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The Role of Cellular Differentiation and Cell Fate in Malignant Melanoma

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1. Introduction

1.1 Defining differentiation and cell fate in cancer

In the setting of embryonic development, terms such as “cell fate” and “cellular differentiation” are relatively easy to grasp, since they refer to a straightforward linear model whereby progenitor cells give rise sequentially to various distinct and identifiable lineages, eventually resulting in a terminally differentiated cell that has until recently been thought to possess very little cellular plasticity or multipotent capacity. The sequences of events that regulate this process are extremely well conserved within a species, and even across species in many instances. In the setting of cancer, cell fate and cellular differentiation are often used descriptively to convey an observed phenotype rather than a defined and well-understood molecular process. How accurate is it to refer to “differentiation” in cancer when the so-called end-point for this process results in cellular heterogeneity that is antithetical to the regulated and predictable differentiated cells that result from embryonic development? What does it mean to refer to “cell fate” during the inherently dysregulated series of events involved in oncogenesis, which may not result in a distinct endpoint even across cells within the same tumor?

Our review uses these terms in reference to the dynamic processes that constantly shape the function and properties of melanoma cells, which coincidentally utilize many of the same pathways involved in the regulated process of differentiation and determination of cell fate during embryonic development. While the label of being a differentiated cell may imply a terminal nature that might be viewed as less tumorigenic or lethal in the setting of cancer, this concept requires further experimental confirmation. Undoubtedly, these terms will become more refined as our understanding of the molecular events underlying melanoma progression comes into clearer focus. For now, they are the best terms available to describe some of the events and processes that help determine the behavior and response of tumor cells, and we use them with the full knowledge that years from now, advances in our understanding of cancer could render these terms woefully inadequate, or worse yet inaccurate.

1.2 Differentiation in nevi and melanomas

At one time, the classification of melanomas at the cellular level was largely limited to cell morphology, the expression profile of selected melanocytic or neuronal markers by

immunohistochemistry, and the presence or absence of phenotypic characteristics such as pigmentation. These early clinical and histological observations provide relevant insight into the role of cellular differentiation in both benign nevi and in melanoma (Barnhill et al., 2004). The phenomenon of “maturation with depth” was described histologically in nevi well before the advent of molecular genetics (Winkelman and Rocha, 1962), and highlights the capacity of certain nevus cells to dynamically evolve and undergo changes in cell fate, either through autologous signaling or in response to stromal factors. Maturation with depth is also seen in certain subsets of melanomas (sometimes termed “nevroid melanomas”), which reflects some of the cellular and molecular plasticity seen across tumors (Schmoekel et al., 1985). Within the past decade, the sequencing of the human genome and the rapid development of technologies such as microarrays and deep sequencing has markedly advanced our understanding of cellular transcriptomes and led to the use of gene expression signatures as indicators of cell fate and cellular differentiation. It has become increasingly clear from studies in melanoma and across all cancers that the gene expression profile of a tumor or cell line can provide important information regarding the origin of the cancer cells, the status of various signal transduction pathways, and even the potential therapeutic susceptibility of cells to specific therapies. Furthermore, these analytic advances have confirmed the heterogeneity of melanoma that was already well-described histologically.

1.3 Objectives

Because cellular phenotype represents a primary measure of the state of melanoma cell differentiation, the focus of our discussion will center on some of the accumulated data regarding the characterization of phenotype by various experimental measures. Our overall goal is to summarize the literature on differentiation and cell fate in melanoma, focusing on the gene expression profiles of both proliferative as well as invasive melanoma cells, along with discussing the mechanisms by which these profiles result in the described phenotypes. We will also review how gene signatures relate to the dynamic process of cellular differentiation in melanoma cells, including the evidence for the existence of phenotype switching, and how this phenomena may contribute to melanoma heterogeneity. Finally, we will examine how genetic mutations, phenotypic instability and therapeutic susceptibility affect efforts to treat this deadly disease. In the end, we hope that this review will stimulate thought and discussion on the viability of directed differentiation in melanoma and other cancers as a potential therapeutic strategy. By exploiting the powerful regulatory effects of the morphogen pathways utilized by cancer cells during oncogenesis, these types of strategies could potentially alter not only the behavior of these cells, but also their susceptibility and response to current and emerging cancer therapies.

2. Global genetic regulation of melanoma differentiation

2.1 Historical observations and recent advances

2.1.1 The challenge of melanoma treatment

Melanoma is the most lethal form of skin cancer, and both the incidence and consequent mortality rates of this deadly disease have increased globally in recent decades (Lens and Dawes, 2004). Death from this cutaneous neoplasm usually occurs as a result of distant metastasis, most commonly affecting the lungs, liver and brain. Its ability to become invasive and metastasize within months of the initial lesion developing makes melanoma one of the most aggressive forms of all human cancers (Miller and Mihm, 2006). Recent

advances in radio-, chemo- and immunotherapies have resulted in improved prognoses and prolonged survival times in many different types of malignancies. Melanoma however, has remained largely resistant to treatment by any combination of these three therapeutic modalities, with current cancer treatment options still resulting in a median survival time of 12 months or less in patients diagnosed with metastatic disease (Tsao et al., 2004).

2.1.2 The intersection between developmental biology and cancer

In recent years, we have gained a deeper understanding of the mechanisms underlying melanoma progression from a primary melanocyte to metastatic disease. Classically, this transformation has been viewed as the step-wise accumulation of genetic and epigenetic aberrations which results in an increasingly more malignant phenotype (Singh et al., 2008). A key feature of this progression model is the explicitly one-way nature of gene mutation. Based on this view of melanoma progression, a theory reminiscent of Darwinian natural selection has been conceived to explain the inevitable development of cellular heterogeneity and therapeutic resistance in melanoma tumors. Specifically, it is commonly believed that melanoma cells are constantly accumulating novel, prometastatic genetic mutations, which inevitably lead to the development of dominant subpopulations of tumor cells with a distinct survival advantage. As new dominant subpopulations are generated from ongoing genomic instability, the constantly evolving tumor is able to both maintain cellular heterogeneity, as well as develop dynamic resistance to chemotherapeutic agents.

As tumor cells acquire more mutations, their properties dynamically change, as measured by changes in global gene expression or by changes in their capacity for relevant functions such as motility, proliferation, or invasion. By convention, these changes in cellular phenotype define a new cell fate, and reflect a process of differentiation (albeit dysregulated) that parallels the observed maturation of non-cancerous cells during development. To appreciate concepts such as differentiation and cell fate in the dysregulated setting of melanoma, one needs to have some contextual understanding of differentiation and cell fate determination in the normal development of cells that may serve as precursors for this deadly cancer. While the cell of origin for melanoma is almost certainly of neural crest origin, and likely in the melanocytic lineage, the recent unexpected finding that murine epidermal melanocytes can arise from a niche of Schwann cell precursors highlights the incomplete nature of our current understanding (Adameyko et al., 2009). This finding suggests that an understanding of Schwann cell biology could be potentially relevant for identifying and treating melanomas that may arise from this distinct precursor population, since these melanomas could conceivably display different cellular phenotypes than melanomas derived from non-Schwann cell-derived melanocytes.

While previous models of development have depicted differentiation as primarily a one-way event, the observation that differentiated Schwann cells can be induced to form progenitor cells that subsequently give rise to glia and melanocytes provides evidence that so-called "terminal differentiation" does not preclude the ability to acquire pluripotency and/or the initiation of broad changes in cellular programming (Dupin et al., 2003). Likewise, the observation that melanocytes can de-differentiate to a precursor cell that gives rise to mature glia (Dupin et al., 2000) may further reflect a plasticity or instability of cell fate that is fundamentally relevant for melanoma and the exceptional resistance of this cancer to treatment.

2.1.3 Insight from gene expression profiling studies

An emerging body of evidence is re-defining the conventional genetic model of melanoma progression. Gene expression profiling studies using multi-center cohorts of patient tumors have previously revealed the existence of two major expression signatures (Hoek, 2007; Hoek et al., 2006). These signatures correlate to two distinct populations of melanoma cells, one with a predominantly proliferative phenotype and the other with a predominantly invasive phenotype. Subsequent transcriptional profiling studies in melanoma cells have revealed two discrete states of differentiation (Hoek, 2007; Tap et al., 2010). The first state results in a phenotype closely resembling primary human melanocytes, while the second results in a phenotype resembling neuronal stem cells (Hoek, 2007; Tap et al., 2010). Further, melanoma cells with a proliferative phenotype tend to be in a melanocytic differentiation state, while cells which acquire an invasive phenotype tend to dedifferentiate into a neuronal state. Several studies have demonstrated that melanoma has the ability to switch back-and-forth between these two phenotypes and/or differentiation states, triggered by factors such as microenvironmental conditions and therapeutic intervention (Hoek et al., 2008; Hoek and Goding, 2010). The ability of melanoma cells to constantly switch phenotypes undoubtedly contributes to the resistance of melanoma to treatment.

2.1.4 Cancer stem cells and melanoma

Traditional chemotherapy operates largely under the premise that all cancer cells have equal malignant potential, with drug therapy focused on decreasing the population of cells within the tumor. Recently, studies looking at melanoma and other cancers have introduced the concept that certain populations within the tumor, often referred to as cancer stem cells (CSCs), have increased tumor initiating capabilities along with increased resistance to traditional chemotherapeutic approaches (Dou et al., 2007; Grichnik et al., 2006; Schatton et al., 2008). Consequently, the ability to identify, study and manipulate cells with the highest tumor-initiating capacity will be critical for developing effective therapeutic strategies.

In the case of melanoma, the exact nature of CSCs remains controversial, with several putative CSC markers having been proposed in the literature (Zabierowski and Herlyn, 2008). These markers include ABCB5 (Schatton et al., 2008), CD271/NGFR (Boiko et al., 2010), and CD34 (Held et al., 2010). However, additional reports have demonstrated that tumor initiating capacity may actually be quite common among melanoma cells, and does not depend on the expression of any of the published putative melanoma CSC markers (Quintana et al., 2010; Quintana et al., 2008). In the context of cellular differentiation, studies have also found that like other cancers, melanoma exhibits similarities to embryonic stem cells (Klein et al., 2007; Postovit et al., 2007). The ability to correlate features of melanoma cells such as gene expression profiles with functional phenotypes such as tumor-initiating capacity (a hallmark of CSCs) will further delineate the role of cell fate in regulating melanoma progression.

2.1.5 Tumor heterogeneity and plasticity

While the cellular heterogeneity of tumors is a long-recognized phenomenon (Fidler, 1978), these recent studies highlight the variability within populations highlight regarding both gene signature and phenotypic plasticity. The concepts of phenotype switching and cancer stem cells in melanoma may both represent what happens within the tumor environment. Cancer stem cells may not be a fixed population, but rather a dynamically changing one

resulting from the phenotype switching of more “differentiated” cells in a tumor. This type of model could reconcile both sets of observations, and implies an inherent plasticity of melanoma cells that would undoubtedly complicate therapeutic efforts and potentially contribute to the variability seen with the use of cell surface markers to isolate CSC populations. While the use of the term differentiated in this model may again conjure up pre-conceptions that this population may be either more terminal/benign or less lethal in the long term, this assumption requires further experimental confirmation.

2.2 The proliferative phenotype of melanoma cells

Since cell fate and differentiation in the setting of cancer most closely parallels phenotype, this review will focus in particular on phenotypic characteristics that have been the center of efforts to understand melanoma at the molecular level. The first of the two major phenotypes expressed by malignant melanoma cells is the proliferative phenotype. As the name suggests, this phenotype is associated with a high rate of proliferation, as well as minimal invasive potential (Hoek et al., 2008; Hoek et al., 2006). Two key features of this group of cells help to account for their proliferative nature, including the activity of microphthalmia-associated transcription factor (MITF) (Levy et al., 2006) as well as the activity of the canonical Wnt/ β -catenin pathway (Chien et al., 2009a).

2.2.1 Microphthalmia transcription factor (MITF)

MITF is a transcription factor that plays a critical role in the differentiation of melanoblasts from other cells derived from the neural crest (Levy et al., 2006), and severe mutations of MITF impede the embryonic development of melanocytes (Goding, 2000). It also plays a critical role in the normal functioning of differentiated primary melanocytes, regulating genes involved in the manufacture of melanosomes and melanin (Hornyak, 2006). This multi-tasking potential has been summarized using a proposed “rheostat model” of MITF function (Carreira et al., 2006; Hoek and Goding, 2010), which describes three different scenarios: MITF function in normal, differentiated melanocytes; MITF function in proliferative melanoma cells; and decreased MITF expression as seen in invasive melanoma cells.

As previously mentioned, the primary role of MITF in a normally functioning melanocyte cell, where MITF expression is highest, is the regulation of genes involved in melanosome and melanin production (Lekmine et al., 2007; Levy et al., 2006). Some examples of the broad range of genes that affect pigmentation, whose expression is regulated by MITF, include melanocortin 1 receptor (*MC1R*), melanocortin 4 receptor (*MC4R*), tyrosinase (*TYR*) and melan-A (Lekmine et al., 2007; Levy et al., 2006). Other genes essential for normal melanocyte development, such as endothelin receptor type B (*EDNRB*) (Sato-Jin et al., 2008), p21^{cip1} (Carreira et al., 2005; Sestakova et al., 2010) and p16^{INK4} (Loercher et al., 2005), have also been shown to depend on MITF expression. Significantly, p21^{cip1} and p16^{INK4} are both regulators of the cell cycle, and are expressed when MITF levels are at their highest.

As melanocytes acquire activating mutations in critical signalling pathways such as the MAPK pathway, the cellular effects of MITF change. Although the exact mechanism of this change is still not clear, it is primarily thought to be the result of altered post-translational modification of MITF, causing MITF to be targeted towards a different set of genes (Hoek and Goding, 2010). According to the rheostat model, overall MITF activity is also thought to be lower in malignant melanoma cells as compared to melanocytes (Hoek and Goding, 2010). Consequently, MITF expression in this cell population results in suppression of

senescence and increased proliferation, as well as decreased invasiveness, both hallmarks of melanoma cells in the proliferative gene expression cluster.

There have been many mechanisms proposed to help explain how MITF expression leads to increased proliferation of melanoma cells. For example, suppression of p27^{kip1}, or cyclin-dependent kinase inhibitor 1B (CDKN1B), is thought to be of primary importance in this process. The main function of p27^{kip1} is to impede cell cycle progression at G1. In melanoma cells with a proliferative phenotype, there is decreased p27^{kip1} expression (Carreira et al., 2006). This is thought to be the result of increased expression of diaphanous-related formin DIA1, a protein which is upregulated by MITF and has a role in the regulation of a wide variety of cellular functions, including actin polymerization and E-cadherin organization. DIA1 in turn increases degradation of p27^{kip1} by S-phase kinase-associated protein 2 (SKP2), a gene which is regulated by DIA1 (Carreira et al., 2006). In addition, other genes upregulated by MITF in proliferative melanoma cells include *BCL2* and *CDK2* (Cheli et al., 2010). While *BCL2* upregulation imparts apoptotic resistance on melanoma cells, increased expression of cyclin-dependent kinase 2 results in cell cycle dysregulation and thus increased proliferation (Cheli et al., 2010).

Besides increasing proliferation, MITF also contributes to the low invasive potential characteristic of melanoma cells in the “proliferative state”. Based on the results of Carreira et al., the ability of MITF to inhibit invasion is likely dependent on its DIA1-mediated regulation of the RHO/ROCK pathway, a known promoter of invasiveness (Carreira et al., 2006). MITF also appears to be a negative regulator of the Notch signalling cascade, which is itself a driver of invasive potential (Thurber et al., 2011). The frequent dysregulation of MITF by gene amplification in melanoma cells (Garraway et al., 2005) further highlights the critical nature of MITF in melanoma progression, and in the regulation of melanoma cell fate.

2.2.2 Wnt/ β -catenin signaling

The Wnt/ β -catenin signaling pathway represents a morphogenic pathway that is critical for development and almost always dysregulated in the context of cancers (Chien et al., 2009a). Constitutive activation of the Wnt/ β -catenin pathway is a common feature of many cancers, and increased activity of this pathway in general is a feature which distinguishes proliferative melanoma cells from invasive ones (Hoek et al., 2006). In the context of cellular differentiation and melanoma, which is neural crest-derived, Wnt/ β -catenin signaling is particularly relevant since this pathway plays a pivotal role in regulating the differentiation of precursor cells into either neurons or melanocytes (Dorsky et al., 1998).

The key mediator of canonical Wnt/ β -catenin pathway signalling is β -catenin, a protein whose degradation is inhibited upon Wnt ligand binding, allowing its translocation to the nucleus where it regulates target gene expression (Chien et al., 2009a). Wnt/ β -catenin and MITF are intimately related in that β -catenin increases MITF expression (Dorsky et al., 2000b), while MITF can interact with β -catenin in cultured malignant melanoma cells to alter its downstream gene-targeting (Widlund et al., 2002). Like MITF, Wnt/ β -catenin signalling is critical to the normal embryonic development of melanocytes, so much so that the Wnt3a ligand is one of only three factors needed to stimulate the development of a pluripotent human embryonic stem cell into a mature melanocyte (Fang et al., 2006).

The exact role of Wnt/ β -catenin signaling in melanoma remains controversial, in part due to some differences in observed patient data and data obtained from murine models. Overall, increased activation of this pathway in approximately one-third of all melanomas is suggested by the presence of increased nuclear localization of β -catenin (Chien et al., 2009b), a surrogate

marker for Wnt/ β -catenin activation. More importantly, the activation of Wnt/ β -catenin signaling has been unexpectedly associated with improved patient survival (Bachmann et al., 2005; Chien et al., 2009b). In addition, the presence of nuclear β -catenin in the majority of benign nevi (Bachmann et al., 2005; Kageshita et al., 2001; Maelandsmo et al., 2003) implicates a role for Wnt/ β -catenin signaling in maintaining normal cellular homeostasis, and suggests that the dysregulation of this pathway may contribute to the process of melanoma progression. The interpretation of these observations in patients is complicated by studies using a mouse model expressing a constitutively-active β -catenin mutant under the transcriptional control of a melanocyte-specific promoter. This transgenic mouse model suggests that increased Wnt/ β -catenin signalling contributes to the initial immortalization of melanoma cells by inhibiting the expression of p16^{INK4a}, a tumor suppressing protein which plays a key role in the induction of G1 cell cycle arrest in response to DNA damage (Delmas et al., 2007). Interestingly, although immortalization is often associated with proliferation, Delmas et al. show that in fact these two processes are effectively uncoupled during the process of malignant transformation in melanocytes (Delmas et al., 2007). However, despite facilitating the immortalization of melanocytes, the presence of a constitutively-active β -catenin mutant was by itself not sufficient to generate spontaneous melanomas, which required concomitant activation of MAPK signaling through *Nras* mutation (Delmas et al., 2007). With regards to the issue of differentiation and questions surrounding the cell of origin for melanoma, the comparison of Wnt/ β -catenin signaling in both human patients and mice raise two interesting points. First, melanomas from the mouse model appear to originate from the bulge region of the hair follicle, which is the proposed niche for melanocytic stem cells (Delmas et al., 2007); by contrast, most patient melanomas arise from interfollicular epidermis rather than from the hair follicle. Second, while these studies utilize a constitutively active mutant of β -catenin (Delmas et al., 2007), these types of activating mutations are quite rare in patient melanomas (Lucero et al., 2010), where Wnt/ β -catenin activation is thought to result primarily from secreted Wnt ligand. Intuitively, the activation of signaling by secreted Wnt ligand is subject to modulation by extrinsic factors including endogenous inhibitors (i.e. DKK1, SFRPs) (Chien et al., 2009a), which would permit a model more akin to the MITF rheostat. Interestingly, the activation of Wnt/ β -catenin signaling through the forced expression of WNT3A rather than a mutant β -catenin results in decreased proliferation in vitro and in vivo, correlating with the increased expression of genes associated with melanocyte differentiation (Chien et al., 2009b).

2.3 The invasive phenotype of melanoma cells

The second major gene expression signature of melanoma cells results in a phenotype characterized by a high degree of invasiveness and relatively lower rates of proliferation. Both MITF as well as canonical Wnt/ β -catenin signalling are markedly downregulated in this population of cells, with a concomitant upregulation of a variety of pro-invasive mediators (Hoek et al., 2006). Notably, invasive melanoma cells are defined by genes suggestive of increased noncanonical WNT5A signalling, increased Notch1 signaling, upregulation of TGF- β and Brn-2 transcription factor as well as loss of the AP-2 transcription factor, all of which are reviewed below.

2.3.1 WNT5A and non-canonical Wnt signaling

Whereas the canonical Wnt pathway is reliant on β -catenin signaling, the noncanonical Wnt pathway uses calcium-dependent mediators such as protein kinase C (PKC) to propagate

the signal of its primary extracellular ligand, WNT5A (Chien et al., 2009a). Normally, WNT5A signalling plays an important role in the regulation of cell fate, embryogenic patterning and cell motility (Chien et al., 2009a). In many forms of cancer, including colon, breast and liver cancer, WNT5A acts as a tumor suppressor (Chien and Moon, 2007). In the context of melanoma cell differentiation, which is assessed largely through gene signatures, the continued appearance of WNT5A as a major determinant of melanoma clusters in transcriptional profiling studies speaks to its likely importance as a genetic marker (Bittner et al., 2000; Hoek, 2007; Hoek et al., 2006; Weeraratna et al., 2004).

Functionally, WNT5A is thought to contribute significantly to the invasive phenotype by regulating cellular migration, both through PKC and the re-distribution of cellular adhesion molecules (Dissanayake et al., 2007; Weeraratna et al., 2002; Witze et al., 2008). In addition, WNT5A signalling has been shown to inhibit the canonical Wnt/ β -catenin pathway and consequently cause the downregulation of the downstream target genes of β -catenin (Chien et al., 2009b; Dissanayake et al., 2008), which may further contribute to its role in melanoma progression. The observation that WNT5A can be involved in the specification of dopaminergic neuronal cells (Castelo-Branco et al., 2003) and specification of axonal or synaptic function (Agalliu et al., 2009; Bodmer et al., 2009; Varela-Nallar et al., 2010) may also indicate a previously under-appreciated role of this pathway in the control of cellular differentiation. Conceivably, the role of WNT5A in neuronal cells may overlap with the phenotypic effects seen with WNT5A activation in melanoma cells, thereby contributing to whether these cells may display a phenotype that is neuronal as opposed to melanocyte-like. Alternatively, the expression of WNT5A in melanoma may simply be a reflection of cellular differentiation state, which would be consistent with transcriptional profiling studies where WNT5A is enriched in melanoma cells with a gene signature suggestive of neuroprogenitor cells (Tap et al., 2010).

2.3.2 Notch signaling

Another pathway implicated in melanoma is the Notch signaling cascade, which is one of the prototypical regulators of cell fate determination during embryonic development (Kopan and Ilagan, 2009). Like other primary transforming mutations in melanoma such as BRAF and NRAS, constitutive activation of the Notch1 receptor was in itself sufficient for the malignant transformation of human melanocytes (Pinnix et al., 2009). In fact, Notch1 signaling is also required for β -catenin-induced proliferation in melanoma cells with a proliferative phenotype (Balint et al., 2005). However, the expression of Notch is negatively regulated by MITF, and thus its contribution to cellular phenotype is most pronounced in invasive melanoma cells where MITF is down-regulated (Thurber et al., 2011). Reports that these Notch effects in melanoma are mediated through regulation of the MAPK and PI3K-Akt pathways (Liu et al., 2006a; Liu et al., 2006b) further highlight the convergence of critical pathways during melanoma progression.

Like Wnt signaling, the Notch pathway plays a critical role in the specification of cell fate during neural crest development, making it particularly relevant to melanoma biology (Cornell and Eisen, 2005). During embryonic development, Notch signaling is thought to regulate both the specification of neural crest, as well as the subsequent determination of secondary cell fate through differentiation into glial-based lineages (Cornell and Eisen, 2005). Engagement of Notch signaling can inhibit neurogenesis and neural differentiation, which may play some part in the role of this pathway in melanoma. Likewise in the context

of stem- and progenitor cells, Notch signaling can have varied roles in regulating either the maintenance of stem cell phenotype or the differentiation of stem cells into mature lineages (Liu et al., 2010). Understanding the interplay between Notch and other signaling pathways will be vital to effectively leveraging this important regulator of cellular differentiation for therapeutic benefit.

2.3.3 Transforming growth factor-beta (TGFB)

The morphogen-based Nodal signaling pathway, representing a subset of transforming growth factor β (TGFB) signaling pathways, was perhaps the initial pathway of interest in melanoma with regards to the concept of 'differentiation therapy' (Hardy et al., 2010; Postovit et al., 2008a; Postovit et al., 2008b; Strizzi et al., 2009). Like many of the other pathways that participate in melanoma biology, Nodal is a morphogen ligand involved the determination of cell fate during development. Melanoma is characterized by the presence of increased levels of Nodal ligand coinciding with decreased expression of the secreted Nodal antagonist Lefty (Postovit et al., 2008a). Typical benign nevi appear to express low levels of Nodal, although a subset of congenital nevi express levels of Nodal by immunohistochemistry that are comparable to what is observed in melanoma (Yu et al., 2010). Analogous to its role in maintaining human embryonic stem cells in the undifferentiated state, Nodal signaling in melanoma is thought to regulate or facilitate the plasticity of tumor cells (Postovit et al., 2008a), thus eliciting considerable interest in the targeting of this pathway for therapeutic purposes.

Overall, the role of TGFB in melanoma pathogenesis is complex, given the variability of its effects based on a cell's stage of progression (Javelaud et al., 2008; Lasfar and Cohen-Solal, 2010). Whereas normal human melanocytes are exquisitely sensitive to the anti-proliferative effects of TGF- β , resistance to this effect begins to develop as malignant transformation occurs. However, proliferative melanoma cells do retain sensitivity to TGF- β , and thus in this population of melanoma cells TGF- β actually acts as a tumor suppressor (Hoek et al., 2006). Hoek et al. demonstrated that melanoma cells which clustered into the invasive, low MITF gene expression group also showed upregulation of many downstream targets of TGF- β (Hoek et al., 2006). Further, specific inhibition of the TGF- β signaling cascade via exogenous Smad7 significantly reduces the capacity of melanoma cells for anchorage-independent growth, a characteristic intrinsic to metastatic potential (Javelaud et al., 2005). An important mechanism by which TGF- β signaling is able to increase invasive potential is likely its ability to decrease MITF expression, which results in upregulation of the pro-invasive RHO/ROCK and Notch signaling cascades (Carreira et al., 2006; Thurber et al., 2011). It also induces the expression of factors which inhibit canonical Wnt signaling (Hoek et al., 2006), promotes angiogenesis through factors such as VEGF, and has broad immunosuppressive effects which may contribute to the therapeutic resistance of TGF- β expressing tumor cells (Javelaud et al., 2008).

A small but accumulating body of literature has also characterized the involvement in melanoma of bone morphogenic proteins (BMPs), which represent another subset of the TGFB superfamily (Hsu et al., 2005). Like TGFB, BMP7 can act in an autocrine fashion to inhibit melanoma cell growth (Hsu et al., 2008). Interestingly, the upregulation of BMP7 with melanoma progression coincides with upregulation of Noggin, an antagonist of BMP (Hsu et al., 2008). This finding parallels the observed acquisition of resistance to autocrine TGFB seen in melanoma cells compared to melanocytes (Krasagakis et al., 1999). In contrast,

others have reported that BMPs including BMP7 promote melanoma cell migration and invasion (Rothhammer et al., 2005), suggesting that the current model for how BMPs affect melanoma is still incomplete. The observation that nevi display relatively low levels of BMP4 and BMP7 may reflect that these cells retain sensitivity to these ligands (Rothhammer et al., 2005), and would be consistent with a potential oncogenic role for BMPs during melanoma progression.

2.3.4 Brn-2

Brn-2 is a POU domain transcription factor which regulates melanocytic growth (Cook and Sturm, 2008; Thomson et al., 1995), and in the setting of melanoma, is upregulated by both the MAPK and β -catenin signaling pathways (Goodall et al., 2004a; Goodall et al., 2004b). Increased expression of Brn-2 is a hallmark of invasive melanoma cells, and its activity contributes to this phenotype through a variety of different mechanisms. For one, Brn-2 is a potent repressor of MITF expression, and downregulation of Brn-2 during melanoblast differentiation to a mature melanocyte is necessary to ensure adequate expression of MITF (Goodall et al., 2004a). In invasive melanoma cells however, Brn-2 is upregulated, leading to MITF depletion and increased metastatic potential. Pinner et al. demonstrated that Brn-2 upregulation is a key feature of invasive and metastatic melanoma cells through intravital imaging of GFP-tagged Brn-2 levels in melanoma (Pinner et al., 2009). The invasive and metastatic tumor cells also showed decreased pigmentation, a surrogate marker for dedifferentiation as a result of a Brn-2-induced decrease in MITF expression. Besides acting as a repressor of MITF expression, Brn-2 increases invasive potential through its role as an activator of the Notch pathway. With siRNA knockdown of Brn-2 in the A2058 melanoma cell line, there is a resultant decrease in the expression of several Notch1-related target genes (Thurber et al., 2011). Interestingly, Brn-2 is one of only three factors necessary to facilitate the conversion of mouse fibroblasts into functional neurons (Vierbuchen et al., 2010), demonstrating the importance of this pathway not only in melanocyte biology, but also in neuronal biology. This observation is not entirely surprising given close links developmentally between melanocytes and other neural crest-derived cell types.

2.3.5 AP-2

The activity of AP-2, a 52kDa transcription factor, is vital to the normal embryonic development of neural crest cells, and also appears to play an important role in the differentiation of adult cells (Bar-Eli, 2001; Tellez et al., 2003). In normal human melanocytes, AP-2 is responsible for the regulation of a host of normal cellular functions, including DNA repair, cell cycle arrest and cell adhesion (Zhuang et al., 2007). However, in melanoma cells, the loss of AP-2 is a prominent feature associated with a switch to an invasive phenotype (Braeuer et al., 2011). It appears that this downregulation is the result of the increased activity of cAMP-responsive element binding (CREB) protein, a common feature in melanoma progression due to dysregulation of the MAPK pathway (Melnikova et al., 2010). Melnikova et al. showed that activation of PKA-dependent CREB signaling downregulates AP-2 in invasive melanoma cells, while re-introduction of AP-2 into these cells restores a non-metastatic phenotype (Melnikova et al., 2010). By our definition, this effect of AP-2 would constitute forced differentiation, resulting in an altered cell fate. Further studies will be needed to address whether AP-2 and its regulation by PKA/CREB can be utilized as a viable therapeutic strategy.

2.4 Classification of melanoma by states of differentiation

2.4.1 Relating gene signatures to the differentiation of cell lineages

Melanocytes develop from embryonic stem cells along two distinct lineages (Dorsky et al., 2000a; Thomas and Erickson, 2008). The classical lineage involves migration of neural crest cells (NCC) from the dorsal aspect of the neural tube. These pluripotent NCC initially have the ability to differentiate into a wide variety of cell types, including smooth muscle cells, peripheral neurons and glia, as well as melanocytes (Dorsky et al., 2000a; Thomas and Erickson, 2008). Certain extrinsic factors are necessary in order to induce melanocytic differentiation of these stem cells, and several have been identified, including mast cell growth factor (*MGF/KITLG*), endothelin 3 (*EDN3*) and *SOX10*.

Intriguingly, in the search for key intrinsic modulators of melanocytic differentiation, it appears as though one transcription factor acts as the universal regulator of this process: *MITF* (Goding, 2000). Mutations of the *MITF* gene cause neural crest cell dysfunction and marked impairment of normal pigmentation, as reflected in patients with a variant of Waardenburg Syndrome (Goding, 2000). Some typical markers of melanocytic differentiation include tyrosinase (*TYR*), melanoma antigen recognized by T-cells 1 (*MART-1*) and transmembrane glycoprotein NMB (*GPNMB*).

Not all melanocytes follow the same path of differentiation from the neural crest. If the transcription factor *HMX1* is expressed in the dorsal root ganglia, NCCs instead migrate along the ventral aspect of the dorsal root, and are induced to differentiate towards a neuronal phenotype (Adameyko et al., 2009; Krispin et al., 2010). These neuronal and glial cells are then directed to expanding branches of peripheral neurons throughout the body. Among this population of ventrally-derived neuronal cells is a group of pluripotent stem cells known as Schwann Cell Precursors (SCPs). Adameyko et al. demonstrated that although these cells are fully capable of differentiating into Schwann cells, SCPs are also capable of differentiating along a melanocytic lineage, under the influence of growth factors such as *IGF-1* and *PDGF* (Adameyko et al., 2009). Melanocytes spawned from this neuronal lineage have a gene expression profile defined by a lack of *MITF* or *Wnt/β-catenin* influence, and thus lack expression of many melanocyte-specific genes (Tap et al., 2010). Thus, the transcriptional profile closely parallels (and in fact may represent) invasive or stem cell-like melanoma cells (Hoek et al., 2006).

2.4.2 Melanoma gene signatures resembling melanocytes or neuronal precursors

Analogous to the two distinct clusters of gene expression profiles representing proliferative and invasive subpopulations of melanoma cells discussed in this chapter until now, there is also a large body of research which has used transcriptional profiling studies to identify two major differentiation states of melanoma cells. These differentiation states have previously been classified by Tap et al. as either the differentiated melanocyte group (DMG), or the neuronal precursor group (NPG) (Tap et al., 2010). Based on our description of the two distinct lineages of melanocytes, the differentiation state of melanoma cells in the DMG resembles melanocytes of the classical lineage, while the differentiation state of melanoma in the NPG resembles melanocytes of a neuronal lineage. The existence of these two distinct melanoma differentiation states is in keeping with the cancer stem cell theory, which is quickly gaining acceptance within the cancer research community as the missing link in our understanding of phenomena such as metastasis and therapeutic resistance (Zabierowski and Herlyn, 2008). This theory proposes that every tumor is made up of cells in various

states of differentiation, with one subgroup made up of quickly dividing tumor cells which remain highly differentiated and another subgroup made up of tumor cells which take on a stem-like phenotype.

In relation to melanoma, the melanocytic subgroup of cells, or DMG, represents a population of tumor cells which express many of the markers of a differentiated melanocyte. These markers include TYR, MART-1, GPNMB, endothelin receptor type B (EDNRB) and neurturin (Tap et al., 2010). Melanoma cells belonging to the DMG are increasingly being referred to as the fast-growing, differentiated counterparts to stem cell-like tumor initiating melanoma cells. As with melanocytes which develop from NCC via the classical lineage under strong SOX10 influence, the well-differentiated population of melanoma tumor cells express a transcriptional profile which reflects the influence of MITF and Wnt/ β -catenin signalling. Tap et al. postulate that melanoma cells belonging to the DMG develop as per the typical pattern of melanocyte development from the dorsal aspect of the neural tube (Tap et al., 2010). It would appear that melanoma cells in the DMG may in fact represent the same subgroup of highly proliferative, minimally invasive melanoma cells described by Hoek & Goding, and referred to as proliferative melanoma cells (Hoek and Goding, 2010; Tap et al., 2010).

The existence of a distinct subpopulation of cells within malignant tumors which retain key stem cell-like properties such as the capacity for self-renewal and differentiation is a hallmark of the cancer stem cell theory, and indeed melanoma tumors do contain populations of de-differentiated cells which resemble neuronal stem cells (Barnhill et al., 2004). Much like the neuronal melanocyte lineage which develops from NCC under the strong influence of the HMX1 transcription factor and a lack of SOX10 signalling, the stem-like NPG melanoma population expresses a relative lack of MITF and Wnt/ β -catenin influence in its transcriptional profile (Tap et al., 2010). Instead, several cellular markers associated with neuronal and not melanocytic cells are expressed, including neural crest nerve growth factor receptor CD271, glial fibrillary acidic protein (GFAP), neurofilament protein (NFP) and synaptophysin (Syn) (Boiko et al., 2010). Further, melanoma cells which express stem-like properties are highly invasive and minimally proliferative. Add to all of this the fact the transcriptional profile of the NPG reflects the strong influence of noncanonical WNT5A signalling, and we come to the reasonable conclusion that the de-differentiated population of neuronal melanoma cells identified by Tap et al. may in fact be equivalent to the invasive melanoma population described by Hoek & Goding (Hoek and Goding, 2010; Tap et al., 2010).

2.5 Phenotype switching and tumor heterogeneity

In contrast to the unidirectional genetic theory of melanoma progression is the fact that the proliferative transcriptional grouping is comprised of cell lines originating from primary tumors as well as from distant secondary metastases, while the invasive grouping predominantly contains pre-metastatic cells taken from the outer margins of a melanoma lesion, or metastatic melanoma cells collected during the actual process of intravascular migration (Eichhoff et al., 2010; Hoek et al., 2008). A Clark-like model of melanoma progression would instead predict that the proliferative grouping would be made up entirely of pre-metastatic cells, while the invasive grouping would contain mainly metastatic and post-metastatic cells (Miller and Mihm, 2006). However, evidence derived from several in vivo studies instead suggests that melanoma cells have the ability to cycle from a predominantly proliferative to a predominantly invasive phenotype, based on such influences as the tumor microenvironment and pharmaceutical therapies (Hoek and Goding, 2010).

One compelling study in support of phenotype switching as a source of melanoma heterogeneity was conducted by Pinner et al., in which it was shown through the use of intravital imaging of melanoma cells that highly motile, intravasated metastatic melanoma cells reverted back to their pre-metastatic, proliferative phenotype once seeding at the site of secondary tumor metastasis occurred (Pinner et al., 2009). Specifically, whereas melanoma cells in the primary tumor as well as the secondary metastatic tumor were shown to have a phenotype defined by low levels of the transcription factor Brn-2 and a high level of pigmentation, highly motile, actively metastasizing melanoma cells were shown to express a predominantly Brn-2-high/pigment-low phenotype (Pinner et al., 2009).

In a patient-matched analysis of both a primary metastatic lesion and distant metastases, Eichhoff et al. demonstrated that, consistent with our description of proliferative and invasive melanoma phenotypes, cells in the primary and distant tumors expressed high levels of *MITF* and Melan-A, while cells in the "unstructured" regions of these tumors (areas from which metastatic cells are most likely to be derived) stained heavily for WNT5A and far less for Mitf and Melan-A (Eichhoff et al., 2010). Strikingly, this study also revealed late-stage metastatic melanoma cells adopted phenotypes and morphologies nearly identical to early-phase cells, indicating that melanoma cells have significant plasticity with regards to their gene expression profile (Eichhoff et al., 2010).

Lending further support to the existence of phenotype switching are the findings of Hoek et al., in which seed melanoma cells of either an exclusively proliferative or exclusively invasive phenotype were injected subcutaneously into immunocompromised mice (Hoek et al., 2008). Regardless of seed cell phenotype, the resultant tumors expressed both major gene signatures, and furthermore the signatures appeared to adhere to a strict geographic localization pattern within the tumor. Specifically, melanoma cells expressing a proliferative phenotype were predominantly found on the periphery of melanoma tumors, while the invasive melanoma cells were found within the core of the tumor. As the tumor invades to deeper levels of tissue, the cells within the tumor core are brought into contact with microenvironmental factors such as Nodal signaling proteins, which in turn drive melanoma invasion (Hoek et al., 2008).

2.6 Cell fate as a determinant of therapeutic response

2.6.1 The advent of targeted BRAF inhibitors for metastatic melanoma

One of the most promising targeted molecular therapies currently under development are small-molecule targeted kinase inhibitors such as PLX4720 (Tsai et al., 2008) and PLX4032 (Flaherty et al., 2010a; Halaban et al., 2010; Yang et al., 2010). This drug works by inhibiting the mutated form of BRAF, a key serine/threonine protein kinase in the mitogen-activated protein kinase (MAPK) pathway (Singh et al., 2008). Up to 40-60% of melanomas harbor activating BRAF mutations (Goel et al., 2006; Gorden et al., 2003; Greene et al., 2009), and of these mutations, most are the result of a single amino acid substitution in the activation loop of exon 15. The most common mutation is BRAF^{V600E} mutation, which results in a 500-fold increase in kinase activity, and is the target of PLX4032 inhibition (Flaherty et al., 2010b). Indeed, the results of Phase I/II trials have been promising, with a demonstrated response rate of up to 80% (Roukos, 2011).

Exposure to drugs such as PLX4720 or PLX4032 results in a variety of anti-melanoma effects through the inhibition of the MAPK pathway and more specifically by mitigating aberrant activation of extracellular signal-regulated kinase (ERK), a downstream target of BRAF. As a

result, the use of PLX4720 or PLX4032 in cells harbouring activating mutations of BRAF such as BRAF^{V600E} demonstrate decreased levels of proliferation, impaired colony-forming capability and the induction of apoptosis (Tsai et al., 2008). Despite obvious excitement over the demonstrated response rate of PLX4032 thus far, talk of accelerated FDA approval and current Phase III clinical trials, questions still remain regarding the potential benefit of this drug on overall patient survival (Roukos, 2011).

2.6.2 The impact of cell fate on response to targeted BRAF inhibition

Despite producing startling initial results in some patients and achieving an overall response rate of around 80%, all melanomas sensitive to treatment with the V600 BRAF inhibitor PLX4032 eventually develop resistance, and some melanomas with the V600 BRAF mutation are resistant from the outset of treatment (Flaherty et al., 2010a). Thus, melanoma cells treated with PLX4032 make for an ideal model of mechanisms underlying the process of therapeutic resistance.

Transcriptional profiling provides some insight into the role of cell fate and PLX4032 resistance (Tap et al., 2010). In general, BRAF-mutant melanomas with a DMG (differentiated melanocyte group) gene expression signature had the least amount of resistance to growth inhibition by PLX4032, while melanoma cells with the NPG (neuronal precursor group) gene expression signature displayed the highest level of resistance to growth inhibition by PLX4032. However, the most interesting findings of this study stem from the characterization of initially sensitive cells which later acquired resistance. Contrary to the obvious conclusion that BRAF must have undergone further mutations thereby rendering PLX4032 unable to maintain its inhibitory effect, what investigators found instead was that the activity of several parallel signaling pathways was upregulated to compensate for the BRAF inhibition. Of note, levels of acquired drug resistance were highest in melanoma cells with mutated NRAS, a GTPase signaling molecule which allows for cross-talk between the PI3K-AKT and MAPK pathways.

In more general terms, it appears as though BRAF-mutated melanoma cells sensitive to PLX4032 uniformly revert to a less differentiated transcriptional profile resembling neuronal stem cells (Tap et al., 2010). Future studies will likely illuminate the exact role of cell fate in regulating the response to targeted BRAF inhibition. Understanding how the exact differentiation state of a cell (reflected by gene signature) affects phenotypes such as dependence on MAPK signaling, drug metabolism, and susceptibility to apoptosis will provide the foundation for developing therapies aimed at manipulating cell fate to therapeutic advantage.

3. Conclusion

Recent developments in our understanding of melanoma are particularly interesting with regards to thinking about forced differentiation as a potential therapeutic approach. Can we employ strong morphogen pathways to forcibly alter or refine melanoma cell fate in a manner that renders them more susceptible to therapies like targeted BRAF inhibition? Are morphogen pathways able to act as a 'master override' within a large tumor cell population to effectively decrease the heterogeneity that arises from dynamic phenotype switching? Is there a "differentiated" cancer cell state that is truly more benign and manageable compared to a parallel "undifferentiated" state?

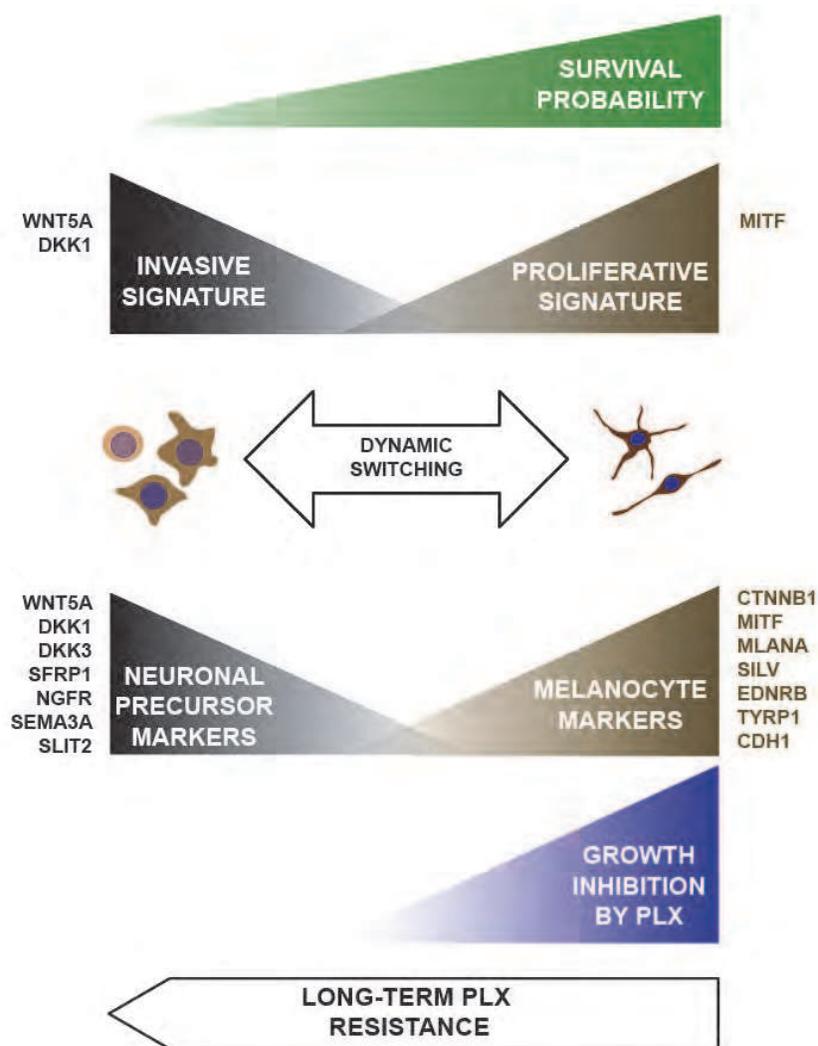


Fig. 1. A summary of cell fate and differentiation in melanoma. This model attempts to reconcile findings in the literature regarding cell fate and differentiation. In the middle, melanoma cells are shown transitioning dynamically between two states: 1) a neuroprogenitor-like state (left) highlighted by gene signatures enriched for inhibitors of Wnt/ β -catenin signaling such as WNT5A and DKK1; and 2) a more melanocyte-like state (right) exhibiting a gene signature consistent with active Wnt/ β -catenin signaling, and reflecting increased expression of melanocytic markers. Note that the continued exposure of cells to PLX4032 can promote the development of cells with largely a neuronal or invasive signature (left). The increased survival denoted on the right side (upper part of panel) reflects the clinical observation that increased Wnt/ β -catenin signaling correlates with increased patient survival (Bachmann et al., 2005; Chien et al., 2009b).

Our traditional understanding of melanoma progression was based on the presumption that once a melanocyte acquired malignant potential, the constant accumulation of novel mutations conferred a progressively more aggressive phenotype as well as an increasing survival advantage to newly generated cancer cells. While it is true that key mutations such as the BRAF mutation targeted by PLX4032 are critical to melanoma pathogenesis, the sequence of intracellular events underlying the processes of tumor proliferation and metastasis in melanoma is far more dynamic than previously thought.

The recent characterization of distinct gene expression signatures in melanoma challenges our traditional model of melanoma progression. For one, two discreet transcriptional profiles would not be predicted by this model. Instead, we would expect a continuous spectrum of signatures representing the accumulation of new genetic mutations as melanoma became more aggressive. Secondly, we would expect that the transcriptional profiles of melanoma cells from secondary metastatic sites would bear less resemblance to cells in the primary tumor. Instead, proliferative melanoma cells from both primary and secondary sites are virtually identical, while the majority of invading and metastasizing melanoma cells express an invasive gene signature. Consequently, cell fate and the process of cellular differentiation are important for understanding the plasticity of melanoma cells and developing effective treatment strategies with durable long-term results.

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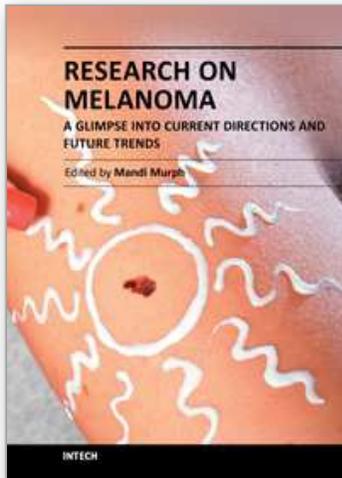
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The book *Research on Melanoma: A Glimpse into Current Directions and Future Trends*, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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