
Receptor tyrosine kinase	Ki23057	Preclinical
	AZD2171	Phase I
Cell cycle inhibitors		
Aurora kinase inhibitors		
	SNS-314	Phase I
	AT9283	Phase I
Polo-like kinase inhibitor		
	GSK461364	Phase I
Cyclin-dependent kinase inhibitor		
	Flavopiridol	Phase I

Table 14.1 Targeted Agents and Clinical Trials for Gastric and Gastroesophageal Cancer—cont'd

Drugs and Their Targets	Agents	Clinical Trials
Downstream inhibitors		
PI3Kinase inhibitors		
	Everolimus	Phase I, II
Heat shock protein 90 inhibitor		
	STA-9090	Phase I
Ubiquitin—proteasome pathway inhibitor		
	Bortezomib	Phase II
Others		
Matrix metalloproteinases (MMPs)		
	Marimastat	Phase III
Histone deacetylase inhibitor		
	Vorinostat	Phase I
Protein kinase C inhibitor		
	Bryostatin	Phase II

VEGFR: vascular endothelial growth factor receptor, EGFR: epidermal growth factor receptor, HER2: human epidermal growth factor receptor type 2, IGF: insulin-like growth factor, FGFR: fibroblast growth factor, PI3K: Phosphatidylinositol 3-kinases, HGF: hepatocyte growth factor.

receptors, VEGFR-1 and VEGFR-2. Serum VEGF concentration has been related to metastasis and worse outcome in GC and GEJ tumors (Karayiannakis et al., 2003; Maeda et al., 1994).

Multiple strategies have been developed to target the VEGF pathway including monoclonal antibodies and tyrosine kinase inhibitors.

2.1.1.1. Bevacizumab

Bevacizumab is a recombinant humanized IgG1 monoclonal antibody against VEGF. It has been extensively evaluated alone and in combination with chemotherapy in many solid tumors. It significantly enhances the antitumor efficacy in colorectal (Hurwitz et al., 2004), lung (Sandler et al., 2006), ovarian (Cannistra et al., 2007), renal cell (Escudier et al., 2007b), and breast cancer (Miller et al., 2007).

Multiple phase II trials have evaluated bevacizumab in the treatment of GC as well as GEJ tumors; combining bevacizumab with irinotecan and

cisplatin in 47 patients with metastatic gastric and GEJ cancer resulted in response rate of 65% in the 34 patients with measurable disease. Median survival was 12.3 months, whereas 25% of the patients had thromboembolic events (Shah et al., 2006).

Another study of oxaliplatin, docetaxel, and bevacizumab was performed in 38 previously untreated patients with locally advanced or metastatic GC and GEJ tumors; median progression-free survival (PFS) was 6.6 months and median survival 11.1 months. Gastrointestinal perforation occurred in three patients (El-Rayes et al., 2010).

Combination of modified DCF (docetaxel, cisplatin, and 5-fluorouracil [5-FU]) and bevacizumab in 44 patients with metastatic GC and GEJ tumors resulted in response rate of 67% and median overall survival of 16.8 months. Venous thromboembolism was seen in 39% of the patients (Shah et al., 2011a).

Another phase II trial combining bevacizumab with 5-FU, leucovorin, and oxaliplatin (FOLFOX) was conducted (Cohenuram & Lacy, 2008); out of the 16 patients enrolled, 10 patients (63%) achieved a partial response (PR) and 6 patients (37%) achieved minor response or disease stabilization. The median time to progression (TTP) and overall survival (OS) were 7 and 8.9 months, respectively. There was no observed bevacizumab-related toxicity such as perforation or thrombotic events. These trials are summarized in Table 14.2.

The promising results of the phase II trials led to the AVAGAST (Avastin in Gastric Cancer) trial (Ohtsu et al., 2011). This was the first multinational, randomized, placebo-controlled trial to evaluate the efficacy of adding bevacizumab to cisplatin-based chemotherapy in the first-line treatment of advanced gastric cancer. As many as 774 patients from 93 centers in 17 countries were enrolled; approximately 50% of the patients were from Asian countries. Median OS was 12.1 months with bevacizumab plus chemotherapy versus 10.1 months with placebo plus chemotherapy (hazard ratio 0.87; 95% Confidence interval CI, 0.73–1.03; $P = 0.1002$). Both median PFS and overall response rate were significantly improved with bevacizumab versus placebo. No bevacizumab-related safety signals were identified. The trial did not reach its primary objective.

In summary, the addition of bevacizumab to chemotherapy is safe and effective in gastric cancer; however, the phase III trial was negative for the improvement in OS.

The heterogeneity of gastric cancer might explain the discordant results between phase II and III trials; it is worth noting that in patients with GEJ

Table 14.2 Clinical Trials Targeting VEGFR in Gastric and GEJ Tumors

Study	Phase	Agent(s)	*n*	ORR	TTP	OS
Shah et al.	II	Bevacizumab + CDDP/CPT-11	47	65	8.3	12.3
El-Rayes et al	II	Bevacizumab + docetaxel/oxaliplatin	8	50	NA	NA
Enzinger et al.	II	Bevacizumab + docetaxel/CDDP/CPT-11	32	63	NA	NA
Kelsen et al.	II	Bevacizumab + docetaxel/CDDP/5-FU	44	67	12	16.2
Jhawer et al.	II	Bevacizumab + docetaxel/CDDP/5-FU	42	64	NA	NA
Ohtsu et al.[a]	III	Bevacizumab + Cisplatin + 5-FU	774	[a]29/38	[a]5.3/6.7	[a]10/12
Bang et al.	II	Sunitinib (second-line)	42	5	4.3	12.7
Moehler et al.	II	Sunitinib (second-line)	38	5	1.5	6.3
Kim et al.	I	Sorafenib + capecitabine/CDDP	21	63	10	14.7
Sun et al.	II	Sorafenib + docetaxel/CDDP	44	39	5.8	13.6

ORR: objective response rate, *n*: sample size, TTP: time to progression, OS: overall survival, CDDP: cisplatin, CPT-11 irinotecan, NA: not applicable, 5-FU: 5-flurouracil.
[a]This was a randomized phase III trial. OR, TTP, and OS for patient without and with Bevacizumab, respectively

tumors in the AVAGST study treated on the bevacizumab arm, response rate was exceptionally high (85%) and survival rate was improved. On the other hand, Asian population showed better outcome in regard to OS and PFS regardless of the treatment received when compared to European and Americans. Selection bias, sample size, and study design might have limited the conclusions of single-arm phase II studies.

In order to identify patients who might benefit from anti-VEGF therapy, a panel of tumor angiogenic factors was evaluated in the AVAGAST study; five angiogenic markers were evaluated: EGFR, VEGF-A, VEGFR-1, VEGFR-2, and neuropilin (NRP) (Shah et al., 2010). Low tumor neuropilin expression was associated with shorter OS in the placebo group. Adding bevacizumab seems to correct this effect: patients with low tumor neuropilin, a coreceptor for VEGF-A, had an OS treatment hazard ratio numerically better than those with high neuropilin (low NRP HR 0.75; 95% CI 0.59–0.97; high NRP HR 1.07; 95% CI 0.81–1.40). It was concluded that neuropilin appeared to be a prognostic and a promising

biomarker candidate with the potential to predict clinical outcome in bevacizumab-treated patients. In addition, lower baseline plasma VEGF-A correlated with longer OS. Further evaluation is ongoing.

A different approach in targeting VEGF pathway is through tyrosine kinase inhibitors which inhibit VEGF receptor among others (i.e., Flt-3, c-kit, RET, etc.).

Several tyrosine kinase inhibitors have been approved for the treatment of solid tumors and some are currently being evaluated in gastric cancer.

2.1.1.2. Sunitinib

Sunitinib is an oral, multitargeted tyrosine kinase inhibitor of VEGFR, platelet-derived growth factor receptors (PDGFRs), c-kit, RET, and Flt3 that has been approved for the treatment of advanced renal cell carcinoma (RCC) and imatinib-resistant or imatinib-intolerant gastrointestinal stromal tumors (GIST).

Several trials have evaluated sunitinib in the treatment of gastric cancer; a phase II trial of single agent sunitinib in 78 patients with advanced gastric and GEJ cancer who received sunitinib as second line showed promising results; 2 patients had partial responses and 25 patients had stable disease for $\geq$6 weeks. Median PFS was 2.3 months and median OS was 6.8 months (95% CI, 4.4–9.6 months). Grade $\geq$ 3 thrombocytopenia and neutropenia were reported in 34.6% and 29.4% of the patients, respectively, and the most common nonhematologic adverse events were fatigue, anorexia, nausea, diarrhea, and stomatitis (Bang et al., 2011). Another phase II study in 52 pretreated patients with advanced GC reported that sunitinib (50 mg/day for 4 weeks, followed by 2 weeks rest) was well tolerated (Moehler et al., 2011a). In the intention to treat population, the objective response rate (ORR) was 3.9%, median PFS was 1.28 months, and median OS was 5.81 months. In subgroup analyses, VEGF-C expression in the tumor was associated with significantly shorter median PFS but there was no difference in tumor control rate.

Similar to other TKIs, sunitinib has multiple drug interactions by enhancing QTc prolongation, and increasing or decreasing the metabolism of CYP3A4 substrates. Common toxicities include hypertension, hand–foot syndrome, and liver dysfunction.

2.1.1.3. Sorafenib

Sorafenib is a potent inhibitor of Raf tyrosine kinase and several other receptor tyrosine kinases, including VEGFR-2, VEGFR-3, and PDGFR-β.

Sorafenib has been approved for the treatment of RCC and hepatocellular carcinoma based on phase III trials (Escudier et al., 2007a; Llovet et al., 2008). In tumor xenografts models, sorafenib effectively inhibited tumor growth and angiogenesis in gastric tumors (Yang et al., 2009).

Sorafenib has been evaluated for the treatment of GC in several studies; when combined with capecitabine and cisplatin in a phase I trial (Kim et al., 2012) as first-line therapy, the objective response rate was 62.5% and the median PFS and OS were 10.0 and 14.7 months, respectively. Another phase II study of 44 patients combined sorafenib with docetaxel and cisplatin; in this trial, the median PFS was 5.8 months and the median OS was 13.6 months (Sun et al., 2010).

Another phase II trial of sorafenib in patients with metastatic GC and GEJ monotherapy is still accruing patients.

2.1.1.4. Vandetanib (ZD6474)

Vandetanib is a dual VEGFR and EGFR tyrosine kinase inhibitor, and it also inhibits RET-tyrosine kinase activity, an important growth driver in certain types of thyroid cancer. In 2011, vandetanib became the first drug to be approved by the Food and Drug Administration (FDA) for treatment of metastatic medullary thyroid cancer. In orthotopic gastric cancer model, vandetanib inhibits tumor growth, decreases microvessel density, and slows down tumor cell proliferation (McCarty et al., 2004).

A recently reported phase I trial (Astsaturov et al., 2012) evaluating vandetanib plus paclitaxel, carboplatin, 5-FU, and XRT induction therapy followed by surgery for previously untreated locally advanced cancer of the esophagus and GE junction found that targeting VEGFR/RET/EGFR in combination with induction chemotherapy is well tolerated and with promising clinical activity warranting further phase II evaluation.

This compound is currently being investigated in a phase I/II trial in combination with docetaxel alone or in combination with oxaliplatin in GC.

2.1.1.5. Telatinib

Telatinib is a potent small molecule oral tyrosine kinase inhibitor that selectively targets the VEGF and PDGF receptor families. Telatinib has showed evidence of activity in gastric cancer in early phase trial which led to a phase II study evaluating telatinib in combination with capecitabine and cisplatin as first-line treatment in patients with advanced cancer of the stomach or GE junction (Ko et al., 2010). The preliminary results were

promising, as activity with the combination has been observed. Final report of the study is still pending.

More studies are still investigating the VEGF pathway inhibition in gastric cancer despite the negative results of the AVAGAST trial. Several prognostic and predictive markers to predict clinical outcome in patients treated with VEGF inhibition are in development and expected to make its way to the patient selection and clinical practice. Investigating VEGF pathway inhibitors in the neoadjuvant setting is ongoing as well. In the United Kingdom, The Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial (MAGIC)-B is evaluating the role of adding bevacizumab to perioperative chemotherapy in operable adenocarcinoma of the stomach and gastroesophageal junction.

Ramucirumab, a newer fully human, IgG1 monoclonal antibody specifically and potently inhibits VEGFR-2, has demonstrated efficacy and tolerability that appears more favorable than commercially available anti-angiogenic drugs. Phase II and III trials using ramucirumab as a single agent and in combination with chemotherapy in several tumor types including gastric cancer are ongoing. A study of weekly paclitaxel with ramucirumab in patients with advanced gastric adenocarcinoma was completed and the results will be available soon. A phase III, randomized, double-blinded study of ramucirumab versus placebo and BSC in the treatment of metastatic gastric or gastroesophageal junction adenocarcinoma in second line setting will be launched soon. Another randomized, multicenter, double-blind, placebo-controlled phase III study of weekly paclitaxel with or without ramucirumab in patients with metastatic gastric adenocarcinoma is also planned.

2.1.2. Epidermal Growth Factor Receptor Inhibition

EGFR is a transmembrane glycoprotein receptor for (EGF) family of extracellular protein ligands (Herbst, 2004) and it is overexpressed in several GI malignancies. Ligand binding to the extracellular domain leads to EGFR activation and phosphorylation of the intracellular tyrosine kinase, leading to the activation of Ras/Raf/mitogen activated protein kinase (MAPK) or the Akt/mTOR pathway (Oda et al., 2005). EGFR overexpression presents in 30–50% of all the gastric and GEJ cancers and is associated with poor outcomes (Galizia et al., 2007; Lieto et al., 2008; Wang et al., 2007). The EGFR gene copy number might be a predictive biomarker in this setting.

The most common approaches to inhibit the EGFR are by inhibition of the EGFR via monoclonal antibodies (i.e., cetuximab, matuzumab, and panitumumab) or tyrosine kinase inhibitors (i.e., gefitinib, erlotinib). Both methods have been examined in patients with GC. Off note, in GC, the *K-ras* gene has been reported not to be a useful biomarker for the response to cetuximab (Park et al., 2010).

2.1.2.1. Cetuximab

Cetuximab is an IgG1 type chimeric monoclonal antibody that binds to the extracellular domain of the human EGFR and competitively inhibits the binding of EGF, other ligands, and ligand-induced tyrosine kinase autophosphorylation. This antibody–receptor interaction prevents receptor dimerization and thereby blocks ligand-induced EGFR tyrosine kinase activation. Cetuximab also induces EGFR internalization, downregulation, and degradation (Martinelli et al., 2009).

Cetuximab is currently approved for the treatment of advanced colorectal (Saltz et al., 2007) and squamous cell head and neck cancer (Vermorken et al., 2008).

Cetuximab has been evaluated extensively in phase II studies in patients with advanced gastric cancer as monotherapy or in combination with chemotherapy (Table 14.3). In patients with untreated or recurrent advanced gastric and GEJ cancer, cetuximab was combined with several chemotherapy regimens in different clinical setting with varying results; when combined with FOLFIRI (5-FU, irinotecan, folinic acid) in 38 patients, ORR was 44% and OS was 16 months (Pinto et al., 2007). In combination with FUFOX/FOLFOX (5-FU, oxaliplatin, folinic acid), cetuximab produced an ORR of 65% and OS of 9.5 months (Lordick et al., 2010). Other combinations have been evaluated (Agarwala et al., 2009; Bjerregaard et al., 2009; Chan et al., 2011; Han et al., 2009; Kim et al., 2011; Moehler et al., 2011b; Pinto et al., 2009; Safran et al., 2008; Tebbutt et al., 2008; Woll et al., 2011; Yeh et al., 2009; Zhang et al., 2008) such as carboplatin/paclitaxel, cisplatin/docetaxel, capecitabine/cisplatin, and XELOX (capecitabine and oxaliplatin); response rates ranged between 6 and 69% with an OS between 4.0 and 16.6 months.

Cetuximab-related adverse events were commonly seen in all these trials with infusion-related reactions, skin toxicity, and diarrhea being the most common. Based on the promising efficacy in several phase II studies, a phase III trial has thus been conducted. EXPAND (Erbitux in combination with

Table 14.3 Clinical Trials of EGFR Pathway in Gastric and Esophageal Cancer

Study	Phase	Agent(s)	*n*	ORR	TTP	OS
Pinto et al.	II	Cetuximab + FOLFILI	38	44%	8	16
Lordick et al.	II	Cetuximab + FUFOX	52	65%	7.6	9.5
Safran et al.	II	Cetuximab + Carbo/ paclitaxel/RT	60	27%	NA	NA
Tebbutt et al.	II	Cetuximab + docetaxel	38	6%	2.1	5.2
Ma et al.	II	Cetuximab + CDDP/ CPT-11/surgery	20	0%	NA	NA
Kanzler et al.	II	Cetuximab + IF	49	42%	8.5	16.6
Han et al.	II	Cetuximab + FOLFOX	40	50%	5.5	9.9
Pinto et al.	II	Cetuximab + CDDP/ docetaxel	48	41%	NA	NA
Woell et al.	II	Cetuximab + oxaliplatin/ CPT-11	51	63%	6.2	9.5
Zhang et al.	II	Cetuximab + CDDP/ capecitabine	49	48%	5.2	NA
Yeh et al.	II	Cetuximab + CIV 5-FU/ LV/CDDP	35	69%	11	14.5
Bjerregaard et al.	II	Cetuximab + CPT-11	31	6%	3:2	NA
Kim et al.	II	Cetuximab + XELOX	44	52%	6.5	11.8
Moehler et al.	II	Cetuximab + FOLFILI	49	46%	9	16.5
Lordick et al.	II	Cetuximab + FOLFOX	52	65%	7.6	9.5
Chan et al.	II	Cetuximab	35	3%	1.6	3.1
Rao et al.	II	Matuzumab + ECX	21	65%	5.2	NA
Rojo et al.	II	Gefitinib	75	NA	NA	NA
Dragovich et al.	II	Erlotinib	70	9	2	6.7
Wainberg et al.	II	Erlotinib + FOLFOX	34	50	NA	11

ORR; objective response rate, TTP: time to progression, OS: overall survival, 5-FU: 5-fluorouracil, NA: not applicable, FOLFILI: biweekly bolus 5-FU/leucovorin, irinotecan, infusional 5-FU, FUFOX: weekly oxaliplatin/leucovorin, infusional 5-FU, CDDP: cisplatin, CPT-11, irinotecan, IF: weekly irinotecan, infusional folinic acid/5-FU, FOLFOX: biweekly bolus 5-FU/leucovorin/oxaliplatin and infusional 5-FU, LV: leucovorin, XELOX: capecitabine, oxaliplatin, ECX epirubicin/cisplatin/ capecitabine.

Xeloda and cisplatin in advanced esophagogastric cancer) trial has been completed and recruited more than 870 patients. PFS will be the primary end point for this trial.

2.1.2.2. Panitumumab

Panitumumab is the first fully human immunoglobulin G_2 monoclonal antibody targeting the EGFR. Clinical benefit has been demonstrated in patients with advanced colorectal cancer who do not harbor the *K-ras*

mutation and are unresponsive to standard therapies (Van Cutsem et al., 2007). In gastric cancer, a randomized trial of epirubicin, oxaliplatin, and capecitabine (EOX) with or without panitumumab (REAL-3) is currently recruiting patients (Okines et al., 2010)

2.1.2.3. Matuzumab

Matuzumab is another humanized IgG_1 monoclonal antibody against EGFR. In a phase I study of matuzumab in combination with ECX (epirubicin/cisplatin/capecitabine) as first-line therapy for patients with EGFR-positive gastric and GEJ cancer (Rao et al., 2008), treatment was well tolerated without major dose limiting toxicities other than grade 3 fatigue. Of the 45 patients screened, 21 (47%) had EGFR-positive tumors. The ORR was 65%, and the median TTP was 5.2 months. The data were not encouraging as the TTP was inferior to the PFS obtained in the original phase III trial of ECX chemotherapy alone (Cunningham et al., 2008).

Clinical trials using tyrosine kinase inhibitors in GC have shown modest efficacy when used as a single agent or in combination with cytotoxic therapy in several settings.

2.1.2.4. Gefitinib

Gefitinib is an orally active EGFR tyrosine kinase inhibitor with promising activity against a range of malignancies in early phase trials. However, in gastric and GEJ cancer, a phase II study of single agent gefitinib was reported. Seventy-five patients with previously treated gastric and GEJ cancer received gefitinib at 250 mg or 500 mg daily. Gefitinib reached the tumors at concentrations sufficient to inhibit EGFR activation; however, it did not translate into clinical benefit. Disease control was achieved only in 18% of the patients (Rojo et al., 2006).

2.1.2.5. Erlotinib

Erlotinib is another oral EGFR tyrosine kinase inhibitor. Erlotinib has been approved in the United States for the treatment of lung and pancreatic cancer. In gastric and GEJ cancers, erlotinib was found to be active in patients with GEJ cancer only. A phase II trial in 70 patients with advanced gastric and GEJ cancer showed a response in 9% of the patients with GEJ cancer. The median overall survival was 6.7 months (Dragovich et al., 2006).

2.1.3. Human Epidermal Growth Factor Type 2 Inhibition

HER-2 is a member of the EGFR family. The HER2 oncogene encodes for a 185 KD transmembrane glycoprotein receptor with intracellular tyrosine kinase activity (King et al., 1985).

HER-2 is involved in signal transduction leading to cell growth and differentiation. It is encoded within the genome by HER-2/neu. None of the EGF family of ligands is known to activate HER-2; however, HER-2 is the preferential dimerization partner of other members of the ErbB family (Olayioye, 2001). The HER-2 gene is a proto-oncogene and is located at the long arm of human chromosome 17 (Coussens et al., 1985).

HER-2 overexpression correlates with poor prognosis in ovarian and breast cancer (Slamon et al., 1989). In GC, HER-2 amplification and HER-2 protein expression by immunohistochemistry was found in 11.9% of the tumors and higher amplification was associated with worse survival in Japanese patients (Yonemura et al., 1991). These results have not been reproduced in follow-up studies.

HER-2 overexpression in gastric cancer ranges from 7 to 34% depending on the population studied. On the other hand, a high concordance of HER-2 amplification, by both IHC and FISH, has been reported in primary tumors as compared to regional lymph node or distant metastases (Bilous et al., 2010; Bozzetti et al., 2011; Marx et al., 2009).

Preclinical studies have shown that anti-HER-2 therapies have significant antitumor activity in both *in vitro* and *in vivo* models of gastric cancer (Matsui et al., 2005; Tanner et al., 2005).

The most common approaches to inhibit HER-2 are by inhibition of the HER-2 via monoclonal antibodies (i.e., Trastuzumab) or tyrosine kinase inhibitors (Lapatinib). Both methods have been examined in clinical trials in patients with gastric cancer.

2.1.3.1. Trastuzumab

Trastuzumab is a humanized monoclonal antibody against HER-2. Trastuzumab has been combined with cytotoxic chemotherapy in patients with gastric and GEJ tumors in several trials; a small phase II study evaluating trastuzumab in combination with cisplatin/docetaxel doublet in HER-2 positive metastatic gastric and GEJ adenocarcinoma showed radiological response in 80% of the patients. The results were preliminary and the final manuscript has not been published (Nicholas et al., 2006). In another unpublished study, 21 patients with HER-2 positive advanced gastric or GEJ adenocarcinoma were treated with cisplatin and trastuzumab; the total

response rate was only 35% (Cortes-Funes et al., 2007). No grade 4 toxicity related to trastuzumab was reported in any of these trials.

Trastuzumab for Gastric Cancer (ToGA) (Bang, et al., 2010) study is an open label, international, phase III, randomized controlled trial undertaken in 24 countries. Patients with gastric or GEJ adenocarcinoma overexpressing HER-2 protein by immunohistochemistry or gene amplification were included. Patients were randomized to receive capecitabine and cisplatin or fluorouracil and cisplatin every 3 weeks for 6 cycles, or chemotherapy in combination with intravenous trastuzumab. HER-2 positivity rate was reported in 22.1% of the patients. Patients who completed 6 cycles of treatment in the trastuzumab arm were allowed to continue on trastuzumab until progression.

The improvement in median survival was 2.7 months in the intent to treat analysis in patients who received trastuzumab (median overall survival 13.8 months compared with 11.1 months with hazard ratio 0.74). Response rate, time to progression, and duration of response were significantly higher in the trastuzumab plus chemotherapy group as well. The median survival in chemotherapy-only arm was higher than expected for this patient population and could be related, at least in part, to the high proportion of Asian patients in the study (55%). A treatment benefit was found in all the predefined subgroups including GEJ tumors.

2.1.3.2. Lapatinib

Lapatinib is a small molecule dual tyrosine kinase inhibitor of EGFR and HER-2. Lapatinib is an oral agent and is effective in trastuzumab-resistant advanced breast cancer (Cameron et al., 2008; Geyer et al., 2006). Lapatinib monotherapy in gastric cancer was evaluated in phase II study and showed limited single agent activity with a 12% response rate (Iqbal et al., 2011). These patients were not selected based on HER-2 overexpression.

Multiple clinical trials are currently evaluating the role of trastuzumab or lapatinib in HER-2 overexpressing GEJ and gastric tumors in the advanced or locally advanced resectable disease. A randomized open label phase III trial is evaluating concurrent chemotherapy and radiation with or without trastuzumab in treating patients with HER2-overexpressing esophageal adenocarcinoma. Other studies are planned or currently recruiting patients to evaluate trastuzumab or lapatinib in metastatic disease in combination with standard chemotherapy as well as other targeted therapy. A phase III global study designed to evaluate clinical end points and safety of chemotherapy plus lapatinib (Lapatinib Optimization Study in HER-2 Positive

Gastric Cancer; LOGIC) is currently ongoing. In addition, a phase III trial is underway to compare the safety and OS between lapatinib plus weekly paclitaxel and weekly paclitaxel alone as second-line treatment (TYkerb with Taxol in Asian gastric cancer; TYTAN).

2.1.4. Insulin-Like Growth Factor-1 Inhibition

The insulin-like growth factor-1 receptor (IGF-1R) belongs to the insulin receptor family (IGF-1 and IGF-2). IGF-1R is expressed on cell surface and phosphorylation of intracellular substrates leads to activation of the MAPK and PI3K/Akt pathways promoting tumor growth, progression, and invasion in several cancers including gastric cancer (Foulstone et al., 2005). IGF-1R signaling has been linked to resistance to cytotoxic therapy and inhibition of IGF-1R signaling enhances tumor cell apoptosis in numerous models. IGF-1R signaling has been also causally linked to *de novo* or acquired resistance to EGFR-targeting agents in several malignancies. In gastric cancer, IGF-1R expression in resected tumors correlates with poor clinical outcomes (Matsubara et al., 2008); in 86 patients with resected gastric tumors, patients with low expression of both IGF-1R and EGFR had significantly longer overall survival compared to those who lack the low coexpression.

The IGF-1R and its associated signaling system have gained significant interest in the treatment of several malignancies. Targeting IGF-1R pathway is through monoclonal antibodies, IGF-1R antisense/siRNA, and receptor tyrosine kinases. In gastric cancer, data on IGF-1R inhibition are still premature; one phase I trial of docetaxel combined with CP-751,871, an IGF-1R antibody, demonstrated promising results (Attard et al., 2006).

2.1.5. c-Met Tyrosine Kinase Inhibitors

Met is a membrane receptor that is essential for embryonic development and wound healing. C-Met is a receptor tyrosine kinase that is expressed in epithelial and endothelial cells. Hepatocyte growth factor (HGF), its ligand, is expressed by cells of the mesenchymal linkage. Overexpression of c-Met and activating c-Met mutations have been widely documented in many tumor types including gastric cancer (Lee et al., 2000) where c-Met deregulation correlates with poor outcomes. In a study of 121 patients with advanced gastric cancer, HGF and c-Met were significantly overexpressed in patients with liver metastases (Amemiya et al., 2002). Coexpression of c-Met

and HER-2 proteins in patients with gastric cancer has been associated with poorer survival (Nakajima et al., 1999).

c-Met inhibition has been evaluated in early phase trials with promising results; two phase I trials of ARQ197, a nonadenosine triphosphate (ATP) competitive small-molecule inhibitor of c-Met, in patients with solid tumors showed disease stabilization in 7 of 11 patients, with prolonged stabilization for >32 weeks in five tumor types, including gastric cancer (Yap et al., 2008a). Another trial of 36 patients reported that 5.5% of the patients achieved a PR, and 53% had stable disease (SD) (Garcia et al., 2007).

A phase II study examined the safety and efficacy of two dosing schedules of foretonib (GSK1363089), an oral small-molecule inhibitor of c-Met and VEGFR-2, as a single agent in patients with metastatic GC. Foretonib was well tolerated in both dosing schedules. The study found that c-Met amplification in metastatic gastric cancer is rarer than anticipated (3/43 patients). Amplification of the Met oncogene was not associated with a higher response rate. However, the lack of a well-validated method to assess c-Met makes any conclusive interpretations premature. A single agent demonstrated minimal antitumor activity in a c-Met-unselected gastric population. Mandatory pre- and on-treatment biopsies to better define c-Met pathway and target inhibition were added to the protocol (Jhawer et al., 2009). Other clinical trials of various c-Met inhibitors (TKIs and monoclonal antibodies) are ongoing.

2.1.6. Fibroblast Growth Factor Tyrosine Kinase Inhibitors

Fibroblast growth factor (FGF) and its signaling receptors have multiple biological activities including cell proliferation, differentiation, motility, and transformation (Grose & Dickson, 2005; Moffa et al., 2004). Fibroblast growth factor receptor 2 (FGFR2) is amplified in poorly differentiated gastric cancers (scirrhous cancer) with malignant phenotypes (Hattori et al., 1996) which makes it a promising molecular target for treatment.

In preclinical models, AZD2171, an oral highly potent VEGF, FGFR1, PDGFRB, and VEFGR2 tyrosine kinases inhibitor among others, significantly and dose dependently inhibited tumor growth gastric cancer xenografts. The most potent antitumor activity was seen in xenografts overexpressing FGFR2. These results suggest that AZD2171 might be clinically beneficial in patients with FGFR2 expressing gastric tumors (Takeda et al., 2007).

Ki23057, a broad-range tyrosine kinase inhibitor of FGFR2, inhibits FGFR1, FGFR2, and VEGF2 tyrosine kinases. It inhibits the proliferation of

gastric scirrhous cancer cells with FGFR2 gene amplification only. Oral administration of Ki23057 inhibits the growth and peritoneal dissemination of gastric cancer cells through FGFR2-RAS/ERK inhibition, rather than through FGFR2–PI3k–AKT signaling inhibition (Nakamura et al., 2006). To our knowledge, no clinical trials are currently available for this compound in gastric cancer.

2.2. Cell-Cycle Inhibition

2.2.1. Aurora Kinase Inhibitors

Aurora kinases (A, B, and C) are serine/threonine kinases that have been recognized as important regulators of cell proliferation from mitotic entry to cytokinesis (Carmena et al., 2009). In normal cells, the aurora kinase protein levels increase from G2 to M phase. Overexpression of aurora kinase A results in chromosomal instability in a variety of tumors, including GC. In addition, aurora kinase A inhibits drug-induced apoptosis leading to drug resistance (Kamada et al., 2004). Aurora kinase A overexpression in upper gastrointestinal cancers indirectly activates HDM2 leading to p53 suppression and cancer cell survival (Dar et al., 2008) which translates into poor clinical outcomes (Macarulla et al., 2008).

Various aurora tyrosine kinase inhibitors are currently under investigation in phase I trials. In a phase I trial of SNS-314, a novel selective inhibitor of aurora kinases A, B, and C, in patients with solid tumors showed no objective response (Robert et al., 2009). In another phase I trial of AT9283, a multitargeted kinase inhibitor including aurora kinases A and B, 33 patients were treated and the best response was a PR in 1 patient and two patients with SD (Kristeleit et al., 2009).

2.2.2. Polo-Like Kinase Inhibitors

Polo-like kinases (PLKs) are a family of conserved serine/threonine kinases, which are involved in signal transduction pathways leading to the formation of, and changes in, the mitotic spindle. As such, they are involved in the regulation of cell-cycle progression through G_2 and mitosis. These enzymes also activate cyclin-dependent kinase/cyclin complexes during the M-phase of the cell cycle. PLK-1 overexpression is seen in various malignancies, including gastric cancer (Takai et al., 2005) and it is associated with the accumulation of proliferation-related genes and oncogenes. Inhibiting PLK-1 leads to cell growth inhibition and apoptosis. Moreover, PLK-1 is a prognostic marker for gastric cancer (Jang et al., 2006); patients with PLK1-positive tumors have more

lymph node metastasis and diffuse growth pattern and thus worse outcome when compared to those with PLK-1-negative tumors (Kanaji et al., 2006).

The inhibition of PLK-1 via small interferences RNA (siRNA) resulted in cdc2 activity, increased cyclin B expression, and accumulation of gastric cancer cells at G2/M, improper mitotic spindle formation, delayed chromosome separation, attenuated procaspase 3 levels, and increased apoptosis.

Phase I trials are currently ongoing to evaluate the role of PLK inhibitors in various tumors, including gastric cancer (Olmos et al., 2011).

2.2.3. Cyclin-Dependent Kinase Inhibitors

Cyclin-dependent kinases (CDKs) comprise a group of protein kinases (cdk1–cdk9) that participate in cell-cycle regulation via the retinoblastoma (Rb) product. The inactivation of the Rb pathway results from either overexpression or amplification of CDKs, from downregulation of negative factors such as endogenous CDK inhibitors or mutations in the Rb gene or its product. This pathway is deregulated in different malignancies, resulting in a disturbed $G_1$to S phase of the cell cycle (Senderowicz, 2000).

Flavopiridol is a synthetic flavone that inhibits *in vitro* tumor cell growth at nanomolar concentrations by blocking cell-cycle progression at G_1 or G_2 (Carlson et al., 1996; Kaur et al., 1992). Flavopiridol is a potent inhibitor of CDKs with respect to the ATP-binding site including cdk-1, cdk-2, cdk-4, and cdk-7, and hypophosphorylation of Rb (Losiewicz et al., 1994). Flavopiridol has also been shown to induce apoptosis, inhibit angiogenesis, and potentiate the effects of chemotherapy by arresting the cell in the G1 or G2/M phase (Melillo et al., 1999; Patel et al., 1998).

In a phase I study of 38 patients with advanced cancer, flavopiridol was administered as continuous infusion. One patient with gastric cancer had a Complete response (CR) lasting more than 48 months (Thomas et al., 2002). Recently, a phase I trial of FOLFILI in combination with flavopiridol in patients with gastric cancer and other solid tumors was reported; clinical benefits were seen in 39% of the patients (Dickson et al., 2009). A phase II study of flavopiridol as a single agent in 16 patients with gastric cancer showed no activity (Schwartz et al., 2001).

2.3. Other Targeted Mechanisms

2.3.1. PI3 Kinase Pathway Inhibition

The PI3K enzymes are involved in the phosphorylation of membrane inositol lipids (Vivanco & Sawyers, 2002). The activation of PI3K generates

the second messenger phosphatidylinositol (3-5)-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). This recruits proteins to the cell membrane, including the Akt/PKB kinases, resulting in their phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) (Yap et al., 2008b), and by PDK2 (Yang et al., 2004).

Deregulation of the PIP3/Akt/mTOR pathway can occur secondary to oncogenic mutations of PIK3CA (Samuels et al., 2004), loss of PTEN function (Suzuki et al., 1998; Yoshimoto et al., 2007), mutation of Akt/PKB isoforms (Bellacosa et al., 2005), or upstream activation through other pathways like IGF-1R. Abnormal expression of the PTEN protein in gastric cancer is found in 11% of the tumors and is related to the tumor differentiation, advanced staging, and chemoresistance (Oki et al., 2005). Upregulation of the PI3k/Akt/mTOR downstream pathway correlates with a worse prognosis and may contribute to the resistance to chemotherapy (Yu et al., 2008). Everolimus, an oral mTOR inhibitor, has been evaluated in gastric cancer.

2.3.1.1. Everolimus

Everolimus (RAD001) is an oral mTOR inhibitor that has shown anticancer activity both in preclinical models (Cejka et al., 2008) and in phase I study in Japanese gastric cancer patients (Okamoto et al., 2010). Based on these promising results, a multicenter phase II study was performed in pretreated patients with metastatic gastric cancer (Doi et al., 2010). Fifty-three patients were assessable. At a median follow-up time of 9.6 months, median PFS was 2.7 months and median OS was 10.1 months. Common grade 3 or 4 adverse events included anemia, hyponatremia, increased gamma-glutamyltransferase, and lymphopenia. The short PFS compared to the relatively long OS is puzzling and requires further evaluation. Based on these results, a phase III trial is now being planned.

2.3.2. Heat Shock Protein 90 Inhibitors

The heat shock protein 90 (HSP90) is a molecular chaperone and is one of the most abundant proteins expressed in cells. Multiple cell-specific oncogenic processes are tightly regulated by binding of the HSP90 (Neckers, 2007; Workman, 2007). In gastric cancer, HSP90 expression correlates with tumorigenesis and lymph node metastasis (Zuo et al., 2003). The downregulation of Hsp90 can increase drug sensitivity of tumor cells. In preclinical studies, HSP90 inhibition reduced the constitutive and inducible activation of extracellular signal-regulated kinase 1/2, Akt, and signal transducer and activator of transcription (STAT3), and decreased the

protein expression of the nuclear hypoxia-inducible factor-1α (HIF-1α) (Lang et al., 2007). Currently, there are several ongoing studies evaluating HSP90 inhibitors in various malignancies.

STA-9090 is a potent, next-generation HSP90 inhibitor. STA-9090 has shown superior activity and an improved safety profile relative to other agents in preclinical models. Two phase I dose-escalation studies of STA-9090 in patients with solid tumors, including gastric cancer, have shown STA-9090 to be well tolerated at dose levels up to 216 mg/m^2 once weekly (Goldman et al., 2010) or 25 mg/m^2 twice weekly (Cleary et al., 2010). The safety profile and activity signals warrants further evaluation of STA-9090 in solid tumors including gastric cancer.

2.3.3. Ubiquitin–Proteasome Pathway Inhibitors

The ubiquitin–proteasome pathway is essential for protein quality control through degradation. It plays an important role in cell-cycle regulation, transcription, signaling, protein transport, DNA repair, and stress responses. Disturbance in proteasome activity leads to the accumulation of poly-ubiquitinylated proteins, endoplasmic reticulum stress, and even cell death (Latonen et al., 2011).

2.3.3.1. Bortezomib

Bortezomib is a potent inhibitor of the proteasome and has prominent effects *in vitro* and *in vivo* against several solid tumors. It has been approved for the treatment of hematological malignancies and its role in solid tumors is not well established. In preclinical models, bortezomib induced apoptosis in three gastric cancer cell lines, SNU638, MUGC-3, and MKN-28 and when combined with cisplatin and docetaxel, bortezomib dramatically decreased tumor cell growth compared with chemotherapy alone (Bae et al., 2008).

The promising preclinical efficacy led to multiple phase II studies; in a phase II study of bortezomib in 16 patients with advanced gastric adenocarcinoma, no patient had objective response and one patient achieved SD (Shah et al., 2011b). In another phase II trial of 44 patients with advanced gastric and GEJ cancer, 28 chemonaïve patients (arm A) received irinotecan in combination with bortezomib, and 12 patients who were previously treated received bortezomib alone (arm B). Response rates of 44% in arm A and 9% in arm B were reported. The PFS and OS were, respectively, 1.9 and 5.4 months in arm A and 1.4 and 4.1 months in arm B (Ocean et al., 2006). In another phase II trial of bortezomib combined with paclitaxel and carboplatin in first-line treatment of 35 patients with

metastatic gastric and GEJ cancer, tumor response rate was lower than anticipated (23%) and the OS was 8.9 months (Jatoi et al., 2008). Further evaluations of bortezomib in 5-FU-based combination are ongoing.

2.3.4. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of highly homologous protein degrading zinc dependent endopeptidases that break down components of the extracellular matrix. This family currently includes more than 25 members and they play an important role in normal growth and repair. They are aberrantly expressed in several solid tumors and are thought to contribute to the invasive potential of these tumors (Chambers & Matrisian, 1997). Based on promising phase I result, a phase III study of marimastat, an MMP inhibitor, versus placebo was undertaken in 396 patients with inoperable/metastatic gastric or GE junction adenocarcinoma (Bramhall et al., 2002). Patients who had received no more than first-line 5-FU based chemotherapy were randomized to receive either placebo or marimastat. At 2-year follow-up, there was a small but statistically significant difference ($p = 0.02$) in median OS (160 vs. 138 days) and 2-year survival (9% vs. 3%) favoring the marimastat group. Despite these promising results, further development of this drug has been halted secondary to poor tolerability because of musculoskeletal toxicity.

2.3.5. Histone Deacetylase Inhibitors

Epigenetic modulation of gene expression plays an important role in regulating cell biology (Jones & Baylin, 2007). Epigenetic silencing of tumor suppressor genes, induced by the overexpression of histone deacetylase (HDAC), plays a crucial role in carcinogenesis. Further understanding of the cancer cell cycle and the role of HDAC inhibition led to the development of several new anticancer agents (Miremadi et al., 2007).

In humans, 18 HDAC enzymes have been identified and categorized into three classes. In gastric cancer, HDAC is thought to be an independent prognostic marker. Moderate to strong expression of HDAC2 was found in 44 (62%) out of 71 gastric tumors and it was associated with tumor aggressiveness (Song et al., 2005) and nodal spread (Weichert et al., 2008).

HDAC inhibitors act by binding to a critical zinc ion required for catalytic function of the HDAC enzyme (Finnin et al., 1999). These compounds have varying potencies and specificities, with variable effects on the acetylation of nonhistone substrates (Beckers et al., 2007) leading to distinct efficacies, toxicities, and therapeutics (Lane & Chabner, 2009).

More than 15 HDAC inhibitors have been tested in preclinical and early clinical studies and the only HDAC inhibitor approved by the FDA is vorinostat in hematological malignancies. In a phase I trial of vorinostat monotherapy in 16 Japanese patients with gastrointestinal cancer, including 10 with gastric cancer, 8 patients had SD as the best response (Chin et al., 2008). Another phase I trial of vorinostat combined with FOLFIRI in patients with upper gastrointestinal tumors has been reported. Among the 8 patients in whom the response was assessable, 2 had a PR and 5 had an SD (Fetterly et al., 2009).

2.3.6. Protein Kinase C Inhibition

Protein kinase C is a family of enzymes that is involved in controlling the function of other proteins. These enzymes work through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues. PKC inhibitors are currently being investigated in both malignant and nonmalignant conditions.

Bryostatin-1, an inhibitor of protein kinase C, has been evaluated in combination with paclitaxel sequentially in esophagogastric tumors (Ku et al., 2008); despite the promising results, the drug has been discontinued secondary to unexpected grade 3/4 myalgia in approximately half of all the patients.

3. CONCLUSION

Gastric cancer is one of the most common malignancies worldwide with approximately 990,000 new cases and 738,000 deaths per year, accounting for about 8% of the new cancers (Jemal et al., 2011). Approximately, 21,000 patients are diagnosed annually in the United States leading to more than 10,000 deaths (Siegel et al., 2011). At diagnosis, approximately 50% of the patients have the disease that extends beyond locoregional confines, and only half of those will have curative resection. Screening is not widely performed outside the high prevalence areas. Further, 5-year-survival rate remains low even following potentially curative treatment. Cytotoxic agents have been the mainstay of systemic treatment for decades with marginal therapeutic efficacy.

During the recent years, several molecular abnormalities underlying gastric carcinogenesis and progression have been identified. This stimulated the search for novel therapeutic approaches. Targeted agents used as monotherapy and/or added to chemotherapy are unlikely to result in any major

advances given the highly complex nature of molecular abnormities and concurrent aberrations in multiple signaling pathways. The inherent redundancies in pathways also preclude effective blockade of proliferation and survival by targeting only one receptor. A multitargeted approach will need to be evaluated in order to move forward but is severely hampered by the limited knowledge on how to combine these agents, the logistical issue of designing multisponsor trials, as well as the added toxicities.

Biomarkers are increasingly used in cancer treatment to predict the effectiveness and toxicity of anticancer agents. The effective use of biomarkers is expected to lead to individualized treatments suited for an individual patient similar to the HER-2 inhibition in gastric cancer. Currently, few biomarkers are used clinically for cancer therapy, and most have not gone beyond laboratory investigation. In clinical trials, selecting patients based on predictive factors, whenever possible, is ideal; however, this may be difficult with the lack of validated biomarkers in gastric cancer and diversity of molecular changes acquired during malignant transformation, recurrence, or metastasis.

Many of the agents discussed in this chapter have poorly defined target in individual patients, which hampers their optimal development. Measuring the effects of these agents on the targeted pathway is critical to further refine their usage. One approach will be to test the new agents in the neoadjuvant setting and obtain multiple biopsies and correlate patient's outcome with whether the target is functionally of importance, and if it was inhibited by the agent. The caveat remains that response rate in the neoadjuvant setting might not translate into survival in metastatic disease as well as the morbidity and inconvenience related to serial biopsies. Evaluating targeted agents in refractory population might not be the optimal way to identify clinical benefits. Combining targeted therapy with cytotoxic agents and or radiation should be based on sound scientific evidence.

Apart from the molecular targeted agents described in this chapter, many other drugs are currently being evaluated in gastric cancer. Clinical research is moving forward and further studies are needed to determine the optimal usage of targeted therapy in clinical practice hoping that the recent success of HER-2 inhibition will be extended to patients with other biological subset of the disease.

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ABBREVIATIONS

GC gastric cancer
GEJ gastroesophageal junction

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CHAPTER FIFTEEN

HSP90 Inhibitors for Cancer Therapy and Overcoming Drug Resistance

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Abstract

Since the initial discovery of heat shock protein 90 (HSP90) as a target for anticancer therapy, tremendous progress has been made in developing a multitude of potent first- and second-generation HSP90 inhibitors. Promising activity has been reported with 17-AAG in combination with trastuzumab in HER2 positive breast cancer refractory to trastuzumab therapy and more recently in ALK-mutated lung cancers. However, the full potential of this class of agents is yet to be realized. This review not only provides an up-to-date overview of the clinical development of HSP90 inhibitors and their companion biomarker assays but also provides insight into the less-understood role of HSP90 in tumor evolution and drug resistance. A better understanding of these important concepts will facilitate the optimal and expedient development of this class of agents, ultimately fulfilling their promise as potent anticancer therapeutics and leading to the regulatory approval of the first-in-class HSP90 inhibitor.

1. INTRODUCTION

Over the past few decades, great progress has been made in identifying a number of molecularly targeted anticancer therapies. Among these promising targets, heat shock protein 90 (HSP90), a 90 kDa ATP-dependent multifunctional chaperone protein, is unique and sought after due to its role in supporting multiple cellular proteins that are critical to tumor proliferation and survival. Unlike the major classes of molecular chaperones that are involved in the primary folding of nascent polypeptides, HSP90 uses repeated cycles of client protein binding, ATP hydrolysis as well as interaction with the HSP90-interacting proteins or cochaperones (HSP70, Cdc37, HOP, p23, Aha1) to modulate the stability and activity of approximately 200 client proteins. Many of these client proteins are

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signaling oncoproteins such as steroid receptors [estrogen, progesterone, and androgen receptors (ARs)], tyrosine kinases (human epidermal growth factor receptor [HER2], epidermal growth factor receptor [EGFR]), metastable signaling proteins (Akt, Raf-1, IKK), and cell cycle regulators (Cdk4, Cdk6) among others (Zhang & Burrows, 2004). Inhibition of HSP90 leads to proteasome-mediated degradation of these oncoproteins, which has the potential to disrupt multiple signaling pathways including feedback loops that can counteract the efficacy of highly selective targeted agents, making HSP90 inhibition a novel and attractive anticancer strategy (Workman et al., 2007; Zuehlke & Johnson, 2010).

In 1994, geldanamycin, a naturally occurring compound was reported as the first HSP90 inhibitor with antitumor potential (Whitesell et al., 1994) and 17-AAG (tanespimycin), a geldanamycin analog was the first HSP90 inhibitor to enter clinical trials in 1999. Since then, there has been considerable progress in optimizing the pharmacological properties of this class of agents and many synthetic small molecule inhibitors are in various stages of clinical development. Although preclinically HSP90 inhibitors have been hypothesized to be active in a wide variety of tumor types, positive clinical results have been reported in only a few cancers. This may be attributable to the following: (1) failure to identify the most susceptible patient populations for this therapy, (2) suboptimal dosing and scheduling, (3) absence of a validated assay to ascertain target modulation (HSP90 inhibition), (4) incomplete inhibition of the target itself vis-à-vis low therapeutic index of available agents, and (5) lack of a clear understanding of the role of HSP90 in the development of drug resistance. In this review, we describe the clinical development of HSP90 inhibitors for cancer therapy, presenting clinical results for the drugs furthest in development. We also review the current research into identifying novel biomarkers of response and target modulation as well as present the rationale and data for combinatorial approaches to optimize the therapeutic efficacy and overcome drug resistance with this class of agents.

2. CLINICAL DEVELOPMENT OF HSP90 INHIBITORS

The HSP90 chaperone consists of three domains: (1) the amino terminal region (N-domain) that contains the ATP, drug-binding site, and cochaperone interacting motifs, (2) a middle (M) domain that participates in forming active ATPase and also serves as a docking site for client proteins

and cochaperones, and (3) the carboxy terminal region (C-domain) that contains a dimerization motif, a second drug-binding site, and interaction sites for cochaperones (Ali et al., 2006; Prodromou & Pearl, 2003).

2.1. HSP90 Inhibitors Targeting the ATP Binding Site of N-Domain

Thus far, all the HSP90 inhibitors in clinical trials work by inhibiting the ATPase activity of HSP90 by binding the drug-binding site on the N-domain (Janin, 2010; Taldone et al., 2009). In general, they can be classified into first-generation inhibitors based on their similarity to geldanamycin or second-generation small molecule synthetic inhibitors that are either resorcinol or purine derivatives except for SNX-5422, which falls outside these designations (Table 15.1). These inhibitors are reviewed in the following sections.

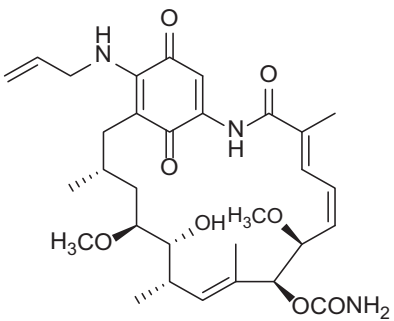
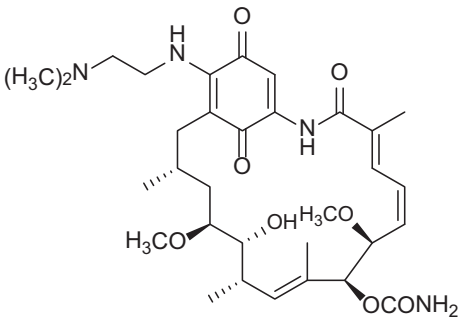
2.1.1. First-Generation Inhibitors: Geldanamycin (GM) Derivatives

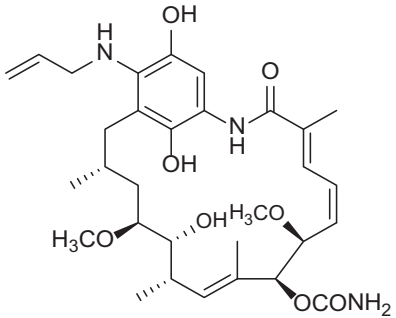
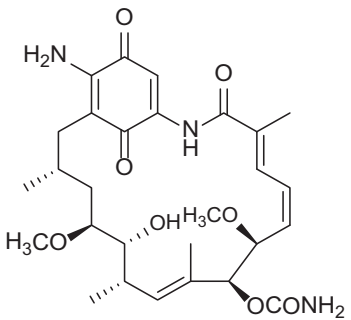
The fundamental understanding of HSP90 inhibition was originally derived from the study of natural compounds like geldanamycin (GM). GM is an ansamycin antibiotic first isolated from the fermentation broth of *Streptomyces hygroscopicus* in 1970 (DeBoer et al., 1970). The seminal paper by Whitesell and Neckers described that GM directly binds to HSP90 and interferes with the HSP90-v-src heterocomplex formation (Whitesell et al., 1994). Further cocrystal structure determination identified that GM competes with ATP for binding to the nucleotide binding site on the N-domain thus inhibiting the ATPase activity of HSP90 (Stebbins et al., 1997). Although GM demonstrated compelling *in vitro* and *in vivo* antitumor activity, its hepatotoxicity limited its use in the clinical setting (attributed to the presence of a quinone ring). Nevertheless, structural variations of the GM compounds paved the way for many analogs including those that were evaluated successfully in clinical trials.

2.1.1.1. 17-AAG (17-Allyl-17-Demethoxygeldanamycin)

Substitution of the nonessential methoxy group on the C-17 of the quinone ring in GM with an amino group led to the formation 17-AAG (17-Allyl-17-Demethoxygeldanamycin). 17-AAG retained the antitumor properties of GM with a more favorable and acceptable safety profile. Various dosing schedules were evaluated in phase I trials of 17-AAG and toxicity in these trials was dose- and schedule dependent (Bagatell et al., 2007; Banerji et al., 2005; Goetz et al., 2005; Grem et al., 2005; Nowakowski et al., 2006;

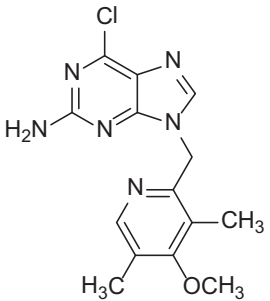
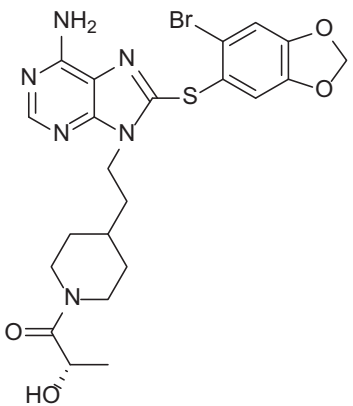
Table 15.1 HSP90 Inhibitors in Clinical Trials

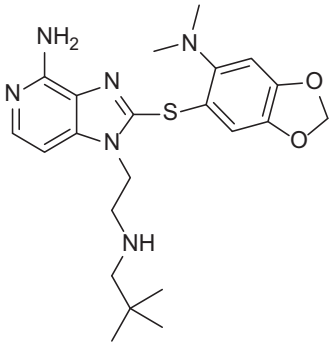
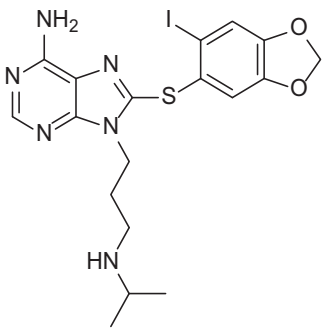
	Inhibitor	Company	Structure	Class	Route	Phase	Current Status
1.	Tanespimycin (17-AAG, KOS-953)	Kosan Biosciences/ Bristol-Myers-Squibb		GM	IV	III	Not being developed
2.	Alvespimycin (17-DMAG)	Kosan Biosciences/ Bristol-Myers-Squibb		GM	IV Oral	I	Not being developed

3.	Retaspimycin (IPI-504)	Infinity Pharmaceuticals		GM	IV	III	Ongoing in combination with docetaxel in NSCLC (NCT0136400)
4.	IPI-493	Infinity Pharmaceuticals		GM	Oral	I	Not being developed

(*Continued*)

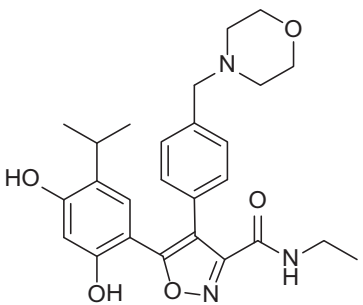
Table 15.1 HSP90 Inhibitors in Clinical Trials—cont'd

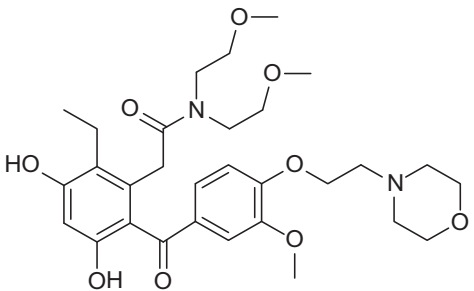
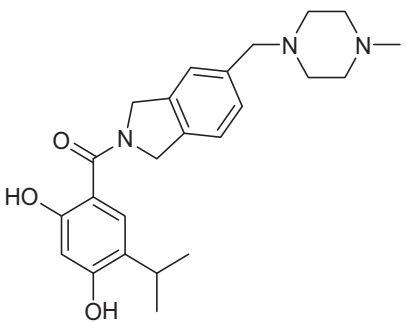
	Inhibitor	Company	Structure	Class	Route	Phase	Current Status
5.	CNF2024/ BIIB021	Biogen Idec		Purine	Oral	II	Not listed in Biogen Idec's pipeline
6.	MPC-3100	Myriad Pharmaceuticals/ Myrexis		Purine	Oral	I	Phase I trial not recruiting

7.	Debio-0932 (CUDC-305)	DebioPharm		Purine-like	Oral	I	Ongoing (NCT01168752)
8.	PU-H71	Samus Therapeutics		Purine	IV	I	Ongoing (NCT01393509)

(*Continued*)

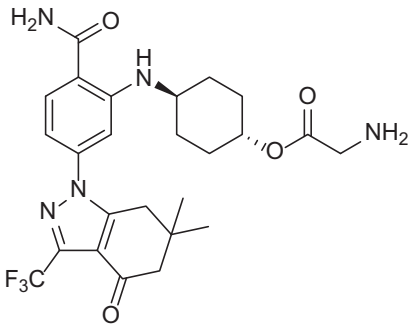
Table 15.1 HSP90 Inhibitors in Clinical Trials—cont'd

Inhibitor	Company	Structure	Class	Route	Phase	Current Status
9. Ganetespib (STA-9090)	Synta Pharmaceuticals	Not reported	Resorcinol-Triazole	IV	II	Multiple trials ongoing (NCT01031225, NCT01273896, NCT0084872, NCT01167114, NCT01227018, NCT01200238, NCT01173523, NCT01039519)
10. NVP-AUY922 (VER-52269)	Novartis		Resorcinol-Isoxazole	IV	II	Multiple ongoing trials (NCT01124864, NCT00526045)

11. HSP990	Novartis	Not reported but claimed as a follow up compound to NVP-AUY922	Not reported	Oral	I	Active not recruiting (NCT 00879905, NCT01064089)
12. KW-2478	Kyowa Hakko Kirin Pharma		Resorcinol	IV	I	Phase I trials completed
13. AT13387	Astex		Resorcinol	IV Oral	I	Multiple trials ongoing (NCT00878423, NCT01245218, NCT01246102)

(*Continued*)

Table 15.1HSP90 Inhibitors in Clinical Trials—cont'd

Inhibitor	Company	Structure	Class	Route	Phase	Current Status
14. SNX-5422	Serenex/Pfizer		Indazol-4-one	Oral	I	Not being developed
15. DS-2248	Daiichi Sankyo Inc	Not reported	Not reported	Oral	I	Ongoing (NCT01288430)
16. XL888	Exelixis	Not reported	Not reported	Oral	I	Phase I trial terminated

GM: geldanamycin; IV: intravenous.

Ramanathan et al., 2005; Solit et al., 2007; Weigel et al., 2007). Hepatotoxicity was the most prominent toxicity with daily administration of 17-AAG, and other common toxicities included diarrhea and fatigue. Pharmacokinetic (PK) studies showed that adequate serum concentrations were achieved with 17-AAG at well-tolerated doses and schedules. Serum concentrations achieved in these trials were higher than those required for depletion of client proteins in *in vitro* and xenograft models. Despite the pharmacodynamic (PD) studies that demonstrated at least partial target modulation in these phase I trials, there were no objective tumor responses, with stable disease (SD) seen as the best response in selected tumor types [melanoma, prostate cancer, breast cancer, and renal cell carcinoma (RCC)] (Banerji et al., 2005; Solit et al., 2007). It has been suggested that the modest activity seen in these studies may have been related to underdosing of patients that was limited by toxicities attributable to the DMSO solvent used in the 17-AAG formulation, which at higher doses is associated with a bad odor, nausea, and anorexia. In addition, the inability to select patients most likely to benefit from this approach and suboptimal target inhibition are other proposed reasons for this result.

Based on this, Kosan Biosciences developed a novel Cremophor-containing injectable formulation of 17-AAG which they called tanespimycin (also known as KOS-953). Using this formulation, they reported the most impressive clinical activity to date with an HSP90 inhibitor in their phase I/II clinical trials of tanespimycin in combination with trastuzumab in patients with metastatic HER2 positive breast cancer refractory to trastuzumab therapy (Modi et al., 2007, 2011). Despite these promising results (Fig. 15.1), further development of 17-AAG was suspended in July 2008 for nonclinical reasons.

2.1.1.2. 17-DMAG (17-Desmethoxy-17-N,N-Dimethylaminoethylaminogeldanamycin)

17-Desmethoxy-17-N,N-Dimethylaminoethylaminogeldanamycin (17-DMAG) was formed as a result of substituting the C-17 methoxy group of GM with N-N-dimethylethylamine. This formulation has increased water solubility and equal or better potency compared to 17-AAG (Hollingshead et al., 2005; Messaoudi et al., 2008). Various dosing schedules of intravenous 17-DMAG were evaluated in multiple phase I trials (Kummar et al., 2010; Lancet, Gojo, et al., 2010; Pacey et al., 2011; Ramanathan et al., 2010). Common toxicities reported in these trials included peripheral neuropathy, renal dysfunction, fatigue, ocular adverse events (including blurred vision,

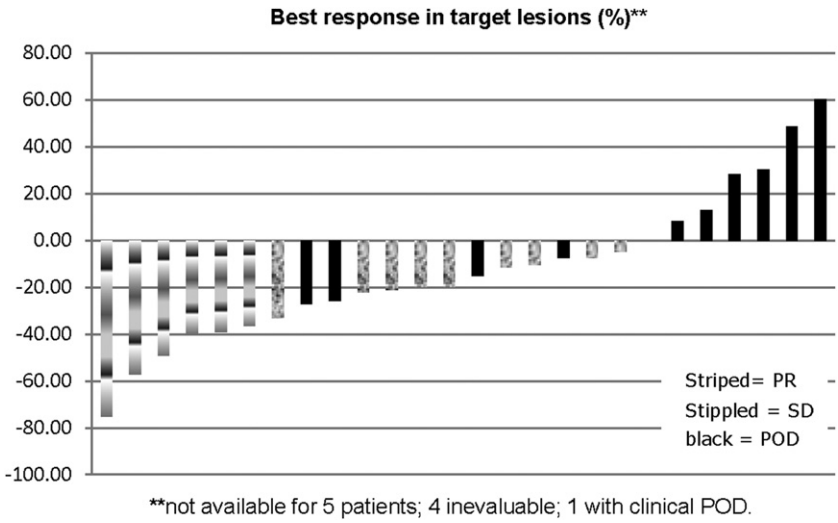


Figure 15.1 Phase II trial of tanespimycin and trastuzumab in metastatic trastuzumab-refractory HER2 positive breast cancer. Patients were treated with tanespimycin at 450 mg/m^2 intravenously and trastuzumab at a conventional dose. The overall response rate in evaluable patients was 22% and the clinical benefit rate (CR + PR + SD) was 59%. Data is depicted as a waterfall plot with best response (%) indicated on the *y*-axis. Partial response is indicated in striped bars, stable disease in stippled bars and progression of disease in black bars.

dry eye, and keratitis), pneumonitis, and thrombocytopenia. Collectively, responses across all phase 1 trials with this agent included 4 complete responses (CR) [1 castrate refractory prostate cancer (CRPC) and 3 acute myeloid leukemia] and 1 partial response (PR) in a patient with melanoma (Lancet, Gojo, et al., 2010; Pacey et al., 2011). Responses in prostate cancer and melanoma were seen with the once weekly dosing. This phase I trial enrolled a total of 25 patients. Also noted were three cases of SD in patients with chondrosarcoma, CRPC, and renal cancer for 28, 59, and 76 weeks, respectively (Pacey et al., 2011). Dose-limiting toxicity (DLT) for the once weekly dosing on this trial occurred at 106 mg/m^2 and included one treatment-related death characterized by rapid onset of grade 4 transaminitis, hypotension, acidosis, and renal failure. The weekly schedule was better tolerated at 80 mg/m^2 but 4 patients experienced grade 1/2 ocular events. HSP72 induction, a surrogate marker of HSP90 inhibition, was observed in peripheral blood mononuclear cells (PBMCs) and client protein degradation was observed in tumor biopsies (Pacey et al., 2011). The other phase I trial enrolled 24 patients with acute myeloid leukemia wherein patients were

administered 17-DMAG twice weekly for 2 out of 3 weeks. Of the 17 patients evaluable for efficacy, CR was noted in 3 patients (Lancet, Gojo, et al., 2010). DLT in this trial was cardiac ischemia in two patients with prior history of cardiovascular events. On the daily (3 consecutive or 5 consecutive days on a 21-day cycle) dosing schedule, reliable degradation of client proteins was not observed in the 24-hour tumor biopsies (Ramanathan et al., 2010). A phase I trial of oral 17-DMAG evaluated two dosing schedules: daily or every other day for 4 out of 6 weeks (Flaherty et al., 2007). No DLT was noted in the 28 patients treated on this trial, and common toxicities included fatigue, anorexia, proteinuria, and peripheral edema. SD was noted in patients with hemangioendothelioma, melanoma, and RCC (Flaherty et al., 2007).

Despite the CRs noted in patients with acute myeloid leukemia and castrate resistant prostate cancer, the development of 17-DMAG was also halted in 2008 to "commit resources to the development of 17-AAG for the treatment of breast cancer as a result of a comparative analysis with 17-AAG based on several factors, including clinical experience to date, strength of intellectual property protection and risk, and time to commercialization" as stated by the Pharmaceutical Sponsor of these agents (Press Release, F. Kosan announces senior management changes and clinical portfolio priorities). The difference in toxicity profiles between these two agents, with tanespimycin having fewer and less diverse toxicities overall, may have been an important factor in this decision.

2.1.1.3. IPI-504 (17-Allylamino-17-Demethoxygeldanamycin Hydroquinone Hydrochloride)

IPI-504 (retaspimycin) is a water soluble, hydroquinone hydrochloride salt derivative of 17-AAG, and the hydroquinone form (IPI-504) is in redox equilibrium with the quinone form (17-AAG). IPI-504 has reached phase II/III trials, with the most notable activity being reported in non–small cell lung cancer (NSCLC) and gastrointestinal stromal tumors (GIST). In a phase II trial of NSCLC, 76 patients were administered IPI-504 at 400 mg/m^2 twice weekly on a 21-day cycle. Nine patients had grade 3 or higher liver function abnormalities. The overall response rate was 20% among patients with EGFR-wild type and EGFR mutant lung cancer. This was also the first trial that showed clinical activity for patients with the oncogenic rearrangement of the anaplastic lymphoma kinase (ALK) gene, consisting of 2 PRs and a 1 SD for 7.2 months duration (Sequist et al., 2010). Promising activity was also seen in GIST, which formed the basis of the phase III

RING (Retaspimycin in GIST) trial. Patients in this trial received the same dose and schedule as those in the NSCLC trial. However, in contrast to the experience with NSCLC and similar to 17-AAG and 17-DMAG studies, hepatotoxicity was prominent in this trial with four on-treatment deaths leading to early closure of the study after 47 of 195 planned patients were enrolled. Three of the four patients had grade 3 or 4 transaminase elevations. Notably, patients enrolled to this trial had received three or more prior therapies since their initial diagnosis and had more advanced disease compared to those in earlier IPI-504 trials. Additionally, 20% had prior hepatic resections, which may have contributed to the excess hepatic toxicity observed (Johnston & Allaire, 2009).

In a phase II trial for patients with HER2 positive advanced breast cancer, patients were administered weekly IPI-504 at 300 mg/m^2 in combination with trastuzumab at 6 mg/kg every 3 weeks. The lower dose of IPI-504 was used in this study based on the experience from the RING trial and overall at this dose, only 1 grade 3 elevation in transaminases was observed and there were no DLTs. Unfortunately, there were no confirmed objective responses but a number of patients achieved disease stabilization (Modi, Saura, et al., 2011). This suggests that the toxicity profile of IPI-504 is dose- and schedule dependent and that the modest results in conjunction with minimal toxicity seen in the breast trial may have been due to insufficient dosing. Currently, the breast program for IPI-504 is temporarily suspended while the safety of a higher dose (450 mg/m^2) weekly dosing schedule in combination with docetaxel is being evaluated in another study of patients with NSCLC (NCT01427946).

2.1.2. Second- and Third-Generation Inhibitors: Synthetic Small Molecules

2.1.2.1. Purine and Purine-Like Analogs

Cocrystal structures of the N-terminal domain of HSP90 with geldanamycin and radicicol has allowed for combinatorial approaches, rational drug design, and high-throughput screening assays to generate promising second-generation synthetic agents. These compounds bind the N-terminal ATPase site of HSP90 with higher affinity than the natural nucleotides and prevent HSP90 from cycling between its ADP- and ATP-bound conformations. The first group of these agents is the purine scaffold series, and based on the prototype PU3 developed by the Chiosis laboratory (Chiosis et al., 2001), numerous other candidates that have entered clinical trials including (1) purines such as CNF2024/BIIB021, MPC-3100, and PU-H71 and

(2) purine-like analog Debio-0932 (CUDC-305). As a group, these compounds demonstrate insensitivity to multidrug resistance, high aqueous solubility, and oral bioavailability (Chiosis et al., 2002; Rodina et al., 2007).

CNF2024, an orally available 9-benzyl purine derivative developed by Conformal Therapeutics and later acquired by Biogen Idec, was tested in phase I trials in chronic lymphocytic leukemia (CLL), lymphomas, advanced solid tumors, and most recently in breast cancer (Jhaveri et al., 2012). Based on its favorable tolerability and early clinical activity, phase II studies were planned with this agent. However, Biogen has elected to halt all clinical trials and plan to license out its further development to potential buyers (Mitchell, 2011). Debio-0932 (NCT01168752) and PU-H71 (NCT01393509) are undergoing phase I evaluation and MPC-3100 (NCT00920205) has recently completed phase I testing and results are pending.

2.1.2.2. Resorcinol Derivatives

As is the case in drug discovery, natural products often pave the way in the discovery of other lead agents. Although radicicol, a macrocyclic lactone antibiotic isolated from the fungus *Monosporidium bonorden* is by itself devoid of *in vivo* activity, its resorcinol core is maintained in multiple agents that are currently being evaluated in clinical trials. These include triazole derivatives such as ganetespib (STA-9090), isoxazole derivatives such as NVP-AUY922/VER52296, and other derivatives like KW-2478 and AT-13387. Of these, NVP-AUY922/VER52296 (developed initially by the scientists at Vernalis and the Cancer Research UK Center for Cancer Therapeutics and now by Novartis) and ganetespib (developed by Synta) are the furthest in development.

AUY922 is currently being evaluated in numerous phase II trials both as monotherapy and in combination with other biologic agents (such as trastuzumab, bevacizumab, bortezemib) across a variety of malignancies. Adverse effects noted in a phase I trial with weekly intravenous injections on a 28-day cycle included nausea, vomiting, and night blindness. DLT included atrial flutter, darkening of vision, diarrhea, and anorexia. Efficacy assessment revealed several cases of disease stabilization. Additionally, cases of metabolic PR were also observed on ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) (Samuel et al., 2010).

Ganetespib is currently being evaluated in phase III trials. When administered as weekly intravenous infusions for 3 weeks of a 28-day cycle, the toxicity profile is similar to AUY922 with the exception of ocular toxicity, which has been infrequently reported with this compound. Other

schedules include twice weekly infusions for 3 weeks of a 28-day cycle and weekly infusions for 4 consecutive weeks; both trials had DLTs of elevated liver enzymes (Cho et al., 2011; Lancet, Smith, et al., 2010). Clinical responses with single-agent ganetespib have been reported in breast cancer (both HER2-positive and triple-negative subtypes), rectal cancer, melanoma, myeloid leukemia, and NSCLC (ALK positive and KRAS mutants) (Jhaveri et al., 2012; Whitesell & Lin, 2012). Diarrhea has been the most common adverse effect, is predominantly grade 1/2 in severity, and is easily reversible. A phase IIb/III trial in NSCLC is currently ongoing (see Section 2.1.4.2.1) and various other phase II trials of single-agent ganetespib in gastric cancer (NCT01167114), GIST (NCT01039519), pancreatic cancer (NCT01227018), and melanoma (NCT01200238) are ongoing.

2.1.2.3. Dihydroindazolone Derivatives

SNX-5422, a glycine prodrug of SNX-2112 is a pyrazole-containing HSP90 inhibitor. A phase I trial of SNX-5422 monotherapy administered orally for 21 days of a 28-day cycle in patients with advanced solid tumors and lymphoma showed no DLT among the 11 patients treated. This trial was ongoing (Bryson et al., 2008) when Pfizer Inc acquired Serenex in March of 2008 (Pfizer Annual report). Subsequently, they discontinued the development of SNX-5422 based on reports of ocular toxicity and irreversible retinal damage (Rajan et al., 2011).

2.1.3. HSP90 Inhibitors as Monotherapy in Molecularly Defined Cancer Subtypes

2.1.3.1. Breast Cancer

In breast cancer, there are multiple known client proteins including the estrogen and progesterone receptor, cyclin D1, Akt, Raf-1, EGFR, and HER2, which is considered the most sensitive client protein amongst these. The mechanism by which HSP90 regulates HER2 is attributed to its role in stabilizing the receptor at the cell's surface such that when HSP90 is inhibited, this induces proteasomal degradation of the unstable HER2 receptor. Recent evidence suggests an additional role for HSP90 as a "molecular switch" in regulating the intrinsic activity of its client proteins, particularly HER2 where it can limit HER2-centered receptor complexes. Furthermore, newer novel clients, such as the intracellular macrophage migration inhibitory factor have also been identified to play a role in inhibiting HER2-driven tumor growth (Schulz et al., 2012). The exact

relevance of this extended role of HSP90 and these newly identified clients is a matter of further investigation (Citri et al., 2004).

Preclinical studies suggest that HSP90 inhibitors may have activity in endocrine resistant breast cancer, HER2 positive, and triple-negative breast cancer (Basso et al., 2002; Caldas-Lopes et al., 2009; Wong & Chen, 2009). Clinically, the most objective tumor regressions have been noted with the first-generation HSP90 inhibitor (tanespimycin) in combination with trastuzumab in HER2-positive disease (Modi, Stopeck, et al., 2011). In order to address the active role of trastuzumab in this combination and assess the activity of HSP90 inhibitors in various subtypes of breast cancer, a phase II trial of ganetespib evaluated the potential of single-agent therapy in a defined cohort of patients with unselected metastatic breast cancer. Of the 22 patients treated on the first stage of the Simon two-stage design, there were 2 PRs noted in patients with HER2 positive metastatic breast cancer who were heavily pretreated. Six additional patients with HER2-positive disease achieved stable disease. Additionally, clinical activity was also reported in a patient with metastatic triple-negative breast cancer who had impressive tumor regressions in the lung, although not meeting strict RECIST criteria for a partial response (Jhaveri et al., 2011).

2.1.3.2. Non–Small Cell Lung Cancer

The progression of NSCLC is associated with an accumulation of molecular abnormalities over time and targeted therapies aimed at these molecular alterations have proven to be an effective treatment strategy. Approximately 5% of the NSCLC patients have ALK rearrangements, particularly the EML4-ALK fusion protein, which is known to be a very sensitive HSP90 client protein (Normant et al., 2011). The clinical translation of this was first reported in a phase II study of IPI 504 conducted in 76 patients with NSCLC after they had progressed on the EGFR TKI therapy. Patients received 400 mg/m^2 of IPI-504 twice weekly on a 21-day cycle. The overall RR was 7% (5 PR) with 4 of these responses seen in patients with tumors that were EGFR-wild type and 1 in a patient with EGFR mutations. Posthoc analysis revealed that 2 out of the 3 patients with ALK gene rearrangement also had a PR and the third patient had SD for 7.2 months with a 24% reduction in tumor size (Sequist et al., 2010). These results were recapitulated in a phase II trial of ganetespib where patients received weekly infusions of 200 mg/m^2 for 3 weeks on a 28-day cycle. Patients were enrolled in this trial based on their mutation status as follows: cohort A: EGFR, cohort B: KRAS, cohort C: EGFR and KRAS wild type (WT), and cohort D: EGFR and

KRAS WT with adenocarcinoma histology. In a subset of patients, additional mutational analysis of BRAF, PIK3CA, ERBB2, and MET as well as FISH analysis for EML4-ALK gene rearrangement was performed. Overall, eight patients in cohort C/D harbored the EML4-ALK gene rearrangement; seven of these (including one patient with crizotinib refractory disease) had disease control lasting at least 16 weeks and 4/8 had objective responses to single-agent ganetespib (Brahmer et al., 2011).

Lastly, recent preclinical studies of mutant KRAS cell lines suggest that these tumors require STK33, a serine/threonine kinase for cell viability and proliferation. Importantly, HSP90 inhibition led to degradation of STK33 and triggered apoptosis in the mutant KRAS cell lines in an STK33-dependent manner. These data form the rationale to explore HSP90 inhibitors in patients who harbor KRAS mutations and utilize STK33 as a biomarker of response (Azoitei et al., 2012).

2.1.3.3. Melanoma

Anywhere from 40 to 80% of the melanoma tumors have activating mutations in the BRAF gene with V600E being the most frequent mutation (Chang et al., 2004). Suppression of the BRAF mutations leads to inhibition of the mitogen-activated protein kinase pathway (MAPK) pathway, which in turn causes growth arrest and promotes apoptosis (Hingorani et al., 2003). Similar to HER2 and ALK, BRAF is an HSP90 client protein. Indeed, changes in biomarkers (c-Raf-1 inhibition, CDK4 depletion, and HSP70 and HSP72 induction) were noted in patients with melanoma who experienced objective responses/prolonged SD in phase I studies with HSP90 inhibitors (Banerji et al., 2005; Pacey et al., 2011). However, contrary to the experience in lung and breast cancer, no objective responses were noted in a phase II trial of single agent 17-AAG therapy (450 mg/m^2 weekly $\times$ 6 weeks) in 15 patients with metastatic melanoma, 9 of which harbored the BRAF mutations. This may be explained by the fact that despite HSP70 induction in the post-treatment biopsies, the effects on Raf-1 kinase expression were short-lived, suggesting a suboptimal target inhibition at this dose and schedule (Solit et al., 2008). Whether optimal target inhibition can be achieved with potent second-generation inhibitors either alone or in combinations is yet to be determined.

2.1.3.4. RCC and Prostate Cancer

Similar to the experience with melanoma, no objective tumor responses were noted in a phase II trial of 17-AAG amongst 20 patients with RCC (12

clear cell and 8 papillary type), where these tumors are known to express client proteins such as hypoxia-inducible factor1-α and c-met, respectively (Ronnen et al., 2006). One explanation for the lack of response in the papillary subtype may be that the met mutation that is found in familial papillary RCC patients is found in only 13% of the sporadic papillary RCC patients (Schmidt et al., 1999) and hence not a major factor in a trial with an unselected group of patients.

Preclinical studies suggest that CRPC may be susceptible to HSP90 inhibitor therapy as the activity of several HSP90 clients including the AR remains critical for disease progression. A phase I trial of 17-DMAG reported a CR in a patient with CRPC (Pacey et al., 2011). However, a phase II trial of single agent 17-AAG failed to show any objective evidence of prostate-specific antigen (PSA) decline or objective responses in CRPC patients (Heath et al., 2008). Another phase II trial of single-agent IPI-504 also failed to report efficacy for this agent in CRPC. In this study, 15 patients with bony metastatic disease had no PSA decline or objective responses. Only 1 out of the 4 patients without bony metastases had a PSA decline of 48% from baseline after 9 cycles (Oh et al., 2009). Interestingly, data in mice suggest that HSP90 inhibition might conversely stimulate the intraosseous growth of prostate cancer due to activation of the osteoclast Src-kinase and Src-dependent Akt activation (Yano et al., 2008). Additionally, the cooperative interactions between the AR and HSP27 (a heat shock protein induced because of HSP90 inhibition) may actually facilitate AR transcriptional activity and enhance prostate cancer survival (Zoubeidi et al., 2007). Perhaps, combining HSP90 inhibitors with agents targeting the AR or with chemotherapy might better target the complex microenvironment of these tumors and provide efficacy that has not been established with monotherapy.

2.1.3.5. Chronic Myelogenous Leukemia (CML) and CLL

The dependence of chronic myelogenous leukemia (CML) on BCR-ABL, another HSP90 client protein, suggests that drug-resistant CML may be an appropriate tumor type to target with HSP90 inhibitors. Indeed, patients with acquired BCR-ABL T315I mutations failing therapy with first- and second-generation ABL TKIs remain sensitive to HSP90 inhibition (Peng, Brain, et al., 2007). ZAP70, another HSP90 client, is expressed in patients with aggressive CLL and can be inhibited with HSP90 inhibitors *in vitro* (Castro et al., 2005). Clinical activity in this setting was reported with BIIB021, an oral HSP90 inhibitor, in a phase I trial (Elfiky et al., 2008).

2.1.4. Combination of HSP90 Inhibitors and Other Anticancer Therapies

Improving efficacy with systemic therapy does not necessarily mean using more lethal drugs. Instead, new strategies that exploit tumor evolution and acquired resistance may be more successful (Cunningham et al., 2011). As tumors progress, they acquire genetic variations and highly advanced metastatic tumors are comprised of genetically and epigenetically heterogenous populations of cells (Merlo et al., 2006). Such epigenetic instability and phenotypic diversity is responsible for the acceleration of tumor invasion, metastatic potential, and drug-resistant biology (Feinberg et al., 2006; Vincent & Gatenby, 2008). Protein homeostasis profoundly influences the relationship between genotype and phenotype (Jarosz et al., 2010) and hence HSP90 may be an important player in determining how the genetic variations in tumors can be translated into phenotypic diversity. In fact, HSP90 has been shown to play a critical role in the evolution of new tumor traits (Jarosz & Lindquist, 2010; Jarosz et al., 2010; Rutherford et al., 2007; Yeyati & van Heyningen, 2008).

Additionally, experiments using fungi have also demonstrated a crucial role for HSP90 in buffering genetic variation and enabling the evolution of drug resistance. In fact, the combination of HSP90 inhibitors with fluconazole and caspofungin have been proposed as a new effective therapeutic strategy for treatment of *Candida albicans* and *Aspergillus fumigatus*, respectively due to their ability to block *de novo* mutation and abrogate acquired mutation due to antifungal treatment (Cowen & Lindquist, 2005; Cowen et al., 2009). Of relevance, other studies of drug resistance in yeast suggest that several anticancer agents are made either less or more potent when combined with HSP90 inhibitors (Lu et al., 2011).

These preclinical studies form the rationale for combination strategies with HSP90 inhibition as a means to not only optimize the anticancer activity of this strategy but also limit the development of drug resistance.

2.1.4.1. HSP90 Inhibitor and HER2 Targeted Monoclonal Antibody Trastuzumab

Preclinical data suggests that HER2 is among the most sensitive HSP90 client proteins. HSP90 inhibitors are therefore being studied in breast cancer both alone and in combination with trastuzumab. In a BT474 xenograft model, the combination of trastuzumab and HSP90 inhibitors produced a superior antitumor effect compared to either drug alone (Chandarlapaty et al., 2010). This could be explained by the weak but prolonged inhibition

of signaling through the HER2 pathway caused by trastuzumab and short-lived but stronger inhibition (via degradation) of the HER2 receptor by HSP90 inhibitors.

Proof of concept was provided by a phase I trial of 17-AAG and trastuzumab in advanced solid tumors where objective responses were noted only in patients with HER2 positive metastatic breast cancer refractory to trastuzumab therapy (Modi et al., 2007). These results were further validated in the phase II trial of this combination with an objective RR of 22% and a clinical benefit rate of 59% (Modi, Stopeck, et al., 2011). However, a phase II trial of IPI-504 and trastuzumab showed modest clinical activity in patients with pretreated HER2+ MBC and did not meet the prespecified criteria for trial expansion (Modi, Saura, et al., 2011). Notably, the dose and schedule of IPI-504 in this trial was significantly lower than that used in the phase II trial in NSCLC and the phase III RING trial for patients with GIST. Additionally, patients on the breast trial were also heavily pretreated with a median of six prior lines of chemotherapy in the metastatic setting, all of which may explain the lack of objective antitumor activity. Various clinical trials continue to explore trastuzumab in combination with newer HSP90 inhibitors for HER2 positive breast cancer (Table 15.2).

Recent clinical data from randomized trials suggests a benefit to continuing trastuzumab beyond progression (Blackwell et al., 2010) which indicates a continued dependence on the HER2 pathway in these tumors. Proposed mechanisms of trastuzumab resistance include the following: expression of a truncated (P95) fragment of HER2 that lacks the trastuzumab-binding epitope, activation of other receptor tyrosine kinases including IGF-1 receptor, and mutational activation of PI3K signaling due to PTEN loss or direct activating PI3K/AKT mutations (Nahta & Esteva, 2006). Because all of these potential mechanisms would be susceptible to the effects of HSP90 inhibition by virtue of the fact that they rely on HSP90 clients, it has been hypothesized that HSP90 inhibitors may be active in trastuzumab-resistant tumors or prevent the development of resistance. To this end, HSP90 inhibitors are being combined with rapamycin and AKT inhibitors and other compounds targeting various components of the PI3K-mtor pathway to either enhance their anticancer activity or circumvent resistance (Francis et al., 2006; Roforth & Tan, 2008). Recently, p95HER2 has been identified as an HSP90 client and trastuzumab-resistant models with high levels of p95HER2 are sensitive to HSP90 inhibition. Chronic treatment with HSP90 inhibitors led to

Table 15.2 Ongoing and Planned Combination Trials of Second-Generation HSP90 Inhibitors with Anticancer Agents

HSP90 Inhibitor + Anticancer Agent	*Company*	*Phase*	*Cancer Type*	*Current Status*
Ganetespib + Docetaxel	Synta Pharmaceuticals	IIb/III	NSCLC	Ongoing (NCT01348126)
Ganetespib + Paclitaxel ± trastuzumab	Synta Pharmaceuticals	I/II	Breast cancer (HER2 and TNBC subtypes)	Planned
Ganetespib ± Bortezemib	Synta Pharmaceuticals	I	MM	Not actively recruiting (NCT01485835)
AUY922 + Trastuzumab	Novartis Pharmaceuticals	I/II, II	Breast Cancer Gastric Cancer	Ongoing (NCT01271920, NCT01402401)
AUY922 + Lapatinib + Letrozole	Novartis Pharmaceuticals	I/II	ER+/HER2+ Breast cancer	Ongoing (NCT01361945)
AUY922 + Erlotinib	Novartis Pharmaceuticals	I/II	NSCLC	Ongoing (NCT01259089)
AUY922 + Capecitabine	Novartis Pharmaceuticals	I	Solid tumors	Ongoing (NCT01226732)
AUY922 + Cetuximab	Novartis Pharmaceuticals	I	KRAS-wild type colon cancer	Ongoing (NCT01294826)
AUY922 ± Bortezemib	Novartis Pharmaceuticals	I/II	MM	Ongoing (NCT00708292)
KW-2478 + Bortezemib	Kyowa Hakko Kirin Pharma, Inc.	I/II	MM	Ongoing (NCT01063907)
AT13387 + Imatinib	Astex Pharmaceuticals	II	GIST	Ongoing (NCT01294202)

ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; GIST: gastrointestinal stromal tumor; MM: multiple myeloma; NSCLC: non–small cell lung cancer; TNBC: triple-negative breast cancer.

sustained loss of both full length HER2 and p-95HER2 with inhibition of Akt activation ultimately leading to complete inhibition of tumor growth (Chandarlapaty et al., 2010).

A key question that remains yet to be addressed is whether upfront therapy with trastuzumab and HSP90 inhibitors can prevent or delay trastuzumab resistance.

2.1.4.2. HSP90 Inhibitor and Chemotherapeutic Agents

Preclinical data from different cancer cell lines and xenograft models indicate that HSP90 inhibitors demonstrate additive or synergistic effects when combined with cytotoxic agents. As HSP90 can protect cells under stress conditions, HSP90 inhibitors have the ability to sensitize cells to the toxic effects of chemotherapy.

2.1.4.2.1. Taxanes While the taxanes disrupt an essential structural component (microtubules) of mitosis, Hsp90 inhibitors impact the regulatory (checkpoint) proteins controlling progression through the cell cycle. In addition, both drugs disrupt other critical facets of cell growth and proliferation, adding to their potential efficacy. Nguyen et al showed 5–22-fold enhancement of paclitaxel cytotoxicity when combined with Hsp90 inhibitors (Nguyen et al., 2001). Hsp90 inhibition can lead to Akt inactivation and sensitize tumor cells to induction of apoptosis by paclitaxel (Solit et al., 2003). Solit et al. also showed synergy between 17-AAG and paclitaxel against breast cancer xenografts when both agents were administered at their submaximally tolerated doses, and the synergy was greatest when both agents were administered sequentially on the same day (Solit et al., 2003). Cells with intact retinoblastoma gene (RB) exposed to this sequential combination underwent G1 growth arrest due to 17-AAG, whereas paclitaxel arrested cells in mitosis. These studies formed the rationale for combination phase I trials of 17-AAG with paclitaxel and docetaxel (Ramalingam et al., 2008; Solit & Rosen, 2006). In a phase II trial, IPI-504 was given in combination with docetaxel to patients with NSCLC (NCT01362400) and ganetespib is currently under evaluation in combination with docetaxel in a phase IIb/III trial for the same patient population (NCT01348126). At Memorial Sloan-Kettering Cancer Center, we are currently planning a phase I/II trial of ganetespib with paclitaxel with or without trastuzumab in patients with HER2 positive and triple-negative metastatic breast cancer (Table 15.2).

2.1.4.2.2. Cisplatin and Gemcitabine Cisplatin cross-links DNA and can consequently trigger apoptosis (Sorenson & Eastman, 1988). Cisplatin can also enhance the activity of HSP90 inhibitors. When HSP90 in inhibited, heat shock factor 1 (HSF1) is released thereby causing a heat shock response, which in turn limits the action of HSP90 inhibitors. Cisplatin blocks the HSF1 mediated heat shock response by blocking the HSF1 binding to the promoter region of the transcription factor (McCollum et al., 2008). Synergistic activity for 17-AAG in combination with cisplatin has previously been reported in colon cancer cell lines (Vasilevskaya et al., 2003, 2004), neuroblastoma and osteosarcoma cell cultures (Bagatell et al., 2005), hepatoma cell cultures, and xenograft models (Liao et al., 2001). Radicicol (a naturally occurring HSP90 inhibitor) also sensitizes colon cancer cells to cisplatin via the interaction between HSP90 and the crucial DNA mismatch repair protein, MLH-1 (Fedier et al., 2005). Synergistic activity may also be related to the effects of 17-AAG on cisplatin induced signaling through the JNK stress-induced and the p53 DNA-damage induced pathways (Vasilevskaya et al., 2003; 2004). Checkpoint kinase 1 (Chk1) has been identified as yet another HSP90 client protein, and when cells are exposed to 17-AAG, it leads to degradation of Chk1, abrogating G1/S arrest induced by gemcitabine (Arlander et al., 2003). These results formed the basis for a phase I trial that evaluated 17-AAG in combination with gemcitabine and cisplatin (Hubbard et al., 2011). Given that there are data, which show that this combination can decrease the toxicity of gemcitabine due to the cytoprotective effects of 17-AAG and cisplatin, both the efficacy and safety results of this trial are highly anticipated (Sano, 2001).

2.1.4.2.3. Cytarabine Cytarabine, a nucleoside analog triggers the sequential activation of ataxia telangiectasia mutated/Rad3-related (ATR) kinase and its substrate Chk1 *ex vivo*, (Mesa et al., 2005) thereby activating the replication checkpoint (O'Connell & Cimprich, 2005). Chk1 activation leads to S-phase arrest of cells. The importance of these events in drug resistance is highlighted by the observation that Chk1 inhibition can enhance nucleoside analog cytotoxicity and overcome Chk1 mediated drug resistance (Mesa et al., 2005) in acute leukemia cells. However, a phase I trial of 17-AAG in combination with cytarabine in patients with relapsed leukemia showed that at clinically tolerable doses of 17-AAG, there was minimal effect on the resistance-mediating client proteins as effective concentrations were achieved only for a brief period *in vivo* (Kaufmann et al., 2011).

2.1.4.2.4. Irinotecan HSP90 inhibitors degrade Chk1, which in turn can enhance the cytotoxicity of the topoisomerase 1 inhibitor, irinotecan, by abrogating the cytoprotective G2-M cell cycle checkpoint (Flatten et al., 2005). A phase I trial reported this combination to be safe with acceptable toxicity. PD assays demonstrated phospho-Chk1 loss, abrogation of the G2-M cell cycle checkpoint, and cell death in tumor biopsies obtained at the maximum tolerated dose (MTD) (Tse et al., 2008).

2.1.4.3. HSP90 Inhibitor and Proteasome Inhibitors

Bortezemib, a first-in-class inhibitor of the 26S proteasome is approved for the treatment of relapsed multiple myeloma and mantle cell lymphoma. Preclinically, bortezemib induces a stress response characterized by transcription of proteasome subunits and molecular chaperones in the heat shock protein family including HSP90 in multiple myeloma (MM) cells (Mitsiades et al., 2002). In this same study, the combination of an HSP90 inhibitor with bortezomib enhanced the bortezomib-triggered apoptosis, even in drug resistant MM cells. Furthermore, this combination induced a prolonged intracellular accumulation of ubiquitinated proteins than either drug alone which was attributed to the synergistic suppression of chymotryptic activity of the 20S proteasome (Mitsiades et al., 2006). Building on this, a phase II trial of tanespimycin and bortezemib conducted in heavily pretreated and refractory MM patients showed an overall RR of 14% (2 PR and 1 minor response) with SD in 10 additional patients (Richardson et al., 2010). Importantly, there were low rates of peripheral neuropathy, especially grade 3 neuropathy with this combination. These findings were in line with the preclinical findings, which demonstrated that tanespimycin has a neuroprotective effect against bortezemib induced peripheral neuropathy (Zhong et al., 2008). These promising results led to the further development of tanespimycin in a phase III trial for the treatment of MM. However, when the clinical development of tanespimycin was suspended, this trial was also affected. (PressRelease, Bristol-Myers Squibb Halts Development of Tanespimycin). A phase I trial of ganetespib with or without bortezemib is currently planned (NCT01485835).

2.1.4.4. HSP90 Inhibitor and Histone Deacetylase Inhibitors (HDAC)

Histone deacetylase (HDAC) inhibitors inhibit deacytelation of many proteins including histones and HSP90. Vorinostat, an HDAC inhibitor inhibits growth and induces apoptosis in various human carcinoma cells (Lane & Chabner, 2009). Furthermore, it affects the expression of various

genes that are necessary for the proliferation of cancer cells. Many HDAC inhibitors are therefore under clinical evaluation as cancer therapeutics (Lane & Chabner, 2009). Synergistic activity due to disruption of the survival pathways and cell cycle progression was observed with the co-administration of 17-AAG and various HDAC inhibitors (George et al., 2005; Rahmani et al., 2003;). Additionally, HDAC inhibition results in hyperacetylation of HSP90, which in turn inhibits the ATP-binding and chaperone activities of HSP90 leading to enhanced degradation of HSP90 clients such as BCR-ABL, HER2, and FLT3. These data suggest potential synergy of HDAC inhibitors with imatinib, trastuzumab, or FLT3 inhibitors in cancers driven by amplified or mutated tyrosine kinases (Whitesell & Lindquist, 2005).

2.1.4.5. HSP90 Inhibitors and Tyrosine Kinase Inhibitors (TKIs)

Several tyrosine kinase inhibitors (TKIs) act synergistically with HSP90 inhibitors in killing tumor cells. Synergy is due to pronounced reduction in the protein level and activity of these kinases, which are HSP90 clients. In NSCLC, tumor-associated activating mutations in the EGFR can identify patients who are likely to respond to the EGFR TKIs (Lynch et al., 2004). However, resistance almost invariably develops after a median of 10–14 months (Oxnard et al., 2011). Several possible mechanisms have been identified, the most common being the T790M gatekeeper mutation which results from a threonine–methionine substitution at position 790 in the exon 20 of the EGFR TK domain in approximately 50% of the cases (Kosaka et al., 2006; Oxnard et al., 2011). These second mutations enable the cancer cells to continue signaling via mutant EGFR. Amplification of *MET* protooncogene is a second mechanism of resistance observed in approximately 20% of the patients who develop acquired resistance following initial treatment with EGFR TKI (Bean et al., 2007; Engelman et al., 2007). Treatment of EGFR mutant cell lines with HSP90 inhibitors (in this case geladanamycin) results in cellular degradation, decreased levels of pAKT/cyclin D1, and increased apoptosis (Yang et al., 2006). Furthermore, HSP90 inhibitors delay tumor growth in nude mice with gefitinib-resistant H1975-xenografts *in vivo* (Shimamura et al., 2008). This has formed the rationale for their use in acquired resistance and a phase I/II trial of AUY922 in combination with erlotinib in NSCLC patients with acquired resistance to EGFR TKIs is ongoing (NCT01259089).

Similarly, molecular analysis of tumor cells of a patient who relapsed 5 months after crizotinib (ALK inhibitor), revealed two novel mutations in the EML4-ALK gene, one of which (L1196M) conferred resistance to

crizotinib. The study also showed that the crizotinib-resistant tumor cells remained addicted to ALK signaling and interestingly retained sensitivity to HSP90 inhibition (tanespimycin). Clinically, this was noted in a NSCLC patient who responded to crizotinib for 1 year before progression and had further objective response after ganetespib therapy (Brahmer et al., 2011).

HSP90 inhibitors also demonstrate clinical activity in AML (Lancet, Gojo, et al., 2010). FLT, a tyrosine kinase and an HSP90 client that is frequently mutated in a subpopulation of AML, is considered a marker of poor prognosis in the elderly (Weisberg et al., 2009). However, FLT3 mutations may have enhanced HSP90 dependence and leukemic cells with this gain-of-function mutation are synergistically and selectively sensitive to 17-AAG and FLT3 inhibitors (George et al., 2004; Yao et al., 2003). Similarly, BCR-ABL (pathophysiologic cause of CML) is degraded in response to HSP90 inhibition (Shiotsu et al., 2000). Drug-resistant CML can therefore be treated with HSP90 inhibitors either alone or in combination with ABL TKIs (Peng, Li, et al., 2007). In fact, low dose HSP90 inhibitor therapy reduces the emergence of BCR-ABL kinase mutants in cells selected for resistance to imatinib (Tauchi et al., 2011). A phase I/II trial is currently evaluating the efficacy of ganetespib in AML, CML and other myeloproliferative disorders (NCT00964873).

Lastly, synergistic activity based on simultaneous disruption of Raf-signaling was the justification for the phase I trial of sorafenib plus 17-AAG, which ultimately showed clinical benefit in RCC and melanoma (Vaishampayan et al., 2010).

2.1.4.6. HSP90 Inhibitors and Death Receptor Ligands: Tumor Necrosis Factor (TNF) and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)

The death receptor ligands TRAIL and TNF are promising candidates for cancer therapy because of their apoptosis-inducing abilities via (1) binding to the death receptors DR4 and DR5 that induce caspase-8-dependent apoptosis and (2) activation of NFκB signaling (Wajant et al., 2005). However, many tumors remain resistant to treatment with TRAIL, which can be correlated with the deregulated expression of antiapoptotic molecules. Survivin, a member of the inhibitor of apoptosis protein family, has been identified as an HSP90 client and is also thought to be capable of inhibiting TRAIL-mediated apoptosis. Survivin is expressed at high levels in glioblastoma and preclinical data suggests suppression of survivin by 17-AAG enhanced TRAIL-mediated apoptosis (Siegelin et al., 2009). Pre- or

coexposure of 17-AAG has also demonstrated induction of apoptosis in TRAIL/TNF resistant prostate cancer LNCaP cells, lung H23, H460, colon HT29, and RKO cells, among others (Day et al., 2010; Solit et al., 2008; Williams et al., 2007). Synergy is also observed with 17-AAG and anti-TRAIL monoclonal antibodies in Hodgkin's lymphoma (Georgakis et al., 2006).

2.1.4.7. HSP90 Inhibitors and Other Treatments

HSP90 inhibitors can enhance the radiosensitivity of multiple tumor cell lines. This is because many HSP90 clients such as Raf-1, AKT, and HER2 are associated with radio response, albeit in a cell type–dependent manner. HSP90 inhibitors can cause degradation of these proteins thus increasing apoptosis and G2 arrest (Yin et al., 2010). These proteins are therefore referred to as the radio-response proteins or proteins that can possibly serve as determinants of radiosensitivity.

HSP90 inhibitors can also act with other drugs such as arsenic trioxide by abrogating AKT activation thereby enhancing the action of arsenic trioxide in leukemia cells (Pelicano et al., 2006).

2.1.4.8. Resistance to HSP90 Inhibitors

While the emergence of acquired target-related resistance seems to be evident with many TKIs, no drug-resistant HSP90-related mutations have been reported thus far. Reduced expression of NADPH/quinone oxidoreductase I (NQ01) has been associated with resistance to 17-AAG; however, no cross-resistance to the nongeldanamycin compounds has been observed thus far (Gaspar et al., 2009). A single point mutation in the N-domain of *Humicola fuscoatra* HSP90 has been reported to confer resistance to radicicol. However, no similar mutation or polymorphism has been reported in the cancer cell HSP90 to date (Prodromou et al., 2009). In addition, HSF1 dependent HSP70 and HSP27 induction frequently occurs in response to HSP90 inhibitors and may lead to diminished drug sensitivity. This represents an important target for improved therapeutic strategies that aim at suppression of the Hsp90 inhibitor-induced heat shock response (McCollum et al., 2008).

2.2. Alternative Methods for Targeting HSP90

2.2.1. HSP90 Inhibitors Targeting the ATP Binding Site of C-Domain

To date, the vast majority of research efforts have been focused on targeting the N-terminal domain of HSP90. However, several new approaches are

being investigated to enhance the cancer cell sensitivity to HSP90 inhibitors. In this regard, a second druggable site has been identified in the C-domain of HSP90. This site can be targeted by the coumarin antibiotics (novobiocin, clorobiocin, coumermycin A1). Unfortunately, these compounds demonstrate a poor affinity for HSP90 coupled with increased affinity for topoisomerase II inhibitors, which has prohibited their clinical development thus far (Donnelly & Blagg, 2008). However, recent efforts with a novobiocin derivative, F-4 has demonstrated increased affinity toward HSP90 and decreased affinity toward the topoisomerase II inhibitors. Additionally, it has shown superior efficacy when compared to 17-AAG, demonstrating increased apoptosis in LNCaP and PC-3 prostate cancer cell lines (Matthews et al., 2010). Another compound, KU135 produced greater apoptosis compared to 17-AAG in Jurkat T cell leukemia lymphocytes (Shelton et al., 2009). Emerging data also favor the C-terminal inhibitors as they cause less-robust HSF1 activation than the geldanamycin compounds (Conde et al., 2009). Together, these data support optimization of the medicinal chemistry and preclinical evaluation of the C-terminal HSP90 inhibitors.

2.2.2. Targeting HSF1, HSP70, and HSP27

Although activation of HSF1 occurs in response to HSP90 inhibition and acts as a PD marker, it also limits the activity of HSP90 inhibitors due to HSF1 dependent transcriptional activation of HSP70, HSP27, which in turn protect the cancer cells from apoptosis. To that end, cells in which HSF1 has been knocked out are much more sensitive to HSP90 inhibition compared to cells with wild-type HSF1 (Bagatell et al., 2000). Similarly, knockdown of HSP27 and HSP70 can increase the sensitivity of the cells to HSP90 inhibition and induce tumor-specific apoptosis (McCollum et al., 2006; Powers et al., 2010). Research efforts are currently focused to identify and validate inhibitors of HSP70, HSF1, and HSP27 either alone or in combination with HSP90 inhibitors (Evans et al., 2010; Hadchity et al., 2009; Powers & Workman, 2007).

2.2.3. Inhibiting HSP90 Cochaperone Interactions

An alternative strategy for HSP90 inhibition is targeting the HSP90 interaction with its cochaperones (such as Cdc37), rather than binding the ATP binding site in the N- and C-terminal domains of HSP90. Recent molecular docking studies and coimmunoprecipitation studies have confirmed celastrol as a natural and unique HSP90 inhibitor that interferes with the HSP90-Cdc37 cochaperone interactions and thus destabilizes several HSP90 clients

(Zhang et al., 2008). Furthermore, celastrol specifically targets the N-terminal domain and the middle HSP90-binding domain of Cdc37, suggesting Cdc37 as an additional target for inhibiting the HSP90-Cdc37 cochaperone complex (Sreeramulu et al., 2009).

3. DEVELOPMENT OF BIOMARKERS AND/OR COMPANION DIAGNOSTIC ASSAYS

As we usher in the era of personalized medicine, the utilization of biomarkers in the clinical development of novel targeted therapies is of paramount importance. Biomarkers not only demonstrate proof- of- target and mechanism but can also help predict responses and stratify patients at an early stage. In HSP90 inhibitor clinical trials, target inhibition has been ascertained predominantly by measuring tumor client proteins pre- and post-HSP90 inhibitor therapy.

3.1. PBMC Assays to Monitor Response to HSP90 Inhibition

Many of the clinical trials of HSP90 inhibitors have evaluated the expression of client proteins like Raf-1, Cdk4, c-KIT from isolated PBMCs taken from patients (Ramanathan et al., 2005; Solit et al., 2007). As discussed earlier, the HSP90 complex machinery is upregulated by HSP90 inhibitors via activation of the transcription factor, HSF1, which has also been established as a PD biomarker. These PD assays performed on surrogate tissue such as PBMCs, although readily accessible and reproducible, do not reflect the true effects of the drug in tumor tissue (Grem et al., 2005; Kummar et al., 2010; Ramanathan et al., 2010). This is due to the fact that HSP90 is expressed in normal tissues in a latent, uncomplexed state of low-affinity binding to HSP90 inhibitors, while in tumor cells, HSP90 exists in a multichaperone complex in an activated high-affinity conformation, driving the selective accumulation and activity of certain HSP90 inhibitors (Kamal et al., 2003). It has been proposed that a functionally distinct HSP90 pool is enriched in tumor cells, referred to as "oncogenic HSP90." This "oncogenic HSP90" (1) specifically interacts with HSP90-dependent oncogenic client proteins to maintain tumor cell survival and (2) is not dictated by HSP90 overexpression alone and predicts the cell's sensitivity to HSP90 inhibition (Moulick et al., 2011). Additionally, induction of HSP70 in PBMC with HSP90 inhibition, although useful for establishing biologically active drug dosing, has failed to predict clinical responses (Kummar et al., 2010).

3.2. Pre- and Post-Treatment Tumor Biopsies to Ascertain HSP90 Inhibition

In a minority of clinical trials, tumor biopsies of patients on HSP90 inhibitor therapy have been undertaken for PD analysis (Banerji et al., 2005; Solit et al., 2008). However, the results have been of limited use due to the difficulty and low yields of biopsy collection as well as the low sensitivity of traditional immunohistochemical staining techniques that can only identify partial but not complete reduction in protein expression. In the phase I melanoma studies, patients who experienced prolonged SD when treated with 17-AAG at 320 mg/m^2/week and 450 mg/m^2/week had evidence of changes in biomarkers such as Raf-1 and CDK4 depletion in their tumor biopsies. However, when 17-AAG was administered at 450 mg/m^2/week × 6 weeks in the phase II trial, the effects on Raf-1 were short-lived with no objective tumor responses suggesting suboptimal target inhibition at this dose and schedule (Solit et al., 2008). The inconsistency in results may be related to the fact that tumor biopsies provide static information of a small part of the tumor and disregard the heterogeneity of metastatic tumor burden; therefore, while they can be helpful in establishing target modulation, there may be other factors, which are missing that may determine the ultimate effectiveness of the therapy.

3.3. Noninvasive Imaging Biomarkers for PK/PD Monitoring and Assess Treatment Response

Molecular imaging biomarkers such as positron emission tomography/computed tomography (PET/CT) are a new emerging class of biomarkers that offer several advantages: they rely on *in vivo* tumor biology thereby allowing response assessment by monitoring changes in the functional/molecular processes, are noninvasive and quantitative, highly sensitive, easily repeated and permit assessment of tumor heterogeneity, and whole tumor burden in the body. In addition, they can provide high-resolution images and accurate data regarding spatial and temporal tumor uptake and retention thus allowing for molecular characterization of the target, measurement of tumor PK and PD changes.

In HSP90 inhibitor trials, ^{18}F-FDG PET has been utilized to assess early treatment response. Specifically, in a phase I trial of IPI-504 in patients with metastatic TKI-resistant GIST where patients received the HSP90 inhibitor on days 1, 4, 8, and 11 of a 21-day cycle, ^{18}F-FDG PET/CT imaging was incorporated in the study design to be performed at baseline, day 11 and day

21. In this trial, there was a reduction in FDG uptake with treatment, reactivation of tumor FDG uptake with planned breaks in drug administration, and decreased FDG uptake with redosing (Demetri et al., 2007). This suggests that at least in high glycolytic tumors, FDG PET may be a useful PD correlate of HSP90 inhibitor antitumor activity. However, because there were no objective responses and only SD in this trial, it has raised the question of whether complete FDG uptake inhibition rather than mere reduction is needed to result in CR or PR. With BIIB021 and NVP-AUY922, metabolic PR and SD were reported using FDG-PET imaging (Modi et al., 2010; Samuel et al., 2010).

Compared to ^{18}F-FDG PET, which measures tumor glucose metabolism, analyses of treatment-induced changes in relevant HSP90 client proteins can be used as PD endpoints to correlate with therapeutic response. To this end, EGFR, AR, HER2, Akt, VEGF, etc. can be targeted with specific small molecular probes, although targets that are membrane proteins can be imaged more easily compared to those that are not. For example, EGFR imaging with ^{64}copper-DOTA-cetuximab has been used for PD monitoring of 17-AAG in PC-3 human prostate cancer xenografts (Niu et al., 2008). Similarly, for HER2-positive tumors, various radiolabeled antibodies have been utilized to monitor HER2 degradation. For example, in the BT474 breast cancer xenograft, about 50% degradation of HER2 could be monitored using ^{68}Gallium-DCHF, a F(ab′2) fragment of the HER2 antibody trastuzumab (Smith-Jones et al., 2004). When ^{68}Gallium PET was compared to ^{18}F-FDG PET, there was no change in tumor uptake visualized on FDG-PET after HSP90 inhibitor therapy (Smith-Jones et al., 2006). Indeed, scanning with F(ab')2-trastuzumab Ga-68 showed uptake to known metastatic sites with no false positives (Fig. 15.2). PET imaging with ^{64}Cu-trastuzumab showed a 64% reduction in tumor uptake in SKOV-3 xenograft models after 24 h of HSP90 inhibitor therapy (Niu et al., 2009). When ^{111}Indium-DOTA-trastuzumab and ^{64}Copper-DOTA-trastuzumab Fab were utilized to image HER2-positive tumors, ^{111}In-DOTA-trastuzumab Fab was more specific than ^{64}Cu-DOTA-trastuzumab Fab for patients with low HER2 receptor density (Chan et al., 2011). More recently, ^{89}Zirconium-trastuzumab was validated preclinically for PD monitoring of HER2 downregulation with 41% decrease in tumor uptake after treatment with NVP-AUY922 (Oude Munnink et al., 2010). Changes in tumor receptor expression of VEGF and EGFR (other known HSP90 clients) have been investigated using, zirconium-89 and copper-64 labeled antibodies (Akhurst et al., 2008; Holland

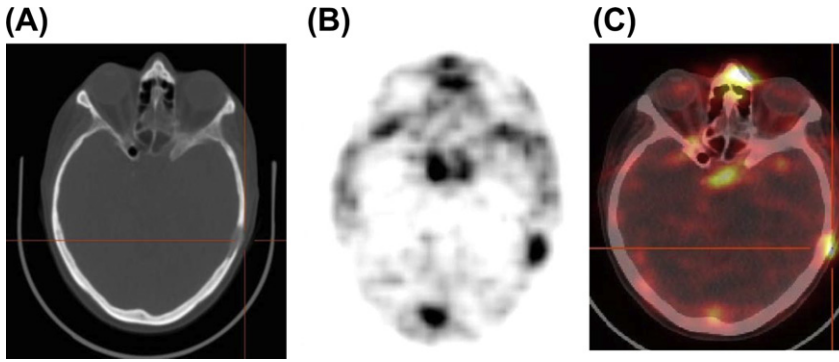


Figure 15.2 Axial images of lytic metastasis in left temporal bone in patient with MBC (with normal uptake in vasculature of ^{68}Ga-F(ab′)$_2$-trastuzumab). **A)** CT Scan; **B)** PET scan with 68Ga-F(ab′)2-trastuzumab; **C)** Fused CT-PET scan with 68Ga-F(ab′)2-trastuzumab. ^{68}Ga: Gallium 68, F (ab′)2: fragment antigen-binding 2, CT: computed tomography, PET: positron emission tomography. For color version of this figure, the reader is referred to the online version of this book.

et al., 2010; Nagengast et al., 2010; Niu et al., 2008). The main difference between these radiolabeled antibodies is their half-life which limits imaging with ^{68}Ga (half-life of 1.13 h) to several hours post-injection, ^{64}Cu (half-life of 12.7 h) to 48 h post-injection, and allows imaging for up to 144 h in case of ^{89}Zr (half-life of 78.4 h). This is particularly relevant as antibodies like trastuzumab accumulate slowly into the tumors and therefore imaging HER2 downregulation days after injection rather than hours after injection would be a rational choice. Results of clinical studies incorporating ^{89}Zr-Trastuzumab PET are eagerly awaited (Schroder et al., 2011). Lastly, although HER2 is thought to be among the most sensitive client protein for HSP90 inhibition, it is not universally expressed in tumor tissue. On the contrary, other client proteins such as VEGF that are universally expressed in tumors have been studied preclinically and are clinically being evaluated using ^{89}Zr-bevacizumab PET (Nagengast et al., 2010; Schroder et al., 2011).

^{18}F-Fluorothymidine (FLT), a marker of cellular proliferation was evaluated at varying doses and time points in spheroid models of BT474 cells treated with HSP90 inhibitor. This study identified a once a week schedule for the HSP90 inhibitor and ^{18}F-FLT as a suitable biomarker to guide dosing schedule and monitor treatment response (Bergstrom et al., 2008).

Another novel strategy for PK/PD assessment is direct radiolabeling of the therapeutic drug itself. For example, PU-H71, a small molecule HSP90 inhibitor, has an endogenous 127-Iodine atom that has been replaced with

a PET radionuclide 124-Iodine to result in the imaging agent, ^{124}I-PU-H71. This PET imaging radioligand is virtually identical to the therapeutic agent, which allows noninvasive assessment of drug biodistribution. A phase 0 trial of ^{124}I-PU-H71 is currently evaluating the biodistribution, metabolism, and radiation dosimetry of this agent in patients with advanced solid tumors (NCT01269593). The purpose of this study is to see if there is tumor uptake by PU-H71 and to identify the duration of retention. These results will allow optimization of the PET agent, which will be incorporated into the planned phase I trial of PU-H71 (NCT01393509) and allow for (1) measurement of tumor PK and (2) investigate tumor dose–response correlations by measuring PU-H71 tumor concentrations for up to 48 h post coinjection of PU-H71 and ^{124}I-PU-H71. Together, these studies will allow for (1) selection of patients who might best benefit from therapy, (2) noninvasive assessment of tumor heterogeneity and whole tumor burden, and (3) useful information that will guide the optimal dosing and scheduling of this class of agents.

3.4. Serum Biomarkers

Serum Biomarkers such as soluble IGFBP2 (insulin growth factor binding protein), extra cellular domain HER2 (Zhang et al., 2006), and serum HSP70 (Dakappagari et al., 2010) have been evaluated preclinically as PD endpoints; however, the clinical utility of these agents is yet to be validated (Eiseman et al., 2007).

3.5. Other Relevant Biomarkers

In HER2-amplified breast cancer and ALK-mutated NSCLC, the expression of HER2 and ALK respectively has been associated with tumor response. Overexpression of HSP90 has also been identified as an independent prognostic marker in breast cancer, being associated with a shorter survival (Pick et al., 2007). In a similar way, low HSP90 expression was correlated to longer overall survival for patients with NSCLC (Gallegos Ruiz et al., 2008). Due to the vast experience with HSP90 inhibitors at MSKCC, we are retrospectively evaluating the role of biomarkers such as HSP90, HSP70, ER, PR, HER2, AR, EGFR, PTEN loss from available archived tissue specimens of patients treated with various first- and second-generation HSP90 inhibitors (17-AAG, 17-DMAG, CNF2024, ganetespib, IPI-504). We will present these results at the upcoming 2012 annual ASCO meeting in June.

HSF1, the transcriptional regulator of the entire heat shock network, is also undergoing evaluation as a potential predictive biomarker in an upcoming metastatic breast cancer trial to determine if patients with evidence of HSF1 activation are more or less likely respond to HSP90 therapy (Whitesell & Lin, 2012).

4. CONCLUSION

Targeting the molecular chaperone, Hsp90 has the potential to simultaneously disrupt multiple oncogenic signaling pathways in cancer cells, making it an appealing therapeutic target (Whitesell & Lindquist, 2005; Workman et al., 2007). Proof of mechanism and proof of concept for therapeutic activity has been reported with tanespimycin, most convincingly for patients with HER2 positive breast cancer. Unfortunately, tanespimycin is no longer in development for reasons unrelated to its clinical potential.

Nevertheless, considerable progress and intense efforts from both industry and academia have resulted in the introduction of highly potent, chemically distinct, second-generation small molecule inhibitors with improved pharmacological and toxicological properties. Despite the multitude of Hsp90 inhibitors in clinical development, none has been approved by the Food and Drug Administration.

So, why are not we there yet? Much of the work on HSP90 inhibitors has focused on identifying tumors that are addicted to a sensitive client that can be degraded by *in vivo* at a nontoxic dose. While this approach has been illustrated to be successful in HER2 positive breast cancer and ALK-mutated NSCLC, focusing only on a specific client/HSP90 interaction, it ignores the actual potential of HSP90 to simultaneously impact multiple oncogenic pathways. Current research efforts are therefore focused on translating the findings from the laboratory into the clinical setting by combining HSP90 inhibitors with chemotherapy or other targeted agents to achieve synergistic antitumor effects. Combination therapies may also serve as an effective strategy to overcome or prevent drug resistance. In this regard, HSP90 inhibitors are currently being pursued in many cancer types including lung cancer, GIST, and melanoma to overcome the resistance to tyrosine kinase inhibitors, and in combination with trastuzumab and inhibitors of the PI3k-Akt pathway in

HER2 positive breast cancer, with the hope that combination therapy upfront one day might prevent drug resistance in the first place (Lamb et al., 2006).

Finally, as with the development of any targeted therapy, judicious exploration and use of biomarkers/companion diagnostic assays are critical in the optimal clinical development of HSP90 inhibitors. Pre- and post-treatment tumor biopsies remain crucial to ascertain target modulation and should be incorporated into future studies. Non-invasive molecular imaging is also emerging as a uniquely promising approach to monitor PD endpoints. Direct molecular imaging using the labeled drug itself (e.g., ^{124}I-PU-H71) can be utilized to measure tumor pharmacokinetics, optimize the dose and schedule of this class of agents, and guide with patient selection. This is particularly necessary in optimizing the dose and schedule of HSP90 inhibitors given that they have a rapid clearance from the plasma compartment but a prolonged retention in tumors themselves (Caldas-Lopes et al., 2009; Cerchietti et al., 2009; Chandarlapaty et al., 2008; Marubayashi et al., 2010; Vilenchik et al., 2004).

Going forward, a greater understanding of the mechanisms underlying tumor selectivity, genetic variation, and its effects on drug resistance, identification of synergistic combinations, along with incorporation of biomarkers/companion diagnostic assays that can ascertain target modulation and serve as a biomarkers of response will surely accelerate the clinical development of HSP90 inhibitors, and ultimately lead to the much anticipated regulatory approval for these molecularly intriguing and therapeutically promising drugs.

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ABBREVIATIONS

CLL Chronic lymphocytic leukemia
CML Chronic myelogenous leukemia
EGFR Epidermal growth factor receptor

FDG Fluorodeoxyglucose
FLT Fluorothymidine
GIST Gastrointestinal stromal tumor
GM Geldanamycin
HER2 Human epidermal growth factor receptor
Hsp90 Heat shock protein 90
MM Multiple myeloma
NSCLC Non-small cell lung cancer
PD Pharmacodynamics
PK Pharmacokinetics
RCC Renal cell carcinoma

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CHAPTER SIXTEEN

Apoptosis In Targeted Therapy Responses: The Role of BIM

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Abstract

The treatment of advanced cancer has undergone a dramatic change over the past 5 years. Laboratory findings have led to the development of newer treatments, often termed "targeted therapies," which are significantly different from traditional chemotherapies in that they aim to disrupt critical processes needed specifically for a cancer cell's growth and survival, therefore, eliminating some of the general toxicities of chemotherapies. Cancers with specific genetic abnormalities, for instance epidermal growth factor receptor (*EGFR*) mutant lung cancers and *HER2* amplified breast cancers, are often sensitive to these new targeted therapies that can specifically inhibit the function of EGFR or HER2. This has led to more routine prospective genetic testing of cancers to determine which patients should get these treatments instead of chemotherapy. However, emerging clinical data have revealed that some cancers with these genetic mutations (that predict a response) are unexpectedly not sensitive to these treatments. There is a growing body of evidence suggesting a deficiency in apoptosis following targeted therapy treatment can lead to this lack of sensitivity. Moreover, the pro-apoptotic protein BIM has emerged as a key modulator of apoptosis following effective targeted therapy, and deficiencies in BIM expression result in targeted therapy resistance. In this chapter, we summarize what is known about the role of BIM in targeted therapy-induced apoptosis, and discuss the implications of deficient BIM in cancers treated with these therapies. We highlight potential pharmaceutical strategies to overcome low BIM expression and sensitize these cancers to targeted therapies.

1. INTRODUCTION

Improvements in the genetic and molecular characterizations of cancers have revealed that some cancers harbor "addicting" mutations, amplifications, or translocations in a particular gene. The proteins coded by these genes drive the growth and survival of the tumor. In some cancers, the mutated genes encode constitutively active protein kinases that result in tumor proliferation. Drugs have been developed that can

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disrupt the function of these particular oncogenes to which the cancers are addicted. Clinical trials utilizing these "targeted therapies" have demonstrated that they can yield dramatic and robust responses in oncogene-addicted cancers such as *HER2* amplified breast cancers, *BCR-ABL* translocated chronic myelogenous leukemia (CML), epidermal growth factor receptor (*EGFR*) mutant lung cancers, *ALK* translocated lung cancers, and *BRAF* mutant melanomas (Baselga et al., 1999; Druker, 2001; Flaherty et al., 2010; Sequist et al., 2011; Shaw et al., 2011). These clinical trial successes have ushered in the generation of personalized medicine to treat human cancer.

Inhibition of the genetically activated kinase in these oncogene-addiction paradigms leads to growth arrest and, in many cases, to cell death, most notably apoptosis. However, not all of these oncogene-addicted cancers appear to undergo apoptosis following targeted therapy treatment. There is increasing evidence that apoptosis is a central component of tumor shrinkage in response to targeted therapies (Brachmann et al., 2009; Ebi et al., 2011; Faber et al., 2009, 2011). Importantly, recent studies have shown that cancers that arrest growth without concurrent apoptosis following targeted therapy treatment have inferior responses than those that also undergo apoptosis. It has become increasingly clear that even among patients whose cancers harbor the same driver oncogene, there is a wide range of clinical responses ranging from complete remissions all the way to progressive disease. The reasons for such heterogeneity in these cancers are largely unknown. Data from a number of laboratories have implicated differential induction of apoptosis as a contributing component to this heterogeneity of clinical benefit (Brachmann et al., 2009; Faber et al., 2009; Ng et al., 2012). The upregulation of the pro-apoptotic Bcl-2 family member, BIM, has been found to be indispensible for targeted therapy-induced apoptosis (Costa et al., 2007; Deng et al., 2007b; Faber et al., 2009, 2011; Paraiso et al., 2011; Rahmani et al., 2009; Takezawa et al., 2011; Wickenden et al., 2008; Will et al., 2010). Some cancers have diminished BIM expression, and these cancers may have suboptimal responses to targeted therapies.

In this chapter, we briefly overview the history of targeted therapies and discuss its main limitations. For the purposes of this review, we will focus on targeted therapies that are kinase inhibitors (KIs) blocking a genetically activated kinase. We will then focus on the current understanding of the role of apoptosis as a critical component of response to targeted therapies, with emphasis on the role of BIM. Finally, we will discuss the utility of BIM as

a functional biomarker to identify cancers that will have the least responsiveness to targeted therapies, as well as potential strategies to resensitize low BIM expressing cancers to targeted therapies.

2. BACKGROUND

2.1. Development of Targeted Therapies

Contemporary genomics have revealed that certain cancers have addicting mutations to driver oncogenes. When the proteins encoded by these oncogenes are inhibited with targeted therapies, there can be impressive clinical responses. The first such instance was found in patients with CML. These cancers have the *BCR:ABL* translocation (also known as the Philadelphia Chromosome) and are addicted to ABL kinase activity. Pharmaceutical disruption of ABL by small molecule ATP mimetics (e.g., imatinib) leads to dramatic remissions in patients with CML (Druker et al., 1996). Imatinib is now first line therapy for CMLs harboring the *Bcr-Abl* fusion gene (Mauro & Druker, 2001; Thiesing et al., 2000).

Following this seminal discovery, a number of oncogene-addicted cancers have been uncovered. Interestingly, many, but not all, of these have been receptor tyrosine kinases (RTKs), similar to ABL, which when aberrantly active, turn on multiple growth/survival pathways. Lynch et al. (2004) found somatic mutations in the *EGFR* in a subset of patients with non–small cell lung cancers (NSCLCs). These cancers were exquisitely sensitive to the EGFR small molecule KI, gefitinib. Subsequently, EGFR inhibitors have been effective at treating patients harboring *EGFR* mutant NSCLCs, yet they are largely ineffective in NSCLCs without *EGFR* mutations (Bell et al., 2005; Jackman et al., 2006; Sequist et al., 2008). Other examples of oncogene-addicted cancers that have had promising clinical responses to targeted therapies include *HER2* amplified breast cancers, *ALK* mutant lung cancers, and *BRAF* mutant melanoma cancers (Baselga et al., 1999; Flaherty et al., 2010; Kwak et al., 2010; Shaw et al., 2009) (Table 16.1).

2.2. Resistance to Targeted Therapies

2.2.1. Intrinsic Insensitivity

Increasing numbers of targeted therapies are being implemented in the clinics as more oncogene-addicted paradigms emerge. Accordingly, routine assessment of sensitizing genetic alterations is increasingly performed to

Table 16.1 Examples of Oncogene-Addicted Cancers and the Kinase Inhibitors They are Treated with

Addiction	Cancer Type	Therapy
Bcr-ABl	CML (chronic leukemia)	Imatinib (Gleevac)
C-Kit	GIST (sarcoma)	Imatinib (Gleevac)
PDGFR mutation	GIST (sarcoma)	Imatinib (Gleevac)
EGFR mutations	Lung	Tarceva Gefitinib (Iressa)
ALK translocation	Lung	Crizotinib
BRAF mutation	Melanoma	Vemurafenib Herceptin
Her2 amplification	Breast	Lapatinib

match patients with the appropriate targeted therapy. However, it has become apparent that some patients with genetic alterations suggestive of oncogene addiction, who would be expected to respond to therapies, do not. For instance, two recent studies have reported that 30–40% of patients with *EGFR* mutant NSCLCs and *ALK* translocated lung cancers failed to achieve RECIST-criteria remissions with EGFR or ALK kinase inhibitors, and some patients progressed within 6 months of starting the therapy (Sequist et al., 2008; Shaw et al., 2009). The heterogeneity of clinical benefits and unexpected lack of responses in a large subgroup of patients remains an outstanding problem. For the purposes of this review, we will refer to this as intrinsic insensitivity, and this remains a limitation to targeted therapies. It is important to note that many cancers do not respond to targeted therapies because they do not harbor the sensitizing genetic mutations (e.g., a *KRAS* mutant lung cancer does not respond to EGFR inhibitors). We will discuss the failure to undergo apoptosis as a key mediator of intrinsic insensitivity in cancers that do harbor the genetic mutations that suggest sensitivity to a targeted therapy (e.g., an *EGFR* mutant lung cancer that does not respond to and EGFR inhibitor). In addition, there are occasionally secondary genetic events that mitigate the efficacy of targeted therapies to be effective. These may include pre-existing resistance mutations as in the case of *T790M* mutations in *EGFR* mutant NSCLCs or other genetic events that impair the ability of targeted therapies to suppress downstream signaling, such as the presence of *PIK3CA* mutations in *HER2* amplified breast cancers (Engelman & Settleman, 2008). In this chapter, we will discuss intrinsic insensitivity that does not arise from the absence of sensitizing mutations or the presence of pre-existing resistance mutations.

2.2.2. Acquired Resistance

Although many cancers have impressive responses upon treatment with targeted therapies, cancers invariably become resistant to therapy. This type of resistance, called acquired resistance, has also been a major limitation to the clinical benefits afforded by targeted therapies.There has been a wide range of acquired resistance mechanisms that have been uncovered to date (Corcoran et al., 2011; Engelman et al., 2007; Engelman & Settleman, 2008; Ercan et al., 2010; Poulikakos et al., 2011; Pricl et al., 2005; Qi et al., 2011; Qin et al., 2011). Mutations altering the ability of KIs to compete at the ATP binding domain of its target have been discovered in CML resistant to ABL inhibitors, *EGFR* mutant lung cancers resistant to EGFR inhibitors, and ALK mutant lung cancers resistant to ALK inhibitors (Choi et al., 2010; Katayama et al., 2012; Kobayashi et al., 2005; Kwak et al., 2005; Pricl et al., 2005). Compensatory activation of other kinases can result in resistance as well, including *MET* amplification in *EGFR* mutant lung cancers resulting in resistance to EGFR inhibitor (Bean et al., 2007; Engelman et al., 2007), *CRAF* amplification in *BRAF* mutant colorectal cancers resulting in resistance to MEK inhibitor (Corcoran et al., 2010), *KIT* amplification in *ALK* translocated lung cancers resulting in resistance to ALK inhibitor (Katayama et al., 2012), and SRC activation in *HER2* amplified breast cancers resulting in resistance to HER2 inhibitor (Rexer et al., 2011). Activation of these bypass tracts results in reactivation of vital downstream pathways that were originally under the sole control of the original oncogene to which the cancer was addicted. Complicating the biology of acquired resistance further, multiple mechanisms of resistance can be found in the same patient. For instance, Katayama et al. (2012) reported both secondary *ALK* mutations and reactivation of EGFR in *ALK* translocated cancers resistant to ALK inhibitor (Katayama et al., 2012), underlying the complexity and treatment challenges in cancers that have become resistant to targeted therapies.

3. CONSEQUENCES OF ONCOGENE INHIBITION IN ONCOGENE-ADDICTED CANCERS

3.1. Signaling Changes

While different oncogene-addicted paradigms have emerged, the need to understand the biological consequences of acute inhibition of the activated

protein encoded by the oncogene has become increasingly apparent. By assessing important biological events underlying initial drug sensitivity, biomarkers of sensitivity and resistance have been developed. In addition, this understanding points to strategies to overcome acquired resistance. Cell culture studies have been informative in defining the signaling changes that accompany successful targeted therapy treatment. For instance, in *EGFR* mutant NSCLCs, several groups have reported that EGFR KIs treatment leads to suppression of phosphoinositide 3-kinases (PI3K)/mTORC and MEK/ERK signaling, followed by increases in the pro-apoptotic Bcl-2 family member, BIM, Cragg et al, Deng et al., (2007b, Costa et al, Faber et al 2009) and decreases in the anti-apoptotic protein Mcl-1 (Faber et al., 2009). The cumulative result is free cellular BIM, which can initiate apoptosis (see below for more details, and Fig. 16.1). When these pathways are not coincidentally inhibited following EGFR therapy, cancers are unresponsive (Engelman et al., 2005, 2007; Faber et al., 2010; Guix et al., 2008; Yonesaka et al., 2011). Furthermore, direct inhibition of these two pathways can recapitulate much of the efficacy of EGFR inhibitors in *EGFR* mutant NSCLCs (Engelman et al., 2005; Faber et al., 2009). Similarly, these two pathways have been reported to be critical for *HER2* amplified breast cancer cells, though these cancers are much more sensitive to PI3K pathway inhibition alone (Faber et al., 2009).

3.2. Growth Arrest

The resultant phenotype following successful targeted therapy has been elucidated. The inability of cancer cells to transverse the cell cycle, leading to accumulation at the G1 phase, is a major component of this phenotype. This growth arresting effect is seen in *BCR-ABL* CML cells, *BRAF* mutant melanoma cells, *EGFR* mutant NSCLC cells, and *HER2* amplified breast cancer cells following the appropriate targeted therapy (Faber et al., 2009; Puissant et al., 2008; Smalley & Flaherty, 2009). Accordingly, the imperative intracellular signaling (i.e., PI3K/mTORC1 and MEK/ERK) is coincidentally blocked. Indeed, treatment with a PI3K/mTORC1 inhibitor alone inflicts comparable growth arrest in both *EGFR* mutant NSCLCs and *HER2* amplified breast cancers as does EGFR or HER2 inhibitors, respectively (Faber et al., 2009). However, it is important to note that induction of growth arrest alone is not sufficient to induce tumor shrinkage using *in vivo* laboratory models; induction of cell death is needed as well (Brachmann et al., 2009; Faber et al., 2009, 2011).

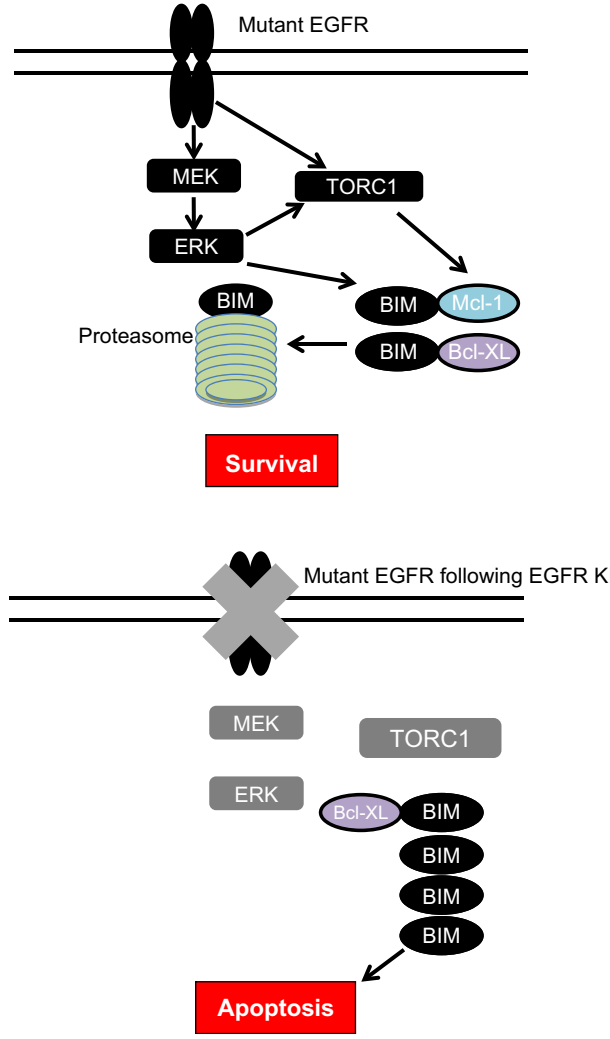

Figure 16.1 ***NSCLC with mutant EGFR activates growth and survival signaling pathways that upon pharmaceutical disruption, results in growth arrest and apoptosis.*** Mutant *EGFR* aberrantly activates PI3K/mTORC1 and MEK/ERK growth factor pathways. Survival is mediated by the Bcl-2 family member proteins, whose expression and localization determines survival or death (apoptosis). ERK signaling promotes proteasome-mediated BIM degradation. In NSCLC *EGFR* mutant cancers, pharmaceutical disruption (marked by "X") leads to loss of PI3K/mTORC1 signaling and MEK/ERK signaling. Loss of PI3K/mTORC1 signaling results in downregulation of Mcl-1, probably through TORC1-dependent translation, and loss of MEK/ERK signaling results in increase expression of BIM, through loss of post-translational modification of BIM that normally leads to proteasome-mediated BIM degradation. Disassociation of BIM/Mcl-1 complexes following mTORC1 inhibition, and accumulation of BIM following ERK inhibition, results in an overall increase in the amount of "free" BIM, leading to apoptosis. For color version of this figure, the reader is referred to the online version of this book.

3.3. Apoptosis

Traditional chemotherapies and radiotherapy have been designed to slow the growth and/or induce toxicity in fast-growing cancer cells. While cancers may be expected to be vulnerable to these therapies because of their high rate of replication, high-resolution data lend pause to that notion because many of these cancers have developed molecular mechanisms to avoid cell death. Indeed, one of the hallmarks of cancer is the unique ability of cancer cells to avoid cell death (Hanahan & Weinberg, 2011).

The term cell death is broad. There are a number of processes that a cell may undergo that leads to its demise. One of these processes, apoptosis, encompasses a fine-tuned, tightly regulated cascade of events designed to minimally impact the host while efficiently eliminating the cell. The intrinsic apoptotic pathway, executed through the mitochondria, has emerged as the major regulator of targeted therapy-induced cellular death. This pathway is governed by Bcl-2 family members, whose balance of expression and localization ultimately determines the integrity of the mitochondria, and as such, the commitment to apoptosis (Warr & Shore, 2008).

BIM is a BH3 domain-only Bcl-2 family member, which binds with uniquely high affinity to all members of the prosurvival Bcl-2 subfamily (Chen et al., 2005). Increases in cellular BIM and coinciding decreases in anti-apoptotic proteins, such as Mcl-1, Bcl-xL or Bcl-2, result in the tipping of the balance of Bcl-2 family members toward the initiation of apoptosis in oncogene-addicted cancers. This leads to the activation of two terminal members of the Bcl-2 family members, Bak and Bax, which then homo- and heterodimerize at the mitochondrial pore (Warr & Shore, 2008; Westphal et al., 2011) (Fig. 16.1). Loss of mitochondrial integrity leads to the release of mitochondrial proteins, formation of the apoptosome, activation of intracellular caspases, and other mechanical and morphological changes unique to apoptosis (Westphal et al., 2011). These changes culminate in the formation of residual apoptotic bodies that are cleared programmatically by phagocytic cells (Westphal et al., 2011).

While the precise mechanisms leading to activation of Bak and Bax remain controversial (Westphal et al., 2011), it is clear by experimentation that the processes altering Bcl-2 family member balance precipitate apoptosis in the context of targeted therapies (Costa et al., 2007; Cragg et al., 2008; Deng, et al., 2007b; Essafi et al., 2005; Faber et al., 2009, 2011; Kuroda et al., 2006; Meng et al., 2010; Rahmani et al., 2009; Tanizaki et al., 2011; Wickenden et al., 2008; Will et al., 2010). BIM stands out amongst the

known pro-apoptotic members of the Bcl-2 family member, because of its ability to bind all Bcl-2 family members with high affinity (a characteristic only shared by PUMA) (Kim et al., 2009; Merino et al., 2011). It has been shown to directly activate both Bak and Bax, changing their conformation to active states, leading to complex formation at the mitochondria and subsequent apoptosis (Gavathiotis et al., 2008), and it is rate-limiting for apoptosis in several contexts (Erlacher et al., 2006). Moreover, several recent studies have converged on BIM as a crucial mediator of apoptosis in response to targeted therapies (Costa et al., 2007; Cragg et al., 2008; Deng, et al., 2007b; Essafi et al., 2005; Faber et al., 2009, 2011; Kuroda et al., 2006; Meng et al., 2010; Rahmani et al., 2009; Tanizaki et al., 2011; Wickenden et al., 2008; Will et al., 2010).

4. BIM

4.1. The Biology of BIM

BIM is found in three main isoforms in human cells: BIM(EL) (~25 kDa), BIM(L) (~17 kDa), and BIM(S) (~13 kDa). These three isoforms retain the vital BH3 binding domain, which is critical for its interaction with other Bcl-2 family proteins (Ewings et al., 2007; Gillings et al., 2009; Wiggins et al., 2011). BIM(EL) mRNA is uniquely spliced so that it retains an ERK docking domain, the kinase responsible for phosphorylation of BIM(EL) at serine residues 59, 69, and 77 (Ewings et al., 2007). Phosphorylation of BIM(EL) by ERK at serine 69 leads to ubiquitin-dependent 26S proteasome-mediated degradation of BIM(EL) (Ley et al., 2003; Luciano et al., 2003). BIM(EL) is also degraded by free 20S proteasomes, which can occur in the absence of ubiquitination (Wiggins et al., 2011). BIM(EL) is thus the only isoform of BIM known to be regulated. post-transcriptionally, and is also the most abundant isoform of BIM (Wiggins et al., 2011). Expression of these three BIM isoforms is also modified through FOX03A-mediated transcription (Essafi et al., 2005). This can be mediated through both the MEK/ERK and the PI3K/AKT pathways, as both can affect FOXO3A-translocation by phosphorylation (Huang & Tindall, 2011).

4.2. BIM in Targeted Therapy-Induced Apoptosis

The role of BIM has extensively been studied in *EGFR* mutant NSCLCs. On inhibition of EGFR signaling, BIM is upregulated through loss of post-

translational modifications and transcriptional regulation (see below for further details). Utilizing siRNA technology, several groups have shown decreasing levels of BIM mitigates apoptosis following EGFR KI therapy (Costa et al., 2007; Deng, et al., 2007b). This protection from apoptosis is (Cragg et al 2007) also offered to *BRAF* mutant melanomas treated with MEK inhibitors (Cragg et al., 2008), *HER2* amplified breast cancers treated with the HER2 KI lapatinib (Faber et al., 2011; Tanizaki et al., 2011), *ALK* translocated lung cancers treated with the ALK KI critzotinib (Takezawa et al., 2011), and *PIK3CA* mutants treated with the PI3K/mTORC dual inhibitor, NVP-BEZ235 (Faber et al., 2011). The latter is particularly noteworthy because there is no increase in protein expression of BIM following treatment, perhaps signifying the robustness of BIM in apoptosis-mediated processes following targeted therapies. Additionally, we have recently found that modulating BIM expression via a tetracycline-inducible expression plasmid can sensitize cancers to targeted therapy-induced apoptosis both *in vitro* and *in vivo* (Faber et al., 2011).

Interestingly, while differential regulation of other Bcl-2 family members is almost certain to be involved in successful targeted therapy-induced apoptosis, the role of these proteins has largely eluded investigators. While we have reported that at least some *EGFR* mutant lung cancers downregulate Mcl-1 following gefitinib (or NVP-BEZ235) treatment (Faber et al., 2009), we failed to uncover any differential regulation of Mcl-1 or other Bcl-2 family members following HER2 TKI in *HER2* amplified breast cancers. Although there is some evidence that the Bcl-2 member Bmf may be involved in *BRAF* mutant melanoma apoptosis following BRAF inhibition (Shao & Aplin, 2010), the role of BIM in BRAF mutant melanoma response to BRAF KI has been more clearly established (Cragg et al., 2008; Paraiso et al., 2011; Shao & Aplin, 2010). Similarly, the only Bcl-2 member consistently reported to be consistently differentially regulated following ALK KI treatment in *ALK* translocated cancer (Takezawa et al., 2011), HER2 KI treatment in *HER2* amplified breast cancer (Faber et al., 2011), or PI3K KI in PIK3CA mutant cancer (Faber et al., 2011) has been BIM.

While apoptosis has been widely reported to follow effective targeted therapy treatment, its precise role in therapeutic efficacy had not been well defined until recently. Brachmann et al. (2009) showed only the *PIK3CA* mutant human cancers that underwent apoptosis following treatment with the PI3K/mTORC1 inhibitor NVP-BEZ235 treatment were the cancers that regressed when grafted into mice. Similarly, we have shown implanted *EGFR* mutant human lung cancers that need to undergo both growth arrest

and apoptosis following targeted therapy to regress in mice (Faber et al., 2009). Targeted therapy that only induced growth arrest could slow tumor growth but did not shrink tumors. In *HER2* amplified cancers, targeted therapy (i.e., HER2 TKI, lapatinib) regressed tumors in high BIM expressing BT-474 xenografted tumors, while low BIM expressing ZR7530 xenografted tumors had minimal response to the same targeted therapy. Both these cell lines suppressed PI3K/mTORC1 and MEK/ERK signaling in response to lapatinib *in vivo*, and growth arrested *in vitro* following lapatinib treatment (Faber et al., 2011). The difference, however, is that BT-474 high BIM expressing cells undergo robust apoptosis in response to lapatinib, while ZR7530 cells do not. In addition, knockdown of BIM in an *EGFR* mutant cancer cell line impaired apoptosis and tumor shrinkage *in vivo* (Faber et al., 2011). These findings as a whole are suggestive that cancers must undergo apoptosis in order to have robust tumor shrinkage in response to targeted therapies and also suggest that lack of an apoptotic response may translate to poor clinical responses. Data from these studies also beg the question as to whether downregulation of BIM occurs during the development of some tumors in order to promote a survival advantage, and if these are the cancers that have the poor apoptotic response to targeted therapies.

4.3. BIM as a Tumor Suppressor

Cellular death proceeding through the mitochondria is a tightly regulated process. An important step in carcinogenesis is the evasion of apoptosis (Hanahan & Weinberg, 2011). There is evidence suggesting that repressed expression of BIM is a mechanism that contributes to this phenotype. For instance, metastatic melanomas have significant repressed expression of BIM compared to dysplasic nevi (Dai et al., 2008), while even in non-malignant dysplastic nevi, *BRAF V600E* mutations are found (Wu et al., 2007).

Egle et al. (2004) reported in 2004 that BIM loss of heterozygosity (LOH) vastly accelerates the development of MYC-driven B cell tumors. One year later Tan et al. (2005) determined that BIM was a *bona fide* tumor suppressor in epithelial cells by grafting baby mouse kidney (BMK) cells into nude mice. BIM-deficient BMK cells formed tumors in mice while those proficient did not, and while BMKs deficient in other pro-apoptotic BCL-2 family members (i.e., PUMA) did not phenocopy BIM deficiency.

In solid tumor cancers, we have recently reported that BIM levels are important determinants to responses to targeted therapies (Faber et al.,

2011). In cancer cell lines, BIM RNA and protein levels predict the amount of apoptosis following targeted therapies including *EGFR* mutant NSCLCs, *HER2* amplified cancers, *PIK3CA* mutant cancers, and *BRAF* mutant colorectal cancers. Interestingly, BIM levels did not predict apoptosis in *EGFR* mutant NSCLCs to traditional cytotoxic therapies: paclitaxel, gemcitabine, or cisplatin. Accordingly, BIM knockdown was protected from targeted therapy apoptosis, but it was much less effective at protecting from chemotherapy-induced apoptosis. The reasons for this differential sensitivity to BIM knockdown may reflect the mechanisms of apoptosis induction when targeted therapies are compared to classic chemotherapies.

4.4. BIM as a Prognosis Factor in Patients

These findings are suggestive that BIM is a tumor suppressor and low levels preclude targeted therapies from inducing robust apoptosis in cell line models (Fig. 16.2). Because loss of other tumor suppressors in human cancers mitigate targeted therapy response (e.g., *PTEN* loss), and BIM mediates an important component of effective targeted therapy (i.e., apoptosis), it would be plausible that BIM may serve as a biomarker for response to targeted therapies in patients with oncogene-addicted cancers. This is an important point for two reasons. First, as routine assessments to identify oncogene-

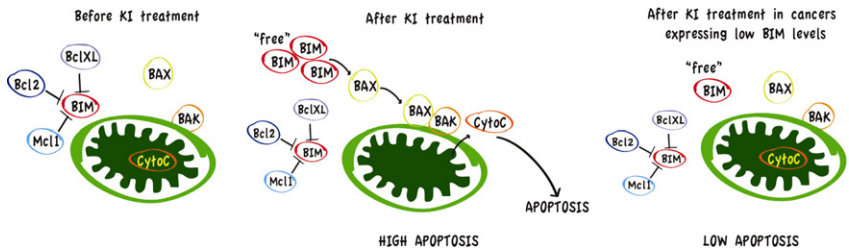

Figure 16.2 ***Treatment of oncogene-addicted cancers with kinase inhibitors results in an altering of the balance of Bcl-2 members, and leads to the initiation of mitochondrial-dependent apoptosis.*** Oncogene-addicted cancers perpetuate anti-apoptotic signaling at the Bcl-2 family level, leading to sequestering of BIM and resulting in cell survival. Treatment with the appropriate kinase inhibitor (KI) often results in a successful change in the balance of these proteins, leading to free "BIM" and the initiation of mitochondrial-dependent apoptosis. However, when there is low cellular basal BIM expression, the amount of free BIM following KI treatment is minimal, and as such, the degree of apoptosis in these cancers is mitigated, leading to intrinsic insensitivity. For color version of this figure, the reader is referred to the online version of this book.

addicted cancers are being implemented, knowing which cancers that will fail to respond to single-agent KIs will be essential. Secondly, if oncogene-addicted cancers with low BIM expression are identified, a novel therapeutic strategy involving the targeted therapy (which will still be expected to induce growth arrest) and an agent that will introduce an apoptotic response can be developed.

BIM expression has been found to be suppressed in a variety of tumors by diverse mechanisms. San Jose-Eneriz et al. (2009) reported that in CML specimens, BIM is often epigenetically silenced, and this is associated with poorer responses to imatinib. Similarly, in pediatric acute lymphoblastic leukemia (ALL), cancers were resistant to glucocorticoids that had epigenetic silencing at the BIM locus (Bachmann et al., 2010). Moreover, when we examined BIM RNA by quantitative (q)RT-PCR, *EGFR* mutant NSCLCs and a small number of HER2 positive breast cancers, we found lower levels of BH3 containing BIM transcripts strongly correlated with reduced progression free survival (PFS) of patients with metastatic lung disease receiving EGFR TKI, or in breast cancer patients receiving the HER2 KI lapatinib. Similarly, Ng et al. (2012) reported in patients with CML, levels of exon 4 BIM transcripts (those containing the BH3 domain) predicted for better response to imatinib. Strikingly, these investigators found that an intronic deletion in BIM caused a shift to production of nonfunctional BIM transcripts in East Asian patients ($n = 203$). These patients did poorer on imatinib as determined by PFS. They also found in patients with *EGFR* mutant lung cancer, patients with these mutations leading to low levels of BH3 containing BIM transcripts had poorer PFS following EGFR KI therapy ($n = 141$). On a whole, these data strongly support the notion that assessment of BIM can predict which patients with oncogene-addicted cancers will respond optimally to therapies, and which patients should be directed to an alternative therapeutic treatment plan.

5. BH3 PROFILING

While this chapter has focused on the role of apoptosis in targeted therapy response, it is worth noting that Anthony Letai and colleagues have discerned much of the importance and biology of the apoptotic response following classic chemotherapies (Del Gaizo Moore & Letai, 2012). This group has developed an assay, termed BH3 profiling, which can determine an individual cancer's likeliness to undergo apoptosis in response to

chemotoxins, or its degree of mitochondrial "priming" (readiness to undergo apoptosis following a given cellular insult). In diffuse large B cell lymphomas, these investigators identified three different apoptotic blocks. Following chemotherapy treatment, some cancers possess what is referred to as "Class A" apoptotic blocks. These blocks occur when the normal generation of BH3 only activators (e.g., BIM) are abnormally inhibited, the analogous situation we find in solid tumor models resistant to targeted therapies. Class B blocks occur when there is aberrant upregulation of anti-apoptotic proteins (e.g., Mcl-1, Bcl-xL, Bcl-2). Class C blocks occur with the loss of terminal BH3 members (Bak or Bax) (Deng et al., 2007a). The latter two scenarios may explain some of our observations in patient samples. That is, even some high BIM expressing cancers failed to achieve good responses to EGFR inhibitor (Deng et al., 2007a; Faber et al., 2011). While BH3 profiling has been primarily done using classic chemotherapies, these data suggest that this method can determine if a cancer that is primed to die, and the subsequent response of the chemotherapy in blood cancers can be predicted with impressive precision (Ni Chonghaile et al., 2011). This also is true for the abt-263 structurally related BH3 mimetic, abt-737 (Ni Chonghaile et al., 2011). Thus, implementing this technology in solid tumor cancers for targeted therapies would be potentially beneficial, though it remains to be determined if these assays would predict for responsiveness to targeted therapies, which induce cell death by mechanisms different from chemotherapies (Faber et al., 2011).

6. CELLULAR MECHANISMS THAT REDUCE BIM IN CANCERS

There is an increasing understanding that BIM is repressed in multiple cancer types. Understanding the underlying mechanisms of BIM repression may inform combinatorial therapeutic strategies that can resensitize oncogene-addicted cancers to targeted therapies.

6.1. Epigenetic Causes

Several groups have recently reported epigenetic regulation at the BIM locus. San Jose-Eneriz et al. (2009) reported BIM loci were hypermethylated in a subset of CML patients that had suboptimal cytogenic and molecular responses. This effect may be mediated by reduced histone H3 tail Lys9 (H3K9) acetylation and increased H3K9 dimethylation. In primary

chemoresistant Burkitt lymphomas (BLs), BIM was silenced through promoter hypermethylation and deaceytlation. These BLs had diminished remission rates (Richter-Larrea et al., 2010). BIM has also been shown to be epigenetically silenced by the IGFR pathway in multiple myeloma (De Bruyne et al., 2010). In pediatric ALL, BIM is epigenetically silenced in glucocorticoid poor-responsive patients (Bachmann et al., 2010). Altogether, these data evidence a strong epigenetic role in diminished BIM expression across a number of oncogene-addicted cancers, the role of epigenetic silencing of BIM in solid tumors remains to be thoroughly explored.

6.2. microRNA

Several microRNAs (miRNAs) have been shown to downregulate BIM in different cancers. Xiao et al. (2008) reported that the miR-17-92 cluster, which is regularly amplified in lymphomas and other malignancies, downregulates BIM (Inomata et al., 2009). miRNAs from the mir-106b-25 cluster can also bind to BIM RNA and reduce their expression (Inomata et al., 2009; Kan et al., 2009; Ventura et al., 2008). In addition, mir106b (Kan et al., 2009) suppresses BIM in cancer cells. In a recent report by Garofola and colleagues, mir30b and mir30c were shown to regulate BIM in lung cancers and mediate resistance to EGFR TKI (Garofalo et al., 2012).

6.3. Genomic Mutations

In addition to epigenetic causes, gross chromosomal alterations have been shown to occur in cancers that result in BIM deletions. Using array-based comparative genomic hybridization to interrogate mantle cell lymphoma patients, Tagawa et al. (2005) reported homozygous deletions in BIM. These findings were later confirmed and extended by Mestre-Escorihuela et al. (2007).

Recently, an elegant study by Ng et al. (2012) utilized massive parallel sequencing of patients diagnosed with CML to explain the presence of intrinsic insensitivity to targeted therapy. They uncovered an intronic deletion in BIM that causes expression of BH3-deleted BIM transcripts in primarily East Asian patients. As discussed above, these patients and those with *EGFR* mutations carrying this deletion had worse outcomes with targeted therapies.

6.4. Other Causes

Other causes of BIM depression have been discovered. Loss of PTEN leads to insensitivity to BRAF inhibitors in *BRAF* mutant melanomas, by blocking the upregulation of BIM (Paraiso et al., 2011). Intriguingly, loss of PTEN did not correlate with loss of growth inhibitory effect, but did correspond with loss of apoptotic response, reinforcing the role of BIM in targeted therapy-induced apoptosis, but not growth arrest.

7. ALTERNATIVE THERAPIES TO OVERCOME LOW BIM EXPRESSION

7.1. Use of Chromatin and DNA Modifying Agents

There are currently efforts to restore BIM levels in cancers with diminished expression. Aberrant epigenetic alterations in BIM may be overcome by therapies that are directed at re-expressing BIM, in combination with the appropriate KI. Bachmann et al. (2010) showed that co-treatment with an HDAC inhibitor could reverse epigenetic silencing at the BIM locus in childhood ALL. Similarly, the addition of decitabine (5-aza-2′deoxycytidine) to imatinib overcomes silencing of the BIM locus in CML (San Jose-Eneriz et al., 2009). Both these strategies may prove useful in solid tumor oncogene-addicted cancers if BIM suppression is mediated by epigenetic mechanisms.

7.2. Use of Bcl-2/Bcl-xL Inhibitors

To overcome genomic loss of BIM or suppression of BIM via miRNA, a different pharmaceutical strategy would have to be implemented. An intriguing class of compounds that have emerged are the Bcl-2 family inhibitor compounds, including navitoclax (abt-263) and the broader Bcl-2 inhibitor, obatoclax. These compounds are BH3 mimetics that bind and neutralize Bcl-2, Bcl-xL, and Mcl-1 (navitoclax is only a weak inhibitor of Mcl-1) (Nguyen et al., 2007; Tse et al., 2008). Navitoclax is relatively well tolerated in patients, with thrombocytopenia reported as the major adverse effect (Gandhi et al., 2011). Investigators have begun testing these compounds in combination with other targeted therapies, although they have not examined them in the context of BIM expression. In *EGFR* mutant NSCLCs and *BRAF* mutant colorectal and melanoma models, Bcl-2/xL inhibitors plus the appropriate KIs yielded an effective, more durable therapy (Cragg et al., 2008). To the extent that low BIM expressing cancers

benefit from this rational combination has not yet been thoroughly investigated, and this will be critical as we identify new treatments for cancers with low BIM expression.

8. CONCLUSION

As the era of personalized cancer medicine is well under way, the number of cancer patients that will be treated with targeted therapies in the future will only increase, and most likely in dramatic fashion. There is an immediate and urgent need for diagnostics and biomarker development to keep pace with the implementation of these drugs, so patients are appropriately directed toward the right targeted therapies, alone or in combinations. Recent work highlighted in this chapter has begun to provide evidence that assessment of BIM expression can predict which patients will have robust and durable responses to a given targeted therapy across a wide range of oncogene-addicted cancers. Even more, these studies may be informing that a cell death response accompanying retardation in cell cycle progression following therapy is necessary for efficacy and in this way can help inform us how to design rational combinatorial treatments to overcome targeted therapy acquired resistance.

These data on the whole also inform that BIM is not only a biomarker for response, but is also very much functional in that response. For instance, manipulation of BIM levels can render a cell sensitive (when overexpressed) or resistant (when suppressed) to targeted therapies. While it may seem disproportional that BIM seems to have such a singularly dominant role over other BH3 proteins in apoptosis following targeted therapy treatment, this may reflect cancers' consistent tendency to actively suppress BIM during their progression.

A major question moving forward will be how to best determine BIM expression in oncogene-addicted cancers. While qRT-PCR is attractive because of the quantitative nature of the assay, there are serious limitations. For one, RNA quality can be highly variable in specimens extracted from patients. Secondly, a significant amount of starting material would be required for accurate and reproducible measurement. Thirdly, qRT-PCR is technically challenging, and variability from laboratory to laboratory could hinder its universality. Lastly, the stroma may contribute significantly to the amount of BIM detected, thereby confounding the data. Immunohistochemistry, on the other hand, is less quantitative, and variability between or

even within laboratories may make it challenging to develop uniform and reproducible scoring. Other possible methods to quantify BIM include RNA in situ hybridization (ISH). This has the advantage of specifically measuring the levels of BIM in the cancer cells using a more quantitative approach.

Despite these obstacles, there is now compelling evidence that BIM is intricately linked to optimal responses in targeted therapies, through its integral role in inducing apoptosis. A greater understanding of the biology leading to BIM repression in some oncogene-addicted cancers will inform alternative treatment options. Overall, a universal, optimal assay to determine BIM expression in these cancers is an important avenue of future investigation as we move forward to improve cancer outcomes in these patients.

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ABBREVIATIONS

CML chronic myelogenous leukemia
EGFR epidermal growth factor receptor
KI kinase inhibitor
NSCLC non-small cell lung cancer
PI3K phosphoinositide 3-kinase
RECIST response evaluation criteria in solid tumors
RTK receptor tyrosine kinase

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