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
(~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 (BRAFV600E) are known to develop drug resistance and experience tumor recurrence (Flaherty et al., 2010; Flaherty,
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 \( \text{BRAF}^{V600E} \) 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 \( \text{BRAF}^{V600E} \) 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\textsuperscript{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.,
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 Iplilimumab or

![Diagram of molecular heterogeneity of melanomas](image-url)
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 BRAFV600E 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 BRAFV600E 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 BRAFV600E expression in tumor cells isolated from different regions of the primary lesions. In that study, the primary melanoma lesion likely harbored mutation positive BRAFV600E 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 $\text{BRAF}^{\text{V600E}}/\text{WT-NRAS}$ and $\text{WT-BRAF}/\text{NRAS}^{\text{Q61R}}$ 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. $\text{BRAF}^{\text{V600E}}$ 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 $\text{BRAF-V600E}$ 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 IL2R\(\gamma_c\) (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\textsuperscript{+} 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\textsuperscript{+} and CD271\textsuperscript{+} 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\textsuperscript{+} melanoma subpopulations derived from patient tissues are more tumorigenic and aggressive than CD271\textsuperscript{-} subpopulations when transplanted in immunodeficient \textit{Rag2\textsuperscript{−/−}\gamma\textsubscript{c}\textsuperscript{−/−}} mice. Many of the melanoma-associated antigens such as MART1, MAGE, and tyrosinase were lost or down modulated in CD271\textsuperscript{+} cells (Boiko et al., 2010). These antigen losses in subpopulation variants are mostly likely linked to the selection of immunologically resistant melanoma cells \textit{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\textsuperscript{+} 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 \textasciitilde1–5\% 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 trametinib) (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 PI3K 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

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**Targeted inhibition of cell signaling or cell cycle pathways and tumor subpopulations**

**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 IGF1 or PDGF 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<sup>V600E</sup> 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
CCND1  cyclin D1
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