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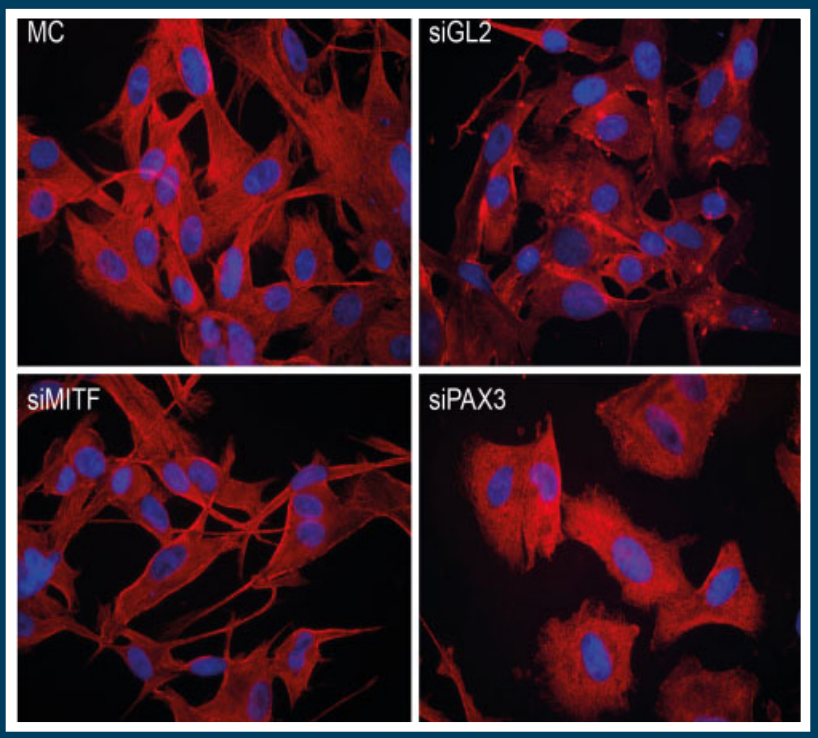

MELANOMA GENETICS/GENOMICS

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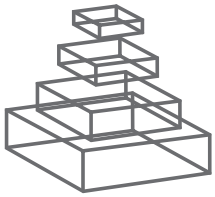

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MELANOMA GENETICS/ GENOMICS

Topic Editor:
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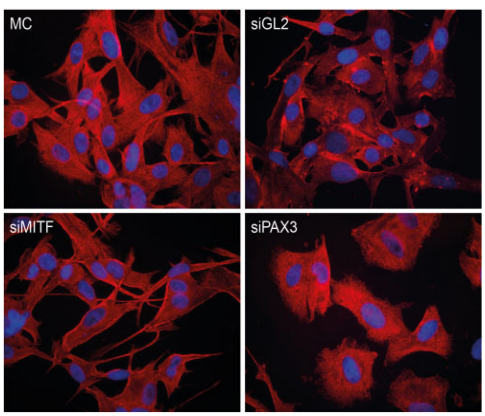

Knockdown of PAX3 or MITF results in differential morphological effects in NZM15 melanoma cells potentially related to invasiveness. NZM15 melanoma cells were grown in media without any transfection (MC), or transfected with siRNA to luciferase (siGL2) as a non-targeting control, siRNA to MITF (siMITF), or siRNA to PAX3 (siPAX3) and then stained after 48 hr with beta-tubulin antibody.

Melanoma is a complex disease driven both by genetic and environmental risk factors, and requires multiple genetic mutations in the evolution from benign melanocyte or nevus into malignant melanoma (MM). Genetic studies of familial and sporadic melanoma have revealed surprising insights into the molecular pathogenesis of this deadly cancer. Collectively, the molecular data show there are four signature pathways involved in melanomagenesis: activation of the RAS/RAF/MEK/ERK and PI3K/AKT pathways and inactivation of the INK4a/RB and ARF/P53 pathways (Chin, 2003). Acting in a coordinated manner, these pathways provide melanocytes the requisite acquired abilities needed to develop into cancer cells: growth-factor independence, insensitivity to anti-growth signals, apoptosis evasion, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Insights into the relative roles and interactions between these pathways have been provided through multiple different experimental approaches and systems, which span from human GWAS and familial studies and/or tumour sequencing to zebrafish and mouse transgenic and knockout models, retroposon mutagenesis studies, cell culture systems, developmental biology, and gene expression studies to name a few.

Table of Contents

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Melanoma genetics/genomics

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Keywords: melanoma, therapeutic targets, biomarkers, genetic pathways, personalized medicine

Gene mutations represent a major driving force in the onset and progression of melanoma. Consequently many genes are being investigated for their role in melanomagenesis, including not only inherited genes but also genetic defects that are acquired due to environmental factors, such as excessive sun exposure. The field of melanoma genetics thus encompasses genes in familial melanoma through to non-inherited genes that increase risk of melanoma. Melanoma genomics on the other hand is the study of genomes of melanoma cells and other cell types and their role in melanoma onset and progression. A "genome" includes not only all the genes of a cell, but also any genetic factors involved in programing the cell and its function.

The present volume aims to provide the reader with a snapshot of current genetic and genomic investigations of melanoma, with special emphasis on targeted treatments, and personalized medicine. A collection of Opinion, Review, Primary Research, Hypothesis and Theory, and Methods articles has been assembled that describes the panoply of genes, therapeutic targets, biomarkers, genetic pathways, and pathogenic mechanisms involved in melanoma onset and metastasis, and of clinical outcomes in patient in response to chemotherapy, immunotherapy, and personalized treatment options.

Much progress has been made in identifying individual genes and pathways involved in melanomagenesis, as outlined in the Review Article by Wangari-Talbot and Chen (1). Indeed, the discovery that melanomas frequently contain somatically acquired mutations in the *BRAF* gene that drive melanoma growth has revolutionized melanoma treatment options, and led to the development of personalized targeted treatments for patients with metastatic melanomas bearing a *BRAF* mutation, reviewed by Klinac et al. (2).

Despite melanomas harboring somatically acquired mutations in genes like *BRAF* or *NRAS*, the response of individual melanoma patients to BRAF inhibitor treatments is very variable, and Stones et al. (3) have investigated gene mutation status with respect to sensitivity to BRAF inhibitors and combination targeted therapies in a panel of New Zealand human melanoma cell lines in their Original Research Article.

Mutations in genes like *GNAQ*, *GNA11*, and *BAP1* are associated with uveal melanoma or blue nevi, and for the first time Hawkes et al. (4) have investigated in their Original Research Article whether inherited mutations in these genes are associated with familial predisposition to uveal melanoma or blue nevi.

Although *BRAF* mutations can be identified from the very earliest stages of melanoma onset, targeted BRAF inhibitor therapies are presently validated for use in advanced stage IV melanomas. Could therapies targeting *BRAF* be successfully used to treat earlier stages of melanoma? This is the subject of an Opinion Article by Ahn and Eccles (5).

With the plethora of genomic information, treatments, and outcome data available from melanoma studies, what is the best way to manage and interrogate all of this burgeoning information? Trevarton et al. (6) describe a web tool integrating multiple sources of genomic information called MelanomaDB in their Methods Article. Then immediately following this is a critique by Reinhold (7) of the advantages and disadvantages of the approach taken by the MelanomaDB article for data integration.

In addition to "driver" mutations in *BRAF*, and the related growth promoting pathways, other pathways are also very likely to be important in melanoma metastasis, including the Hippo pathway, which is discussed in the Hypothesis and Theory Article by Kim et al. (8).

An Original Research Article by Kim et al. (9) investigates the role of epithelial-mesenchymal transition marker expression in human melanocytes and melanoma cell lines. In a similarly themed article Eccles et al. (10) suggest that switching of melanoma cells from a proliferative to an invasive phenotype during metastasis has parallels with developmental mechanisms, which could be under genetic control. They propose a genetic switch theory, which they hypothesize is involved in the transition of melanoma cells to an invasive phenotype in their Hypothesis and Theory Article.

Biomarkers of melanoma progression and metastasis are expected to help with further stratification of patients with poor prognosis following melanoma diagnosis, as discussed by Dye et al. (11) in their Review Article. Expression of one factor called GLIPR1 was found to correlate with the invasive potential in melanoma cells, as demonstrated in an Original Research Article by Awasthi et al (12).

Metastasis generally involves the dissemination of circulating melanoma cells, as discussed in an Opinion Article by Joshi et al. (13), but frequently melanomas metastasize to the brain, which is discussed in an Opinion Article by Yashin et al. (14). The potential for targeted therapy of melanoma brain metastasis through *in vivo* modeling and molecular characterization is the subject of a Review Article by Gaziel-Sovran et al. (15).

This collection of articles clearly demonstrates the impact that melanoma genetics and genomics has had on targeted treatments and improved outcomes of melanoma patients in the past decade, and of the promise yet to come, but melanoma remains an important public health issue in Western societies. This is especially so

in New Zealand and Australia, where the recorded incidence rates are the highest in the world (41.2 per 100,000 population in New Zealand, age standardized to the Segi world population, 2004, and 37.2 per 100,000 in Australia, as compared to, for example, 11.9 per 100,000 in Western Europe. Clearly much work still needs to be done to address these high incidence and mortality rates of melanoma.

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Genetics of melanoma

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Genomic variation is a trend observed in various human diseases including cancer. Genetic studies have set out to understand how and why these variations result in cancer, why some populations are pre-disposed to the disease, and also how genetics affect drug responses. The melanoma incidence has been increasing at an alarming rate worldwide. The burden posed by melanoma has made it a necessity to understand the fundamental signaling pathways involved in this deadly disease. Signaling cascades such as mitogen-activated protein kinase and PI3K/AKT have been shown to be crucial in the regulation of processes that are commonly dysregulated during cancer development such as aberrant proliferation, loss of cell cycle control, impaired apoptosis, and altered drug metabolism. Understanding how these and other oncogenic pathways are regulated has been integral in our challenge to develop potent anti-melanoma drugs. With advances in technology and especially in next generation sequencing, we have been able to explore melanoma genomes and exomes leading to the identification of previously unknown genes with functions in melanomagenesis such as *GRIN2A* and *PREX2*. The therapeutic potential of these novel candidate genes is actively being pursued with some presenting as druggable targets while others serve as indicators of therapeutic responses. In addition, the analysis of the mutational signatures of melanoma tumors continues to cement the causative role of UV exposure in melanoma pathogenesis. It has become distinctly clear that melanomas from sun-exposed skin areas have distinct mutational signatures including C to T transitions indicative of UV-induced damage. It is thus necessary to continue spreading awareness on how to decrease the risk factors of developing the disease while at the same time working for a cure. Given the large amount of information gained from these sequencing studies, it is likely that in the future, treatment of melanoma will follow a highly personalized route that takes into account the differential mutational signatures of each individual's cancer.

Keywords: melanoma, MAPK, PI3K/AKT, *GRM3, PREX2, BRAF, RAC1*

INTRODUCTION

The incidence of melanoma has been rising at an alarming rate in both men and women especially in the Caucasian population (Purdue et al., 2008). According to the American Cancer Society, the lifetime risk of developing melanoma currently stands at 2% in whites, 0.1% in blacks, and 0.5% in Hispanics (American Cancer Society, 2012). It has been proposed that this increase is a result of correction in underreporting through the Surveillance, Epidemiology, and End Results (SEER) program (Cockburn et al., 2008), increased surveillance and diagnosis (Jemal et al., 2001), and an increase in risky behaviors such as indoor tanning (Armstrong and Kricker, 2001; Lazovich et al., 2010). Regardless of the cause of rise in incidence, an increase in survival after a diagnosis of metastatic melanoma has also been noted with the development of new therapies. Targeted therapies such as vemurafenib (Chapman et al., 2011) have emerged from advances in genetic profiling of molecular targets and it is expected that as new targets are identified, novel therapies will continue to emerge. Three key molecular pathways have been found to be highly deregulated in melanoma: mitogen-activated protein kinase (MAPK), as a result of mutations in *RAS*, *RAF*, and *KIT*; PI3K/AKT, as a consequence of mutations in *RAS*, mutations or loss of *PTEN* (phosphatase and tensin homolog) and dysregulated expression of *AKT*, and p16INK4A due to mutations in *CDKN2A*, *ARF*, and *p53*. Various strategies of targeting melanoma have emerged based on the information gained from analyses of these pathways with varying success. Molecular genome screens of tumor samples have been instrumental in identifying novel targets in melanoma. In this review, we will discuss the aforementioned pathways as well as novel emerging targets identified in large-scale tumor genome profiling studies.

MITOGEN-ACTIVATED PROTEIN KINASE (RAS/RAF/MEK/ERK) PATHWAY

The MAPK pathway is a highly conserved signaling cascade involved in various cellular functions including cell proliferation, differentiation, and migration. This pathway can be activated by the stimulation of upstream signaling molecules including growth factor receptors and G protein-coupled receptors (Wellbrock et al., 2004a; Gray-Schopfer et al., 2007). The aberrant activation of the classical MAPK pathway with extracellular signal-regulated kinase (ERK) as the terminal kinase is a frequent event in human cancer and is often the result of activating mutations in the oncogenes; *BRAF* (7%; Davies et al., 2002) and *RAS* (15–30%; Bos, 1989)

based on analyses of all cancer types. It is interesting to note that mutations of *RAS* and *RAF* are mutually exclusive in associated malignancies including melanoma (Brose et al., 2002).

RAS

The RAS proteins (H, K, and N-RAS) are small GTPases localized on the inner leaflet of the plasma membrane where they serve as critical mediators of cell growth, proliferation and differentiation (Trahey and McCormick, 1987; Lowy and Willumsen, 1993). RAS activity is controlled through cycling between a guanosine diphosphate (GDP)-bound state (inactive) and a guanosine triphosphate (GTP)-bound state (active; Downward, 1996; Scheffzek et al., 1997). The cycling between GDP- and GTP-bound state is partially controlled by the intrinsic GTPase activity of RAS, the activity of GTPase-activating proteins (GAPs) which promote the formation of inactive RAS–GDP complexes, and guanine-nucleotide exchange factors (GEFs) that accelerate the formation of RAS–GTP complexes (Cales et al., 1988; Herrmann et al., 1996). Mutations in the *RAS* genes abolish the intrinsic GTPase activities of these molecules and also reduce sensitivity to GAPs by preventing the dissociation of GTP (Trahey and McCormick, 1987; Scheffzek et al., 1997; Wittinghofer et al., 1997). GTP-bound RAS is able to activate its effector molecules such as RAF (Marais et al., 1995) and phosphatidylinositol-3-OH kinase (PI3K; Rodriguez-Viciana et al., 1994), and it is through the activation of these effectors that *RAS* is able to regulate proliferation, survival, and processes linked to tumorigenic cell transformation. The MAPK pathway can also be stimulated by phosphorylation of RAF by RAS (Marais et al., 1995; Weber et al., 2001), which in turn phosphorylates and activates MAPK kinases 1 and 2 (MEK1 and MEK2), which then phosphorylate and activate ERK1 and ERK2 (Rubinfeld and Seger, 2004; Rapp et al., 2006). Activated ERK1/2 phosphorylates numerous transcription factors that control gene expression such as *ELK1* (Babu et al., 2000), *FOS* (Monje et al., 2005), and *c-JUN* (Lopez-Bergami et al., 2007). RAS can also activate the PI3K/AKT signaling cascade through its interactions with the p110 catalytic subunit of PI3K (Rodriguez-Viciana et al., 1994; Pacold et al., 2000) leading to activation, translocation to the membrane, and conformational changes of the lipid kinase. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to produce phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], a second messenger that binds to a large number of proteins such as AKT/protein kinase B (PKB; Haslam et al., 1993; Datta et al., 1995; Franke et al., 1995) through pleckstrin homology domains. AKT is a modulator of oncogenic transformation (Mirza et al., 2000), cell survival (Edinger and Thompson, 2002), apoptosis (Cheung et al., 2008), cell cycle progression (Liang et al., 2002), and glycogen synthesis (Cross et al., 1995).

N-RAS is the most commonly mutated *RAS* isoform in human melanoma and melanocytic nevi (Der et al., 1986; Trahey and McCormick, 1987; Trahey et al., 1987). Mutational analyses have shown that ∼56% of congenital nevi exhibit *RAS* mutations in comparison to 33% of primary and 26% of metastatic melanomas (Albino et al., 1989; Jafari et al., 1995; Demunter et al., 2001). Activating *RAS* mutations are associated with sun and UV exposure and are more common in tumors under continuous UV exposure (56%) than tumors from intermittently or non-sun-exposed sites (21%; Ball et al., 1994; Jafari et al., 1995; van Elsas et al., 1996). The most frequent observed mutations are in codons 12, 13, and 61 and they lead to the loss of the intrinsic GTPase activity of RAS resulting in constitutive signaling and activation of downstream cascades (Der et al., 1986; Trahey and McCormick, 1987; Trahey et al., 1987). This improper signaling has been shown to promote aberrant cell proliferation (Dumaz et al., 2006), metastasis (Ackermann et al., 2005), inhibition of apoptosis (Kodaki et al., 1994; Eskandarpour et al., 2005), and chemoresistance (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994).

Activating mutations of *K-RAS* in melanoma appear to be an extremely rare event occurring in only 2% of cases, with the most common missense mutation found in codon 12 (Shukla et al., 1989; Milagre et al., 2010). This mutation has been shown to induce anchorage-independent growth in melanocytes transformed with *K-RAS*^{G12V}; however, it is less tumorigenic compared to cells transformed with *N-RAS*^{G12V} indicating that *K-RAS* may be a weaker oncogene than *N-RAS* in melanocytes (Whitwam et al., 2007). *H-RAS* mutations are also rare, detected only in 1% of melanomas (Milagre et al., 2010), especially sporadic melanomas and Spitz nevi likely from amplification of its genomic locus on chromosome 11p and oncogenic point mutations (Bastian et al., 2000). In animal models, tumorigenicity of mutant *H-Ras*^{G12V} has been shown to be enhanced in mice with deletions in *p16Ink4a* (Chin et al., 1997, 1999), mutation of *p53* (Bardeesy et al., 2001), or UV exposure (Hacker et al., 2005).

Given the role that RAS plays in cancer, various therapeutic strategies for targeting this oncogenic protein have emerged. Most challenging however, is the search for small molecule inhibitors that can directly target RAS through binding to active sites or binding pockets (Gysin et al., 2011). Several small molecule inhibitors that can suppress RAS activation by preventing guanine exchange through inhibition of RAS–GEF interactions have been identified (Taveras et al., 1997; Colombo et al., 2004; Peri et al., 2005). These small molecules bind to a cleft on the switch 2 region (residues 60–76) but their therapeutic potential is unknown. Inhibitors that target post-translational modifications of RAS have also been explored for therapeutic purposes. The attachment of a farnesyl isoprenoid group to RAS proteins is required for localization to the plasma membrane and activity (Kohl et al., 1995). Several farnesyltransferase inhibitors have been identified through rational design strategies (Dinsmore and Bell, 2003) and compound library screens (Sebti and Hamilton, 2000). These inhibitors have been shown to suppress the activity of mutated, constitutively active RAS in vitro (Kohl et al., 1995; Sebti and Hamilton, 2000) and tumor growth in vivo (End et al., 2001; Gunning et al., 2003). Despite these promising results, clinical validation of several of these inhibitors did not show objective responses in most solid tumors (Sharma et al., 2002). In melanoma, a phase II clinical trial of the farnesyltransferase inhibitor, R115777 (tipifarnib) as a single agent did not show any benefit (Gajewski et al., 2006). Furthermore, in a recently completed trial, tipifarnib in combination with sorafenib or temsirolimus did not show any activity to justify continued use (Margolin et al., 2012). Failures of farnesyltransferase inhibitors in vivo and in clinical trials have been attributed to RAS prenylation and reactivation via geranylgeranyl

transferase type 1 (Britten et al., 2001; Lobell et al., 2001). The specificity of R115777 is to the rarely mutated H-RAS, instead of the more frequently mutated N-RAS or K-RAS, and has also been speculated to be a major cause of the reduction in efficacy (James et al., 1996; Baines et al., 2011). Success in targeting melanomas with RAS mutations may be achieved by inhibiting RAS effector pathways through combined targeting of BRAF, MEK, and PI3K/AKT/mammalian target of rapamycin (mTOR) due to the integral role of these effectors in RAS driven transformation as well as the availability of clinically tested small molecule inhibitors (Davies et al., 2007; Engelman et al., 2008; Fasolo and Sessa, 2008; Lee et al., 2010; Gysin et al., 2011).

BRAF

BRAF is a serine/threonine kinase, a component of the MAPK pathway downstream of RAS and when activated, triggers phosphorylation of MEK (Johnson and Lapadat, 2002). Mutations in *BRAF* are prevalent in human cancers (7%) with the highest incidences found in malignant melanoma (27–70%), papillary thyroid cancer (36–53%), colorectal cancer (5–22%), and serous ovarian cancer (30%; Davies et al., 2002; Kumar et al., 2003; Pollock et al., 2003a; Young et al., 2005). Of the over 40 *BRAF* activating mutations identified, the $BRAF^{V600E}$ mutation is the most common, and accounts for 92% of *BRAF* mutations in sporadic melanomas and 82% of benign nevi, implying that it might be involved in the progression from a benign to a cancerous state (Davies et al., 2002; Kumar et al., 2003; Pollock et al., 2003a). A single-base missense transversion (T to A at nucleotide 1,799) changes valine to glutamic acid in codon 600 (V600E) of exon 15, and results in constitutive activation of the RAF kinase (Davies et al., 2002; Garnett and Marais, 2004; Wan et al., 2004). Given the presence of the $BRAF^{V600E}$ mutation in benign melanocytic nevi (Pollock et al., 2003a), pre-malignant colon polyps and early stage colorectal cancer (Yuen et al., 2002; Ikehara et al., 2005), the oncogenic potential of mutated BRAF has been under investigation. $BRAF^{V600E}$ was shown to transform NIH3T3 fibroblasts and mouse melanocytes resulting in increased proliferation *in vitro*, stimulation of ERK and tumorigenesis *in vivo* (Houben et al., 2004; Ikenoue et al., 2004; Wan et al., 2004; Wellbrock et al., 2004a). Interestingly, benign melanocytic nevi with *BRAF* mutations exhibit growth arrest characteristics including the expression of the senescence marker, β-galactosidase (Michaloglou et al., 2005; Gray-Schopfer et al., 2006; Dhomen et al., 2009). This might suggest that other mutations are required to drive oncogenesis in nevi, which is supported by studies such as those showing that loss of *p53* results in the progression to melanoma (Patton et al., 2005). However, it is still possible that the benign nevi with mutated *BRAF* can escape the oncogene-induced senescence and become melanomas, which might explain the high percentage of this mutation in sporadic melanoma (Wellbrock et al., 2004b; Dhomen et al., 2009). The effects of other less frequent observed BRAF mutations have also been investigated. Among melanomas with mutated BRAF, the $BRAF^{V600K}$ mutation is observed in 12% of cases while $BRAF^{V600R}$ and $BRAF^{V600D}$ are each observed at a frequency of ∼5% (Lovly et al., 2012). These mutations, similar to $BRAF^{V600E}$ result in an increase in BRAF kinase activity and increased MEK and ERK phosphorylation (Wan et al., 2004).

The high prevalence of the $BRAF^{V600E}$ mutation in melanoma has made it a popular target in drug development. Small kinase inhibitors have yielded mixed results with some showing greater efficacy than others. Sorafenib (Nexavar, Bay 43-9006), was initially produced as a specific inhibitor of *CRAF* and was found to also have inhibitory activity toward *BRAF* (Lyons et al., 2001; Wilhelm et al., 2004). Further investigation showed that sorafenib not only inhibited wild-type BRAF, but mutant BRAF as well. Additionally, it also asserts inhibitory activity toward various receptor tyrosine kinases critical in cancerous processes including vascular endothelial growth factor receptor (VEGFR) 1/2/3, platelet-derived growth factor receptor β (PDGFR-β), fibroblast growth factor receptor 1 (FGFR-1), c-KIT, FLT-3, and RET (Wilhelm et al., 2004; Carlomagno et al., 2006; Lierman et al., 2006; Chang et al., 2007). Various studies have shown the potential of sorafenib in inhibiting the growth of a host of malignancies including melanoma, leukemia, hepatocellular carcinoma, esophageal carcinoma *in vitro* and *in vivo* (Wilhelm et al., 2004; Sharma et al., 2005), and is successfully utilized in the treatment of renal cell carcinoma (Escudier et al., 2009). Single agent sorafenib for melanoma treatment has been largely unsuccessful, with efficacy improved when used in conjunction with chemotherapy or adjuvant immunotherapy (Eisen et al., 2006; McDermott et al., 2008; Amaravadi et al., 2009; Augustine et al., 2010; Ott et al., 2010; Egberts et al., 2011).

Small molecule inhibitors with greater specificity to mutant $BRAF^{V600E}$ than the wild-type protein have been developed. SB590885 (GlaxoSmithKline, Collegeville, PA, USA) was shown to have 100-fold more activity than sorafenib in inhibiting *BRAF* activity (King et al., 2006). Sorafenib stabilizes the inactive conformation of the kinase while SB590885 stabilizes the active *BRAF* conformation, which explains the difference in activity and might make SB590885 a better candidate for clinical development (King et al., 2006). Vemurafenib (PLX4720/RG7204), a novel *BRAF* inhibitor with high specificity to $BRAF^{V600E}$ has potent cytotoxicity against melanoma cells *in vitro* and *in vivo* and clinically has improved survival of melanoma patients (Tsai et al., 2008; Yang et al., 2010; Chapman et al., 2011; Young et al., 2012). It also appears that similar to the $BRAF^{V600E}$ mutations, the $BRAF^{V600D}$, $BRAF^{V600K}$, and $BRAF^{V600R}$ mutations are also responsive to inhibition by vemurafenib in pre-clinical trials (Rubinstein et al., 2010; Yang et al., 2010). In clinical trials, $BRAF^{V600K}$ and $BRAF^{V600E}$ both show better responses to the MEK inhibitor, trametinib compared to dacarbazine therapy and also when compared to patients with wild-type BRAF tumors (Flaherty et al., 2012).

During a phase I clinical trial of vemurafenib, 81% of patients with $BRAF^{V600E}$ mutations demonstrated significant shrinkage of liver, bowel, and bone metastases and progression-free survival of 7 months (Flaherty et al., 2010). The follow-up phase II trial showed a response rate of 52% (Bollag et al., 2010). Meanwhile, 48% of patients showed a partial response in a phase III trial, with 0.9% complete responses observed (Chapman et al., 2011). The limiting factor in patient treatment with vemurafenib appears to be innate and acquired resistance. Furthermore, it appears that there are alterations in signaling after BRAF inhibitor exposure that may promote cell growth indicating that meticulous selection of treatment candidates is necessary. This is especially important

because some patients treated with vemurafenib present with dermatological side effects that include keratoacanthomas and squamous cell carcinomas (Oberholzer et al., 2012; Su et al., 2012). Reports indicate that BRAF inhibitors induce ERK signaling and increase growth in wild-type BRAF cells (Heidorn et al., 2010; Poulikakos et al., 2010). Further studies have shown that exposure to BRAF inhibitors results in increased binding of BRAF to CRAF, especially in RAS mutant cells leading to hyperactivation of CRAF, and elevated ERK signaling (Hatzivassiliou et al., 2010). Subsequent analysis showed that this increase was as a result of transactivation of RAF dimers by BRAF inhibitors (Hatzivassiliou et al., 2010; Poulikakos et al., 2010). The binding of a BRAF inhibitor to one protomer within a RAF dimer was found to result in loss of the catalytic activity of the inhibitor-bound RAF and transactivation of the other protomer. This transactivation of RAF homo- and heterodimers is likely responsible for induction of MEK/ERK phosphorylation by RAF inhibitors in cells with wild-type BRAF. The keratoacanthomas and squamous cell carcinomas observed in vemurafenib treated patients show a high rate of RAS mutations and increased ERK signaling despite having the $BRAF^{V600E}$ mutation and treatment with the drug suggesting that the RAS mutations may pre-dispose the patients to these dermal lesions. Acquired resistance mechanisms are also under investigation. Recently, it has been shown that innate resistance to vemurafenib can be attributed to the secretion of hepatocyte growth factor (HGF) by the tumor micro-environment (Straussman et al., 2012). This results in the activation of the HGF receptor, MET, which can reactivate the MAPK and PI3K/AKT pathways (Straussman et al., 2012). Other mechanisms of acquired resistance have also been attributed to reactivation of the MAPK and PI3/AKT pathways via development of *N-RAS* mutations (Nazarian et al., 2010), activation of AKT (Shao and Aplin, 2010), up-regulation and enhanced activation of the receptor tyrosine kinases PDGFR-β (Nazarian et al., 2010), COT/MAP3K8 (Johannessen et al., 2010), insulin-like growth factor 1 receptor (IGF-1R), FGFR3 (Yadav et al., 2012), emergence of an aberrantly spliced BRAF variant [p61BRAF(V600E); Poulikakos et al., 2011] and increases in $BRAF^{V600E}$ copy number (Shi et al., 2012). Other *BRAF* inhibitors such as GDC0879 (Hoeflich et al., 2009; Wong et al., 2009) and GSK2118436/dabrafenib (Anforth et al., 2012; Hauschild et al., 2012) are currently in the development and testing phase to determine their efficacy in melanoma treatment. In clinical testing, dabrafenib was shown to improve progression-free survival with durable responses at 6 months (Falchook et al., 2012b; Hauschild et al., 2012).

To circumvent the innate and acquired resistance problem, combinations of *BRAF* inhibitors with inhibitors of other kinases and pathways that promote melanoma growth are being investigated. Co-inhibition of $BRAF^{V600E}$ with MEK (Flaherty et al., 2012; Shi et al., 2012), PI3K/mTOR (Greger et al., 2012), metabotropic glutamate receptor 1 (Lee et al., 2011; Mehnert et al., 2012), histone deacetylases (Lai et al., 2012), Hsp90 (Catalanotti and Solit, 2012), and cytotoxic T lymphocyte antigen 4 (CTLA-4; Weber et al., 2012) are actively being pursued. The combination of vemurafenib and the CTLA-4 blocker, ipilimumab, is thought to be especially promising as evidence suggests that BRAF inhibitors and immunotherapy may act synergistically (Ascierto et al., 2012). Pre-clinical studies indicate that exposure to high concentrations of PLX4720 does not affect the viability and function of lymphocytes (Comin-Anduix et al., 2010). Furthermore, other studies have shown that PLX4720 treated cells become better targets for immunotherapy due to increased expression of melanocyte differentiation antigens which confer enhanced antigen-specific recognition by CTLs (Boni et al., 2010).

MEK1/2

MEK1/2 are kinases that phosphorylate tyrosine and threonine residues on ERK1/2 kinases (Roskoski, 2012). MEK mutations are rare in human cancers with minimal mutated cases detected in lung cancer (Marks et al., 2008; Sasaki et al., 2010) and ovarian cancer (Estep et al., 2007). Analyses of human melanoma tumors have also shown a low incidence (3–8%) of somatic mutations in MEK (Murugan et al., 2009; Nikolaev et al., 2012). Regardless, MEK inhibitors have emerged as an effective strategy to target drug resistant $BRAF^{V600E}$ melanomas in patients with or without previous exposure to BRAF inhibitors (Gilmartin et al., 2011; Wagle et al., 2011). Trametinib (Falchook et al., 2012a; Flaherty et al., 2012) and selumetinib (Boers-Sonderen et al., 2012) have emerged as potent MEK inhibitors. Pre-clinical studies show that cells with mutated BRAF are sensitized to AZD-6244/selumetinib (Prickett et al., 2011; Dahlman et al., 2012), TAK-733 (Dahlman et al., 2012). Furthermore, clinical studies have also shown that MEK inhibitors increase sensitization to BRAF inhibition with improved survival achieved in patients treated with combination MEK and BRAF inhibitors compared to either drug alone (Flaherty et al., 2012).

PI3K/AKT PATHWAY

Activation of the PI3/AKT pathway is one of the most frequent events in cancer. This pathway is a critical player not only in normal physiological processes but also in tumorigenic development through the positive regulation of G1/S phase progression, inhibition of apoptotic cell death, and increased survival (Cully et al., 2006; Jiang and Liu, 2008; Yuan and Cantley, 2008). When activated by any one of a variety of mechanisms including activated receptor tyrosine kinases (Domchek et al., 1992), interactions with growth factor receptor-bound protein 2 (GRB2) adaptor protein (Pawson, 2004), or RAS (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994; Chan et al., 2002), the second messenger lipid $PtdIns(3,4,5)P_3$ is generated. $PtdIns(3,4,5)P_3$ in turn recruits both phosphatidylinositol-dependent kinase 1 (PDK1) and AKT/PKB to the membrane where PDK1 phosphorylates and activates AKT/PKB and indirectly activates the mTOR (Hay and Sonenberg, 2004; Sarbassov et al., 2005). Activated AKT has multiple functions including increased oncogenic transformation, survival, proliferation, insulin metabolism, and cell cycle regulation (Stambolic et al., 1998; Mirza et al., 2000; Shin et al., 2002, 2010; Stahl et al., 2004). AKT can also directly phosphorylate mTOR through phosphorylation (and inactivation) of tuberous sclerosis complex 2 (TSC2), an inhibitor of mTOR (Ma et al., 2005). The activation of mTOR has been shown to be involved in regulation of glucose availability in the cell and tumorigenesis (Kim et al., 2003; Sarbassov et al., 2005). Dysregulation of the PI3K/AKT pathway in cancer can occur as result of mutations in the gene encoding the p110 catalytic subunit of PI3K, PI3KCA subunit (Samuels et al.,

2004), loss of the tumor suppressor PTEN, a negative regulator of PI3K/AKT pathway (Li et al., 1997) or molecular alterations in AKT (Staal, 1987; Bellacosa et al., 1995; Cheung et al., 2008). In melanoma, PTEN loss and AKT amplification are common events and have been well documented.

PHOSPHATASE AND TENSIN HOMOLOG

The tumor suppressor on chromosome 10, *PTEN* (deleted on chromosome 10) acts as a negative regulator of the phosphatidylinositol 3-kinase (PI3K) signaling pathway and has been implicated in a multitude of cancers. PtdIns(3,4,5)P_3 is a key cell signaling molecule catalyzed from PtdIns(4,5)P_2 by PI3K (Salmena et al., 2008). PTEN hydrolyzes the 3-phosphate on PtdIns(3,4,5)P_3 to generate PIP_2, and thereby negatively regulates PtdIns(3,4,5)P_3-mediated downstream signaling (Stambolic et al., 1998; Carracedo and Pandolfi, 2008). Upon *PTEN* loss, PtdIns(3,4,5)P_3 accumulates and promotes the recruitment of a subset of proteins that contain a pleckstrin homology domain to cellular membranes, including the serine/threonine kinases AKT1, AKT2, AKT3, and PDK1 (Stambolic et al., 1998). Deletion, mutation, or inactivation of *PTEN* results in aberrant activation of PI3K pathway effectors (Stambolic et al., 1998; Suzuki et al., 1998). Various alterations in *PTEN* have been identified in melanoma including allelic loss in 20% of melanomas, altered expression in 40% of tumors and hemizygous deletions and inactivation in 57–60% of melanoma cell lines (Pollock et al., 2002; Goel et al., 2006; Li and Ross, 2007; Yin and Shen, 2008). Ectopic expression of *PTEN* in melanoma cells lacking functional protein has been shown to inhibit AKT phosphorylation, increase apoptosis, and decrease cell proliferation (Stewart et al., 2002). siRNA knockdown of wild-type PTEN has been shown to result in increased phosphorylation of AKT3 and radial growth reinforcing its involvement in melanoma pathogenesis (Stahl et al., 2004). The lack of functional *PTEN* also appears to regulate cell survival by increasing *BCL-2* expression and promoting insensitivity to chemotherapeutic agents (Wu et al., 2003; Stahl et al., 2004; Madhunapantula et al., 2007). In melanoma, the loss of *PTEN* is thought to occur early in melanomagenesis as shown in primary lesions harboring loss of one allele of *PTEN*, or *PTEN* haplo-insufficiency due to the loss of the entire chromosome 10 (Parmiter and Nowell, 1988; Bastian et al., 1998; Wu et al., 2003). Several studies have shown that *PTEN* loss can interact with other melanoma mutations. Bosenberg's group elegantly demonstrated that in a genetically modified mutated *BRAF* transgenic mouse model, the deletion of a functional *PTEN* can drive the development of malignant melanoma (Dankort et al., 2009). Furthermore, other studies have identified functional redundancy between *PTEN* loss and *RAS* mutation and have shown that these two genes are mutually exclusive in melanoma development due to redundant activation of the PI3K/AKT pathways (Tsao et al., 2000, 2004). *De novo Ras* mutations have been observed in a mouse model of $Pten^{+/+}$ mice while $Pten^{+/-}$ melanomas showed a decreased incidence of *Ras* mutations, while $Pten^{-/-}$ mice completely lacked Ras mutations (Mao et al., 2004). Furthermore, Tsao et al. (2000) observed similar results in human melanoma cell lines where cells with *PTEN loss* lacked *RAS* mutations. Similarly, a mouse model of *Tyr-H-RAS*^{V21G}*ink4a/Arf*$^{-/-}$ in a $Pten^{+/+}$ or $Pten^{+/-}$ background showed that inactivation of one copy of *Pten* led to earlier onset of melanoma whereas mice without activated *Ras* in the $Pten^{+/-}$*Ink4aArf*$^{-/-}$ background did not give rise to animals with melanoma (Nogueira et al., 2010). Taken together, these studies suggest that activation of *Ras* and loss of *Pten* cooperates in a subset of melanomas. However, exceptions in the reciprocity of *NRAS* mutations and *PTEN* loss have been noted. In the study by Tsao et al. (2000), they found that one cell line in their cohort had concurrent loss of *PTEN* with an *NRAS* mutation. Similarly, Nogueira et al. (2010) found that ∼14% of the human melanomas they analyzed had an *NRAS* mutation in addition to loss of *PTEN*. It is possible that a small population that harbors both *RAS* and *PTEN* mutations has escaped from signaling through the PI3K pathway and instead its tumorigenic properties are driven by the MAPK pathway.

AKT

Phosphatidylinositol (3,4,5)-triphosphate directly binds to PDK1 which can phosphorylate and activate AKT (Alessi et al., 1997; Currie et al., 1997). *AKT* has three isoforms; *AKT1*, *AKT2*, and *AKT3* with each encoded for by different genes which share a high degree of structural similarities (Staal, 1987; Nakatani et al., 1999). Upon PtdIns(3,4,5)P_3 binding, PDK1 induces AKT kinase activity 30-fold by phosphorylating it on the catalytic domain on residue threonine 308, or through phosphorylation on the carboxy-terminal hydrophobic motif on serine 473 by PDK2 (Alessi et al., 1997; Toker and Newton, 2000). Phosphorylation of both sites has been shown to be essential for maximal activation of AKT (Alessi et al., 1996). These activated AKT serine/threonine kinases, in turn are thought to phosphorylate ∼9,000 proteins with the minimal recognition sequence: R-X-R-X-X-S/T in both the cytoplasm and the nucleus (Lawlor and Alessi, 2001). These proteins are involved in regulating the cell cycle, preventing apoptosis, and triggering cellular growth (Manning and Cantley, 2007).

Expression of these three *AKT* isoforms has been shown to be differential among tissues. *AKT1* is ubiquitously expressed in most organs and tissues at high levels; *AKT2* expression is preferentially elevated in insulin-sensitive tissue such as the liver, muscle, and adipose tissue while *AKT3* is predominantly expressed in the brain and testis (Dong et al., 1999; Zinda et al., 2001; Franke, 2008); expression however does not always imply activation (Stahl et al., 2004). All three isoforms of *AKT* have been linked to cancers of the stomach, breast, pancreas, and ovary (Staal, 1987; Cheng et al., 1992, 1996; Bellacosa et al., 1995). Dysplastic nevi and melanomas display increased *AKT* phosphorylation in contrast to normal or slightly dysplastic nevi (Dhawan et al., 2002). *AKT2* and *AKT3* have emerged as the predominant forms that are dysregulated in melanoma. Activated AKT3 has been detected in 43–60% of sporadic metastatic melanoma when compared to normal melanocytes, an observation attributed to increased copy number of the *AKT3* gene (Stahl et al., 2004). Additionally, levels of phosphorylated AKT3 were found to correlate with melanoma progression suggesting that AKT3 might have a role in the aggressiveness of melanomas (Stahl et al., 2004). In addition to the increase in copy number that leads to improper *AKT3* activation, loss of *PTEN* has also been shown to contribute to *AKT3* up-regulation. siRNA knockdown of *PTEN* led to enhanced AKT3 phosphorylation in both melanocytes and human melanoma cells

(Stahl et al., 2004). siRNA-mediated down-regulation of AKT3 conversely resulted in a decrease in cell survival and tumor growth (Stahl et al., 2004; Tran et al., 2008). AKT3 has also been shown to participate in resistance to BRAF inhibitors and suppression of AKT3 may lead to increased clinical responses with BRAF inhibitors (Shao and Aplin, 2010). *AKT2* over-activation has also been identified in melanoma, breast, and ovarian cancer (Arboleda et al., 2003; Yuan et al., 2003; Nogueira et al., 2010; Shin et al., 2010). Expression of *AKT2* in melanoma has been established in several different models of melanoma; a mutant *Ras* background (Nogueira et al., 2010) and one with ectopic expression of metabotropic glutamate receptor 1 (*Grm1*; Shin et al., 2010). In the metabotropic glutamate receptor model (Pollock et al., 2003b; Namkoong et al., 2007), examination of primary, nodal and in-transit metastasis yielded AKT2 and not AKT3 as the predominant activated isoform. In subsequent studies, Akt was shown to be a downstream target of Grm1 (Shin et al., 2010). Modulation of Akt2 expression levels in an inducible siRNA system lead to growth suppression *in vitro* and *in vivo* (Shin et al., 2010). Furthermore, siRNA knockdown of GRM1 in human melanoma cell also resulted in a decrease in AKT2 phosphorylation corroborating that AKT2 is a downstream target of GRM1 (Wangari-Talbot et al., 2012). Nogueira et al. (2010) have also shown that *PTEN* loss in a mutant *RAS* background can result in the selective activation of *AKT2*. This up-regulation of AKT2 was found to contribute to the increase in cell transformation, invasiveness of melanoma cells and a reduction in E-cadherin expression. In addition, using a complementary genetic approach, a dominant negative mutant of AKT2 led to a decrease in the invasiveness of the melanoma cells (Nogueira et al., 2010). Regardless of which AKT isoform is involved in melanoma, the PI3K/AKT pathway is an important therapeutic target in melanoma.

Several studies have pointed to the potential use of PI3K/AKT inhibitors in suppressing tumor growth *in vitro*, *in vivo* as well as in chemo-sensitization (Brognard et al., 2001; Stassi et al., 2005; Sinnberg et al., 2009; Hirai et al., 2010; Isosaki et al., 2011). PI3K inhibition by the irreversible inhibitor wortmannin or LY294002, can block AKT activation as well as compensatory mechanisms and has been used widely in mechanistic studies to dissect the mode of action of this pathway (Vlahos et al., 1994; Wymann et al., 1996; Garcia-Echeverria and Sellers, 2008). These two compounds however have pharmaceutical limitations such as off-target activities that prevent them from transitioning from the bench to the clinic (Bain et al., 2003; Knight and Shokat, 2007). Based on the wortmannin model, compounds with fewer limitations such as PWT-458 and PX-866 have been developed but neither of them have entered clinical trials yet (Garcia-Echeverria and Sellers, 2008). ZSTK474 a novel potent PI3K inhibitor with anti-tumor efficacy is undergoing safety assessment in solid malignancies (Yaguchi et al., 2006). Other AKT inhibitors such as isoselenocyanates, API-2, SR13668, BI-69A11, GSK690693, and MK-2206 have been shown to have anti-tumor activity in suppressing tumor growth and are undergoing further testing (Forino et al., 2005; Karst et al., 2006; Rhodes et al., 2008; Sharma et al., 2009; Hirai et al., 2010). In a clinical trial however, treatment with the AKT inhibitor perifosine/keryx showed no objective responses in patients with metastatic melanoma and had significant gastrointestinal side effects (Ernst et al., 2005). AKT inhibitors however may be helpful in patients with $BRAF^{V600E}$ melanomas as Akt activation has been shown to cooperate with the mutant B-Raf to promote progression and chemoresistance (Tran et al., 2008; Shao and Aplin, 2010). It is therefore not surprising that combinatorial therapies utilizing an AKT inhibitor such as MK-2206 and the MEK inhibitor, AZD-6244, in patients with relapsed $BRAF^{V600E}$ positive melanomas (clinical trial NCT01510444) are in clinical testing. Another possibility in targeting the AKT pathway in melanoma is through inhibition of mTOR signaling using rapamycin or rapamycin analogs. These mTOR inhibitors show anti-tumor properties *in vitro*, *in vivo* and the ability to improve sensitivity to chemotherapeutic agents (Faivre et al., 2006; Sinnberg et al., 2009). Treatment of melanoma patients with the mTOR inhibitor sirolimus in combination with carboplatin and paclitaxel displayed significant tumor regression (Meier et al., 2009). Promising results have also been observed with another mTOR inhibitor, evolorimus (Hainsworth et al., 2010; Si et al., 2012).

CDKN2A/P16^{INK4A}/ARF

Familial melanomas account for 8–12% of diagnosed melanomas (Greene and Fraumeni, 1979; Fountain et al., 1992). Genetic studies in large melanoma-prone families have demonstrated that loss of heterozygosity or mutations at the p16 locus co-segregate with melanoma susceptibility in familial melanoma kindred (Hussussian et al., 1994; Kamb et al., 1994; Berwick et al., 2006). The 9p21 locus encodes two distinct proteins; p16INK4A and p19Arf in mouse/p14ARF in humans) and has been shown to undergo frequent recombination and deletions in both spontaneous and familial melanoma (Kamb et al., 1994; Quelle et al., 1995). Exon 1α and 1β of the *CDKN2A* gene are driven by two different promoters which results in two alternate transcripts that share exons 2 and 3. The 1α transcript encodes the p16INK4A protein while the 1β transcript encodes the p19Arf protein (Serrano et al., 1993; Quelle et al., 1995). p16INK4A is involved in the regulation of the cell cycle through its control of the RB-regulated G1–S transition (Serrano et al., 1993; DePinho, 1998; Sherr and Roberts, 1999), while p19Arf acts as a tumor suppressor by stabilizing and enhancing p53 levels through the blockade of MDM2-mediated p53 ubiquitination and degradation (Chen et al., 1998; Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998). Population-based studies have been performed in an attempt to elucidate the lifetime risk of developing melanoma in families with these mutations (Bishop et al., 2002; Berwick et al., 2006; Goldstein et al., 2007; Harland et al., 2008; Cust et al., 2011). A study based on 80 melanoma-prone families consisting of 402 melanoma patients and 713 non-affected family members from North America, Europe, and Australia was used by the Melanoma Genetics Consortium to calculate the lifetime projected risk of developing the disease in CDKN2A carriers (Bishop et al., 2002). By age 80, the projected risk of developing melanoma in North America was 76%, 91% in Australia, and 58% in Europe. Analysis of the same sample for comparative risks conferred by p16INK4A or p14ARF did not yield statistical significant differences in the melanoma risk between the two mutations (Bishop et al., 2002). Germ line *INK4A* mutations (Hussussian et al., 1994; Kamb et al., 1994), polymorphisms in the 5′ and 3′ untranslated

regions (UTRs) that alter translation or regulate mRNA stability of *p16INK4A* and promoter mutations of *p16INK4A* are all genomic alterations that have also been identified in association with 9p21-linked familial melanoma (Liu et al., 1999; Kumar et al., 2001). Studies have shown that inactivation of *p16Ink4a* increased susceptibility to both spontaneous and carcinogen-induced melanoma (Krimpenfort et al., 2001; Sharpless et al., 2001). p16INK4A has also been reported to cooperate with other oncogenes to promote melanomagenesis (Serrano et al., 1993; Chin et al., 1997; Ackermann et al., 2005). The combination of *p16INK4a* deficiency with activated *H-Ras* (Serrano et al., 1993; Chin et al., 1997), *N-Ras* (Ackermann et al., 2005), and *K-Ras* (Monahan et al., 2010) in mouse models have been shown to promote highly penetrant melanomas with short latency. Recently, p16INK4A has also been shown to have a role in regulating cellular oxidative stress. In response to potential DNA oncogenic stress such as UV exposure, melanocytes were found to upregulate the expression of p16INK4A mediated by the p38 stress-activated protein kinase (SAPK) pathway (Naidu et al., 2009; Jenkins et al., 2011). In p16INK4A-deficient cells, an increase in intracellular reactive oxygen species (ROS), was noted even in the absence of exogenous oxidative stress with restoration of p16INK4A found to restore ROS levels to normal levels (Jenkins et al., 2011). Interestingly, regulation of ROS by p16INK4A was found to be independent of both its functions in cell cycle control as well as the retinoblastoma protein. Other studies have reported on possible roles of p16INK4A outside of its cell cycle control functions. For example, Becker et al. (2001) have shown that some p16INK4A mutants still retain their ability to bind CDK4. The precise mechanism through which p16INK4 regulates ROS remains elusive.

p19Arf controls the stability of the *p53* tumor suppressor whose activity is abrogated by point mutations in many tumors during carcinogenesis (Greenblatt et al., 1994; Hollstein et al., 1994). In melanoma, the pathological role of *p53* is highly controversial as primary and metastatic melanomas have been found to have low incidences of *p53* allelic loss or point mutations (Yang et al., 2001). However, cases of highly penetrant and aggressive melanomas involving *p53* inactivation in mouse models have been reported (Bradl et al., 1991). Bardeesy et al. (2001) have shown that a transgenic mouse model, *Tyr-RAS/Trp53*$^{+/-}$, characterized by the loss of a *p53* allele but with retention of *p19Arf* develops melanoma. Interestingly, a *p19Arf* deficiency in the *Tyr-RAS;Ink4a/Arf*$^{-/-}$ mouse model with functional *p53* was also found to develop melanoma (Chin et al., 1997). This illustrates a reciprocal role of *p53* inactivation and loss of *Arf* suggesting that they have related functions and that *Arf* may serve as a regulator of *p53* (Sharpless and Chin, 2003). Various therapeutic strategies for restoring wild-type p53 activity are under investigation. Small molecules that stabilize p53 in its active biological conformation and antibodies that bind the p53 carboxyl-terminus and restore its DNA binding function have been shown to have apoptotic and chemosensitization activity (Hupp et al., 1992, 1995). Additional strategies involve the reactivation of p53 through inhibition of MDM2 using small molecules such as nutlin (Vassilev, 2004; Vassilev et al., 2004). These strategies have had mixed results as CP-31398, a compound found to stabilize wild-type p53 and rescue mutant p53 was found not to increase chemosensitivity in human melanoma cells (Luu and Li, 2003). Recent studies have shown that *p53* dysregulation in melanoma can also occur due to the up-regulation of a negative regulator of *p53*, MDM4 in a significant proportion of stage I–IV melanomas (65%; Marine and Jochemsen, 2005). Targeting the MDM4–p53 pathway using the small peptide SAH-p53-8 that binds MDM4 and disrupts MDM4–p53 complexes was shown to result in tumor growth inhibition and sensitization to chemotherapeutics including BRAF inhibitors (Gembarska et al., 2012).

Although the insight obtained from studies on these pathways in melanoma has led to significant improvements in drug development, treatment, and patient survival, complete cure still remains elusive. This is driving cutting edge research into discovering novel drug targets that may lead to greater improvements in design of therapies. Genomic sequencing of tumor genomes and exomes has led to the identification of genes with unexpected roles in melanoma formation, progression, and resistance to therapy. In the next section, we will discuss some of the novel targets identified from next generation sequencing high throughput screens that allow the sequencing of random DNA fragments with large coverage of the cancer genomes. Various changes such as rearrangements, copy number variations, base substitutions, and small indels have been identified with sufficient coverage to identify most somatic mutations in an individual cancer genome (Pleasance et al., 2010).

GENOMIC SEQUENCING OF MELANOMA

Whole genome sequencing has allowed the identification of mutational signatures in multiple tumor types including melanoma (Ley et al., 2008; Pleasance et al., 2010; Link et al., 2011; Puente et al., 2011; Welch et al., 2011). Pleasance et al. (2010) reported on the first comprehensive somatic mutation screen of melanoma performed in the COLO-829 melanoma cell line. A total of 33,345 somatic base substitutions, 292 of them in protein coding sequences were recognized. Two of these somatic substitutions were identified in *SPDEF*, an ETS transcription factor family, which has been associated with progression of breast and prostate cancer (Sood et al., 2007). Further sequencing of 48 additional melanoma biopsy samples confirmed the presence of these base pair substitutions as well as a third somatic mutation in *SPDEF*. A missense mutation was also identified in *UVRAG*, a putative tumor suppressor that complements the ultraviolet sensitivity of xeroderma pigmentosum group C cells and also has a role in autophagy (Kim et al., 2008). In addition, an 8- to 12-fold increase in copy number on chromosome 3p which contains four complete genes: *RARB*, *TOP2B*, *NGLY1*, and *KS (OXSM)* and a four- to sixfold increase on chromosome 15 containing *MKRN3* and *NDN* genes were noted. It is important to point out that this was the first instance that these amplified candidate genes were implicated in cancer development. This study also identified a high rate of C to T transitions in the tumor samples that have been reported to be signatures associated with UV exposure (Daya-Grosjean and Sarasin, 2005; Pfeifer et al., 2005), suggesting that UV-induced DNA damage could have resulted in the pathogenesis of COLO-829 melanoma cells (Pleasance et al., 2010).

Turajlic et al. (2012) also performed whole genome sequencing on primary acral melanoma and matched lymph node

metastasis from the same patient. A total of 12,661 base substitutions were identified in the primary acral melanoma while 11,711 base substitutions were identified in the metastatic specimen. Several single nucleotide polymorphisms were identified in *IFNA16*, which is within the melanoma susceptibility locus on 9p21, *MSH2*, *APC*, and *MEN1* and novel variants of *BRCA1* and *ERCC2* with the later two genes involved in DNA repair. Genomic amplification of several chromosomal regions; 4q12, 11q13, 11q14, 17p11, and 20q11 as well as of the receptor tyrosine kinase gene, *KIT*, were detected in both primary and metastatic samples. Other additional findings were the common C to T transitions at the 3′ base of pyrimidine di-nucleotides (TpC or CpC) associated with UV exposure (Daya-Grosjean and Sarasin, 2005; Pfeifer et al., 2005) indicating that similar to cutaneous melanomas, acral melanomas are just as susceptible to UV-induced DNA damage that contributes to melanoma development (Turajlic et al., 2012). Another genomic screen of acral melanomas likewise showed a high prevalence of UV associated C to T transitions in tumor samples consistent with melanomas arising from chronic sun exposure (Berger et al., 2012). A significant chromosomal rearrangement was found in the *PREX2* locus, which encodes a PtdIns(3,4,5)P_3 RAC exchange factor recently shown to interact with and modulate the function of *PTEN* (Fine et al., 2009). In addition to the nine somatic rearrangements detected near the *PREX2* locus, amplification of *PREX2* was also identified in the tumor samples. Sequencing of another tumor cohort in the evaluation of *PREX2* mutations found a 14% frequency in non-synonymous mutations. Functional significance was assessed using truncation mutants and non-synonymous point mutations of *PREX2*. In comparison to wild-type *PREX2*, the over-expressed mutants showed accelerated tumorigenicity suggesting that some melanoma cells may gain oncogenic activity through *PREX2* mutations (Berger et al., 2012).

Exome screenings are another mechanism being used to examine melanoma tumor mutations. Wei et al. (2011) performed exome sequencing on 14 matched pairs of normal and metastatic tumor DNAs from untreated individuals with melanoma and focused on genes altered in more than two tumor samples. The common $BRAF^{V600E}$ mutation was detected in 7 out of the 14 samples, while 9 other genes harboring recurrent mutations were also identified. One of these genes, *TRRAP* encodes a transformation/transcription domain-associated protein and functions as a component of a multi-protein co-activator complex possessing histone acetyltransferase activity that is central to the transcriptional activity of *p53*, *c-MYC*, and *E2F1*. *TRRAP* had a recurring serine to phenylalanine mutation at amino acid residue 722 in 6 out of the 14 samples suggesting that this might be mutational hotspot in melanoma. The clustering of this mutation is similar to the clustering of activation mutations found in *BRAF*, *NRAS*, or *PIK3CA* in melanoma suggesting it might be an oncogene. To assess the consequences of these substitutions on melanoma cells, knockdown of mutated *TRRAP* in melanoma cells resulted in increased apoptosis suggesting that these *TRRAP* mutations might be essential in the survival of melanoma cells. This screen also uncovered mutations in *GRIN2A*, an ionotropic (*N*-methyl-D-aspartic acid, NMDA) glutamate receptor subunit ε-1 in 6 out of the initial 14 samples as well as in 25.2% of additional melanoma biopsies and cell lines analyzed. The number of C to T transitions observed in *GRIN2A* was also significantly higher than the number of the other nucleotide substitutions. Two mutational clusters, and three recurrent mutations were found in evolutionarily conserved domains which by SIFT analysis are predicted to have protein function (Wei et al., 2011). The identification of this glutamate receptor supports the data by Chen and colleagues who have shown that an aberrantly expressed metabotropic glutamate receptor (*Grm1*) can result in melanocytic transformation *in vitro* and tumorigenesis *in vivo* (Zhu et al., 1998; Pollock et al., 2003b). In addition, significant subsets of human melanoma tumors express the human form of the receptor, GRM1 (Namkoong et al., 2007; Lee et al., 2011). In two completed clinical trials, targeting the glutamatergic signaling mediated by *GRM1* expression led to mixed clinical responses, pointing to the need of a better understanding of glutamatergic signaling and melanoma (Yip et al., 2009; Mehnert et al., 2011, 2012). Activating mutations in another metabotropic glutamate receptor *GRM3*, was also identified in an exon capture screen of G protein-coupled receptors in melanoma (Prickett et al., 2011). The initial screen showed that *GRM3* had a 16.3% mutation rate with 18 non-synonymous mutations in 13 of 80 tumors while a screen of an additional tumor cohort of 57 samples detected a 15.7% mutation rate. Among the mutations detected in *GRM3*, the Glu870Lys mutation was identified in 4 samples suggesting that this is likely a mutational hotspot in this gene. Functional screens performed with cells transformed with mutated *GRM3* showed enhanced activation of MEK1/2, increased migration *in vitro* and pulmonary metastasis in xenograft models. Interestingly, it was also shown that cells with *GRM3* activation mutations are more responsive to treatment with the MEK inhibitor AZD-6244 than *GRM3* wild-type cells (Prickett et al., 2011). *GRM3* might turn out to be an important player in melanoma as an independent exome screen from the Halaban group also identified it as one of the genes with a high mutation burden in sun-exposed melanomas (Krauthammer et al., 2012). Furthermore, given the low success rates observed with MEK inhibitors, *GRM3* activating mutations could be a predictor of MEK inhibitor responsive tumors (Prickett et al., 2011).

Krauthammer et al. (2012) performed an exome sequencing of 147 primary and metastatic tumors which was a significantly bigger sample size than analyzed previously by other groups. Comparison of the 147 melanomas with matched samples revealed 23,888 missense mutations, 1,596 non-sense mutations, 399 splice-site variants, and 282 insertions/deletions. Comparative analysis of sun-exposed versus sun-shielded melanomas showed that sun-exposed melanomas found on the trunks, arms, legs, and head had a higher prevalence of somatic mutations compared to the sun-shielded acral, mucosal, and uveal melanomas. In addition, tumors from older patients were found to contain more mutations than those in younger people with the primary lesions of the older patients found in the head and neck, which is indicative of melanomas arising due as a result of chronic sun damage. Based on sun exposure and mutation burden, the investigators were able to classify the tumors into three distinct groups corresponding to the number of mutations present namely, high, medium, and low mutation count. These mutations likely

originated in lesions from chronically exposed, intermittently sun-exposed and sun-shielded skin regions, respectively. Similar to other exome sequencing studies, a significant proportion of the single base pair mutations included C > T transversions associated with UV-induced DNA damage. Furthermore, they identified a motif, TTTCGT, enriched in sites where three or more mutations were found on sun-exposed skin suggesting a potential hotspot for the formation of cyclobutane pyrimidine dimers which are associated with lesions arising after UV exposure. Of the genes found to be frequently mutated, *BRAF* and *NRAS* featured prominently in lesions found on sun-exposed areas. Most interesting, a novel recurrent mutation was also identified in these sun-exposed melanomas. The recurrent mutation identified in seven of the tumor samples was a substitution of a proline for a serine at amino acid 29 in RAC1 (Ras-related C3 botulinum toxin substrate 1; $RAC1^{P29S}$), a small Rho GTPase family protein with roles in proliferation, migration, and cytoskeletal rearrangements. Analysis of an additional set of 364 tumors detected the $RAC1^{P29S}$ mutation in 20 of the samples (9.2%) and also in 4 out of 76 cell lines (5.3%) derived from sun-exposed tumors. There was no difference in the frequency of the mutation in primary versus metastatic tumors. Of note however, is the higher frequency in men (12.8%) versus women (2.4%) attributed to higher rates of UV exposure in men than women. In *in vitro* assays, $RAC1^{P29S}$ was shown to be a gain of function mutation, 4.5-fold more active in its GTP-bound state compared to the wild-type protein. In transiently transfected cells, $RAC1^{P29S}$ was shown to exhibit increased binding to the downstream effectors PAK1 and MLK3, enhance ERK phosphorylation, cell proliferation, and migration in comparison to the wild-type protein. In addition, it appears that $RAC1^{P29S}$ frequently associates with the netrin 1 receptor, *DCC*, a tumor suppressor which can mediate signals that promote proliferation and migration. It is possible that $RAC1^{P29S}$ and *DCC* loss cooperate in a manner similar to that of PTEN loss and mutations in BRAF or RAS in promoting melanoma tumor growth. In addition, they also found several mutated genes in sun-shielded melanomas. Mutations in *DYNC1I1* dynein, cytoplasmic 1, intermediate chain 1, which encodes a protein with roles in microtubule motor activity, progression through the spindle assembly checkpoint, and normal chromosome segregation were found in 3 of 17 acral melanomas. A second RAC1 mutation, due to a substitution in amino acid 65, Asp65Asn, was found also found in acral melanomas. In six uveal melanomas, mutations in BAP1 were also identified. Thus it appears that distinct mutational signatures exist in lesions depending on the amount of sun exposure and the resulting UV-induced DNA damage. Further, the newly identified $RAC1^{P29S}$ may have therapeutic potential given its cancer-related signaling.

Chin and colleagues similarly reported on a whole exome sequencing study in which they examined paired tumor and normal DNA from 135 melanoma patients in a challenge to differentiate passenger mutations from driver mutations (Hodis et al., 2012). Over 83,000 mutations were identified, with most of them non-synonymous which may suggest that they are passenger mutations and not drivers. In this study, and similar to the previously discussed reports mutation signatures associated with UV exposure were highly predominant. Permutation based framework was used to identify non-silent mutations with predicted functional significance which identified eleven genes with high significant mutation burdens that included *BRAF*, *NRAS*, *TP53*, *PTEN*, *P16INK4A*, and *MAP2K*, as well as new candidates that included RAC1, *PPP6C*, *SNX31*, *TACC1*, and *STK19*. It is important to note that *RAC1* and *PPP6C* were also identified in the screen by Krauthammer et al. (2012). In this study, $RAC1^{P29S}$ was also shown to have increased effector binding as well as increased association with GTP compared to the wild-type protein. In addition, they also identified MAP2K1 as a mutated gene in melanoma, with a recurrently mutated hotspot which confirmed a prior report (Nikolaev et al., 2012). It is important to note that despite converging on some of the same genes using different analysis methodology, there are disparities with genes identified in one screen and not identified in another which may be due to the filters applied for each analysis. Regardless, the permutation framework applied by Chin and colleagues for this analysis may be especially useful for screening bigger sample sizes (Hodis et al., 2012).

Whole exome sequencing is also been used to investigate acquired resistance resulting in drug relapse in patients treated with BRAF inhibitors such as vemurafenib (Shi et al., 2012). In a study by Shi et al. (2012), 20 sets of matched pre- and post-vemurafenib treatment biopsy samples were subjected to whole exome sequencing. An increase in $BRAF^{V600E}$ copy number (2- to 14-fold) was noted in patients who initially responded then relapsed with disease progression. In addition, an increase in mutant *BRAF* to wild-type *BRAF* ratio was also noted in the patient samples that showed increased $BRAF^{V600E}$ copy number suggesting the possible selection for the mutant genotype during the resistance acquisition process. This selection was confirmed in experiments performed in vemurafenib resistant human melanoma cell lines derived from $BRAF^{V600E}$-vemurafenib responsive cells lines under continuous drug exposure. Furthermore, they showed that drug saturation of the mutant $BRAF^{V600E}$ protein could be achieved by increasing the dose as copy number gain conferred resistance to a lower concentration (1 μM) but not a higher concentration (10 μM) implying that dose escalation of vemurafenib or other *BRAF* inhibitors might overcome the acquired resistance (Shi et al., 2012).

Genomic studies have played significant roles in improving treatment protocols for melanoma by expanding our ability to design targeted therapies. In addition, we have also gained insight on how to modify these therapies to achieve maximal results through different combination therapies. Monotherapies for melanoma have been shown to slow disease progression and also increase survival with varying success. Combination therapies have emerged as means to increase survival and long-term remissions. Importantly, it is now easier to predict whether a patient is likely to respond to a particular form of therapy due to the mutational signatures of their tumors. Next generation sequencing and other high throughput screens also continue to uncover genes with novel oncogenic properties in melanoma which open opportunities for drug design. Furthermore, algorithms and permutations may make the process of analyzing large samples and sorting mutations based on significance and potential functions a less complex. The clinical potential of some of these novel melanoma candidate

genes, such as *GRM3* are already clear and given the speed at which modern science is advancing, we can speculate that the information gained from these sequencing studies will in the future be applied toward clinical medicine. Moreover, it is important to also take note of the not so surprising revelations of these sequencing projects especially as they relate to UV exposure and its role in DNA damage and melanoma formation. With an increase in sun seeking behavior and tanning, it is critical that this information is shared with the general public population in the hope that behavior modification will occur in order to reverse the rising incidence of melanoma.

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Advances in personalized targeted treatment of metastatic melanoma and non-invasive tumor monitoring

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Despite extensive scientific progress in the melanoma field, treatment of advanced stage melanoma with chemotherapeutics and biotherapeutics has rarely provided response rates higher than 20%. In the past decade, targeted inhibitors have been developed for metastatic melanoma, leading to the advent of more personalized therapies of genetically characterized tumors. Here we review current melanoma treatments and emerging targeted molecular therapies. In particular we discuss the mutant BRAF inhibitors Vemurafenib and Dabrafenib, which markedly inhibit tumor growth and advance patients' overall survival. However this response is almost inevitably followed by complete tumor relapse due to drug resistance hampering the encouraging initial responses. Several mechanisms of resistance within and outside the MAPK pathway have now been uncovered and have paved the way for clinical trials of combination therapies to try and overcome tumor relapse. It is apparent that personalized treatment management will be required in this new era of targeted treatment. Circulating tumor cells (CTCs) provide an easily accessible means of monitoring patient relapse and several new approaches are available for the molecular characterization of CTCs. Thus CTCs provide a monitoring tool to evaluate treatment efficacy and early detection of drug resistance in real time. We detail here how advances in the molecular analysis of CTCs may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management.

Keywords: metastatic melanoma, personalized treatment, targeted therapy, drug resistance, circulating tumor cells

INTRODUCTION

Melanoma is an aggressive cutaneous cancer that arises from melanocyte cells within the basal layer of the epidermis. This aggressive malignancy accounts for more than 80% of skin cancer deaths and its incidence is increasing worldwide (Linos et al., 2009). Cutaneous melanoma arises from the transformation of melanocytes by the accumulation of mutations in genes that regulate cell differentiation and proliferation. The disease manifests itself as clinically and genetically distinct subgroups indicating the need for patient-specific treatment strategies.

In the past decade, since the discovery of key mutations and activated pathways that drive the development of melanoma (Davies et al., 2002), new targeted therapies have been developed, with mixed success. In the fore front of these is a molecule that specifically inhibits the mutated $BRAF^{V600E}$ kinase, Vemurafenib, which was approved by the FDA in 2011 as a therapeutic option for treatment of unresectable metastatic melanoma (Chapman et al., 2011). Given the success of this treatment and other treatment advances detailed below, new guidelines for the treatment of melanoma are evolving (Fox et al., 2013). Moreover, deep sequencing analyses have revealed new potential targets and much has been learned about the molecular basis of melanoma genesis. A clearer landscape of the mutation profile of melanoma is emerging and with it new potential therapeutic targets.

MUTATIONS IN MELANOMA

The most commonly observed recurrent mutations in melanoma reside within the MAPK pathway. The MAPK/Extracellular signal-regulated kinase (ERK) signaling pathway is commonly activated in melanoma by mutations in BRAF (in 50% of melanomas), NRAS (10–20%), and less frequently in MEK1 and MEK2 (∼8%) (Davies et al., 2002; Curtin et al., 2005; Murugan et al., 2009; Dutton-Regester and Hayward, 2012). Around 70–95% of all BRAF mutations are a V600E substitution, with an alternative V600K in 5–30% of the cases. BRAF and NRAS mutations are usually exclusive with a Q61R substitution in ∼60% of NRAS mutated cases (Colombino et al., 2012).

Mutations in upstream tyrosine kinase receptors such as KIT (10%, mainly in acral and mucosal melanoma), ERBB4 (∼19%) (Prickett et al., 2009), and FGFR2 (∼10%) (Gartside et al., 2009), can activate both the MAPK/ERK and the PI3K/AKT pathways.

Activating mutations in the kinases PI3K (∼3%) and AKT (∼1%) have also been reported, albeit at lower frequencies (Davies et al., 2002; Omholt et al., 2006). More common are mutations or deletions in the tumor suppressor gene PTEN (∼10–27%), responsible for the negative regulation of the PI3K/AKT pathway (Paraiso et al., 2011). Mutations in PREX2 (14%), a negative regulator of PTEN, have been described recently (Berger et al., 2012).

Another tumor suppressor gene commonly altered in melanoma is CDKN2A (∼50%) which regulates the pRB and p53

pathways (Flores et al., 1996). Additional driver mutations in TP53 (∼20%), CDK4 (∼3%), and RB1 (∼3%) have also been described, as well as a hot-spot in the adapter protein TRRAP (4%) (Wei et al., 2011). Furthermore, many mutations have been reported in other components that control cellular proliferation, angiogenesis and apoptosis, including glutamate receptors GRIN2A (33%) (Wei et al., 2011) and GRM3 (16%) (Prickett et al., 2011), G-protein GQNA (50% malignant blue nevi and 46% of uveal melanomas) (Van Raamsdonk et al., 2010), and the kinases MAP3K5 (9%) and MAP3K9 (15%) (Stark et al., 2012). Other genomic aberrations include amplifications in MITF (4%), CDK4 (3%), CCND1 (11%) and TERT (13%), and deletions in CDKN2A (38%) (Hodis et al., 2012).

A recent study described five new genes containing potential driver mutations, PPP6C, RAC1, SNX31, TACC1, STK19, and ARID2. The serine/threonine phosphatase PPP6C which negatively regulates the CCND1 oncogene, appears mutated in 12% of sun-exposed melanomas (Krauthammer et al., 2012), with the R264C substitution in 3% of cases (Hodis et al., 2012). RAC1, a RAS-related member of the Rho family of GTPases which regulate cytoskeleton rearrangements, contains the P29S substitution in around 4% of melanomas (Hodis et al., 2012). SKT19, a predicted kinase of known function, contains a D89N mutation in around 5% of melanomas.

Taken together, these recent *tour de force* studies reveal the complex array of mutations and genetic aberrations associated with melanoma genesis. Nevertheless it seems apparent that no other single mutation will have the same level of frequency as $BRAF^{V600E}$, which is mutated in approximately 50% of human melanomas (Davies et al., 2002). Further analyses to discern driver from passenger mutations as well as their mechanisms of action are required to clarify the intervention targets and rational combination strategies likely to provide the most successful outcomes. What is abundantly clear, however, is that future therapies will require previous knowledge of the patient's mutational status to guide the most appropriate intervention in a personalized fashion. So far only the targeted inhibitor of $BRAF^{V600E}$ Vemurafenib has been approved for treatment of melanoma, however we foresee in the near future that an arsenal of therapies will be available based on the tumor genotype. Thus, it is envisaged that tumor specimens will in future, be subjected to targeted sequencing of all the potential mutation hot-spots for which there are therapeutic targets or which affect treatment outcome. However given the inter- and intra-tumor heterogeneity analysis of circulating melanoma cells may provide a comprehensive and sensitive tool for determining the overall mutation status of a patient's tumors.

CLINICAL ADVANCES IN MELANOMA TARGETED THERAPIES

$BRAF^{V600E}$ INHIBITORS

Developments in molecular targeted therapies (**Figure 1**; **Table 1**) have predominantly focused on targeting the BRAF, MEK, or c-KIT kinases located within the MAPK pathway. Two

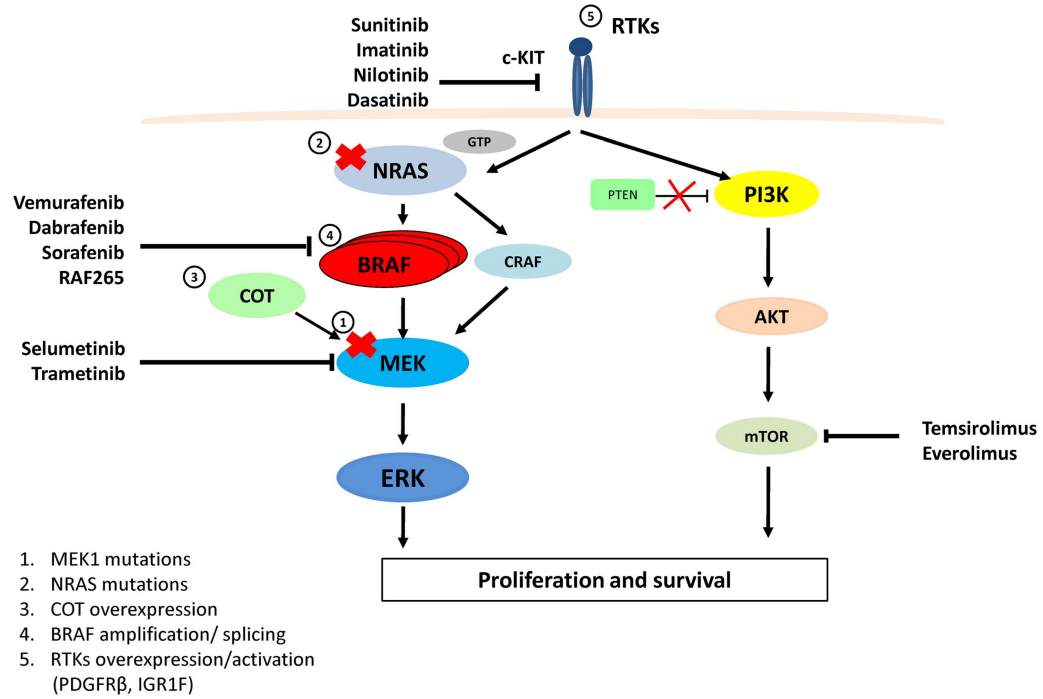

FIGURE 1 | MAPK and PI3K/AKT pathways, therapeutic targets for melanoma and resistance to Vemurafenib. Vemurafenib and Dabrafenib are specific for $BRAF^{V600E}$, while Sorafenib and RAF-265 are pan-RAF inhibitors. Imatinib, Nilotinib, Dasatinib, and Sunitinib target and inhibit c-KIT. Selumetinib and Trametinib inhibit MEK activity. Temsirolimus and Everolimus inhibit the mTOR protein. Resistance to Vemurafenib arises from MAPK pathway reactivation by (1) a $MEK1^{C121S}$ mutation, (2) $NRAS^{Q61R/K}$ mutations, (3) COT1 overexpression, (4) alternatively spliced variants of $BRAF^{V600E}$ or amplification of the mutant BRAF allele, (5) Overexpression or activation of RTKs (PDGFRβ or IGF1R) bypasses mutant BRAF and activates ERK via CRAF-MEK or through independent ERK mechanisms by activating the PI3K/AKT pathway.

Table 1 | Anti-cancer inhibitors undergoing testing for treatment of cutaneous melanoma.

Pathway	Treatment type	Target protein	Specific mutation	Trial	Effectiveness
MAPK	Vemurafenib (PLX4032)	BRAF	V600E/K	Phase I/II (Chapman et al., 2011) NCT01006980 (completed)	CR–PR = 1.8–98% RR = 48% PFS = 5.3 months OSR = 84% at 6 months
				Phase III – (updated BRIM-3) (Chapman et al., 2012)	RR = 48.4% Hazard ratio PFS = 0.26 (95% CI 0.2–0.33)
				NCT01006980 (ongoing study)	OSR = 55% at 13.2 months
				Phase II (Sosman et al., 2012) NCT00949702 (completed)	CR–PR = 6–47% OR = 53% PFS = 6.8 months OS = 15.9 months
				Phase II NCT01586195 (recruiting participants)	N/A
				Phase II NCT01474551 (recruiting participants)	N/A
	Dabrafenib (GSK2118436)	BRAF	V600E/K	Phase I (Falchook et al., 2012b) NCT00880321 (completed)	CR–PR = 50–70% RR = 69% PFS = 5.6 months OSR = 47% > 6 months
				Phase II (Long et al., 2012) NCT01266967 (ongoing study)	PFS = 4 months OS > 7.8 months
				Phase II NCT01153763 (ongoing study)	N/A
				Phase III (Hauschild et al., 2012) NCT01227889 (ongoing study)	CR–PR = 3–47% OR = 50% PFS = 5.1 months Hazard ratio OS = 0.61 (95% CI 0.25–1.48)
	Sorafenib (BAY43-9006, Nexavar)	ARAF, BRAF, CRAF, VEGF2/3, KIT PDGFR	Not specified	Phase I (Pecuchet et al., 2012) (Completed)	OR = 21% at 10 months PFS = 3.6 months OSR = 33% at 11 months
				Phase I NCT01303341 (recruiting participants)	N/A
				Phase I NCT00565968 (recruiting participants)	N/A
				Phase I NCT01078961 (recruiting participants)	N/A
	RAF-265 (CHIR-265)	ARAF, BRAF, CRAF, VEGFR	Not specified	Phase I/II NCT00304525 (ongoing study)	N/A
				Phase Ib NCT01352273 (ongoing study)	N/A
	Selumetinib (AZD6244, PD0325901)	MEK	BRAF V600E	Phase II NCT00888134 (ongoing study)	N/A
				Phase II NCT00936221 (ongoing study)	N/A
				Phase II NCT01519427 (recruiting participants)	N/A

(Continued)

Table 1 | Continued

Pathway	Treatment type	Target protein	Specific mutation	Trial	Effectiveness
	Trametinib (GSK1120212, JTP-74057)	MEK	BRAF V600E/K	Phase II (Kim et al., 2011) NCT01037127 (ongoing study) Phase III (METRIC) (Robert et al., 2012) NCT01245062 (ongoing study) Phase I/II trial NCT01584648 (recruiting participants) Phase II NCT01619774 (recruiting participants) Phase III NCT01597908 (recruiting participants)	CR–PR = 4–30% SD = 47% RR = 33% OR = 24% PFS = 4.8 months Hazard ratio OS = 0.53 (95% CI 0.3–0.94) N/A N/A N/A
PI3K/AKT	Sunitinib (CGP57148, Gleevec, Glivec)	c-KIT	Not specified	Phase I/II NCT00859326 (recruiting status unknown)	N/A
	Imatinib (ST1571)	c-KIT	Not specified	Phase II NCT00424515 (ongoing study) Phase II NCT00470470 (recruiting participants)	N/A N/A
	Nilotinib (AMN107)	c-KIT	Not specified	Phase II NCT01168050 (recruiting participants) Phase II NCT01099514 (recruiting participants)	N/A N/A
	Dasatinib (BMS-354825, Bosulif, Sprycel)	c-KIT	KIT exon 11 and 13	Phase II NCT01092728 (recruiting participants)	N/A
	Temsirolimus (CCI-779)	mTOR	Not specified	Phase II (Margolin et al., 2005) California cancer consortium (completed) Phase II (Dronca et al., 2010) NCT00521001 (completed)	N/A PR = 8% PFS = 2.4 months OS = 8.6 months
	Everolimus (RAD001)	mTOR	Not specified	Phase II NCT00976573 (recruiting participants)	N/A
Immuno-suppression blockage	Ipilimumab (MDX-010, BMS-734016)	CTLA-4	Not specified	Phase I (Hodi et al., 2010) NCT00094653 (completed) Phase III (Robert et al., 2011) NCT00324155 (ongoing study) Phase II (Di Giacomo et al., 2012) NCT01654692 (ongoing study) Phase I/II NCT01400451 (recruiting participants)	CR–PR = 0–13% OR = 10.9% PFS ∼ 30% at 12 weeks OS = 23.5% 2 years CR–PR = 1.6–13.6% OR = 15.2% PFS ∼ 35% at 12 weeks OS = 28.5% 2 years CR–PR = 10–30% RR = 40% PFS > 5 months OS = 50% > 1 year N/A

(Continued)

Table 1 | Continued

Pathway	Treatment type	Target protein	Specific mutation	Trial	Effectiveness
	MDX-1106 (BMS-93558 or ONO-4538)	PD-1	Not specified	Phase Ib (Topalian et al., 2012) NCT00730639 (ongoing study) Phase I NCT01621490 (recruiting participants) Phase I NCT01176474 (recruiting participants) Phase III NCT01721772 (recruiting participants)	CRR = 28% for 1 year PFS at 24 weeks = 41% N/A N/A N/A
	MK-3475	PD-1	Not specified	Phase I (Hamid, 2012) NCT01295827 (recruiting participants)	RR = 51% CR = 9%
	BMS-936559	PD-L1	Not specified	Phase I (Brahmer et al., 2012) NCT00729664 (recruiting participants)	OR = 17% PFS at 24 weeks = 42%

PR, partial response; RR, response rate; CR, complete response; OR, overall response; PFS, progression-free survival; OS, overall survival; OSR, overall survival rate; SD, stable disease; N/A, data not available.

selective $BRAF^{V600E}$ inhibitors Vemurafenib (commonly known as PLX4032, RG7204, or Zelboraf) and GSK2118436 (Dabrafenib) have demonstrated significant anti-tumor activity (Anforth et al., 2012; Falchook et al., 2012b; Long et al., 2012).

Vemurafenib inhibits the mutant $BRAF^{V600E}$ protein and also has inhibitory actions against other kinases, including CRAF, ARAF, and wild-type BRAF (Bollag et al., 2010). The phase III clinical trial (NCT01006980) compared the effect of oral Vemurafenib treatment (960 mg twice daily) to Dacarbazine (1000 mg/m^2 intravenous every 3 weeks) in a total of 675 metastatic melanoma patients with the $BRAF^{V600E}$ mutation. Response rates of more than 48% were observed in Vemurafenib treated patients compared to a 5% response rate in the Dacarbazine arm. The estimated median PFS (progression-free survival) for Vemurafenib was 5.3 months with an 84% overall survival at 6 months, compared to a median PFS of 1.6 months with a 64% overall survival at 6 months for Dacarbazine (Chapman et al., 2011). As a result of this study, Vemurafenib was approved by the US FDA in August 2011 as a new treatment standard for patients with unresectable or metastatic melanoma with a $BRAF^{V600E}$ mutation (US Food and Drug Administration, 2011).

A separate phase II clinical trial of Vemurafenib treatment for patients with an activating $BRAF^{V600}$ mutation (NCT00949702) included 132 previously treated melanoma patients. Patients were assessed for response rate, duration of the response, and overall survival after Vemurafenib treatment (Sosman et al., 2012). Patients received oral Vemurafenib at a dose of 960 mg twice daily. A complete response was reported in 6% ($n = 8$) of patients and a partial response was achieved in 47% ($n = 62$) of individuals with an overall response rate of 53%. Stable disease was noted in 29% ($n = 38$) of patients, while 14% ($n = 18$) of subjects demonstrated progressive disease. At the time of data analysis, patients demonstrated a median PFS of 6.8 months and an overall survival of 15.9 months (Sosman et al., 2012).

Common adverse events related to Vemurafenib treatment included fatigue, skin rash, joint pain, photosensitivity, nausea, and development of cutaneous squamous cell carcinomas (SCC) or keratoacanthoma (KA). From the 130 patients that presented adverse reactions to Vemurafenib, 34 patients developed a Grade III SSC or KA. More recently, Su et al. (2012a) reported the paradoxical activation of the MAPK pathway by Vemurafenib; Vemurafenib accelerates the growth of pre-existing cancerous lesions (SSC and KA) via upstream MAPK signaling, such as through $HRAS^{Q61L}$ (Su et al., 2012a).

At the 2012 ASCO Annual Meeting, results were reported of the ongoing phase III (BRIM-3) randomized trial (NCT01006980) comparing Vemurafenib with Dacarbazine in previously untreated patients with $BRAF^{V600E}$ melanomas (Chapman et al., 2012). In this trial, a total of 675 patients were randomly assigned to receive either 960 mg of oral Vemurafenib twice daily or Dacarbazine 1000 mg/m^2 intravenously every 3 weeks. The median overall survival with Vemurafenib was 13.2 months compared to 9.6 months with Dacarbazine. The 12-month overall survival rates were 55% for Vemurafenib and 43% for the Dacarbazine patients. The hazard ratio for death was 0.62 in favor of the Vemurafenib patients. This study confirms the finding that a targeted therapy, Vemurafenib, improves overall survival rates for patients relative to treatment with a chemotherapeutic agent, Dacarbazine (Chapman et al., 2012).

Dabrafenib (GSK2118436) is a reversible, potent ATP-competitive inhibitor that blocks $BRAF^{V600E}$ kinase fivefold more effectively that it does CRAF or wild-type BRAF. A phase I dose-escalation trial (NCT00880321) reported active inhibition of melanoma and brain metastases in response to Dabrafenib treatment (Falchook et al., 2012b). A total of 156 patients with metastatic melanoma were involved in the study; 3 of these patients were BRAF wild-type with the other 153 presenting with various $BRAF^{V600}$ mutations. Overall, 47% of metastatic melanoma patients with a $BRAF^{V600E}$ mutation maintained successful treatment for more than 6 months. A partial or complete response to Dabrafenib (dosage of 150 mg twice daily) was also noted in 18 $BRAF^{V600K}$ mutation positive melanoma patients who were

given varied doses of Dabrafenib (100–150 mg either once daily or twice daily). Of these, 39% ($n = 7$) demonstrated a partial response to treatment and 22% ($n = 4$) had a complete response to treatment. The median PFS for eight patients receiving Dabrafenib 150 mg twice daily was 5.6 months. For three patients with wild-type BRAF, PFS was 1.5 months. The PFS for patients presenting complex BRAF mutations (K601 and V600-K601insdelE) was 1.8 months. For $BRAF^{V600E}$ patients who did not respond to treatment, PFS was 4.2 month. This study found Dabrafenib to be an effective inhibitor of mutant $BRAF^{V600E/K}$ in metastatic melanoma patients with brain metastases and other solid tumors (Falchook et al., 2012b).

A follow-up phase II multicenter trial (NCT01266967) was conducted over six countries, with a total enrollment of 172 metastatic melanoma patients with confirmed $BRAF^{V600E}$ ($n = 139$, 81%) or $BRAF^{V600K}$ ($n = 33$, 19%) mutations and a brain metastasis. Patients were divided into two cohorts: cohort A consisted of patients who had not received previous treatment for brain metastases and cohort B, subjects had progressive brain metastases after previous treatment. All patients received 150 mg of oral Dabrafenib twice daily. In both $BRAF^{V600E}$ and $BRAF^{V600K}$ patients, overall survival was greater than 7.8 months. Interestingly, the overall response was lower amongst patients with a $BRAF^{V600K}$ melanoma than it was in $BRAF^{V600E}$ patients. For example, in cohort A intracranial responses were achieved in 39.2% ($n = 29$) of $BRAF^{V600E}$ patients compared to the 6.7% ($n = 1$) response obtained in $BRAF^{V600K}$ melanomas (Long et al., 2012).

An ongoing phase III randomized controlled trial (NCT01227889) reported recently showed an overall improved PFS for patients with $BRAF^{V600E}$ mutant metastatic melanoma treated with Dabrafenib compared with Dacarbazine (Hauschild et al., 2012). A total of 187 patients received Dabrafenib (150 mg twice daily) and 63 patients were given intravenous Dacarbazine (1000 mg/m2 every 3 weeks). The median PFS for the Dabrafenib patients was 5.1 months compared to 2.7 months for the Dacarbazine patients. The complete response rate for the Dabrafenib patients was 3% ($n = 6$) compared with a 2% ($n = 1$) response rate for the Dacarbazine group. A 47% ($n = 87$) partial response rate was reported for the Dabrafenib subjects with a 5% ($n = 3$) partial response rate observed in the Dacarbazine group. As this clinical study is ongoing, the current overall survival hazard ratio reported is 0.61 (95% CI 0.25–1.48) in favor of Dabrafenib (Hauschild et al., 2012) but in contrast to the Vemurafenib phase III trial, in this trial all patients randomized to Dacarbazine were given the opportunity to cross over to Dabrafenib on progression masking any overall survival difference. Interestingly, Dabrafenib treatment showed less phototoxic reactions and proliferative epidermal lesions (SCC and KA) in only 6% of patients, compared to 11% under Vemurafenib treatment. On the other hand, inflammatory syndromes with fever, rare with Vemurafenib (6%), were recorded in 20% of Dabrafenib treated patients (Hauschild et al., 2012; Sosman et al., 2012).

Overall, treatment with Vemurafenib or Dabrafenib confers a survival advantage in metastatic melanoma patients and presents an encouraging treatment option. However, response to these two inhibitors is restricted to only a proportion of melanoma patients. Efforts to treat metastatic melanoma patients with broad spectrum multi-kinase inhibitors, as detailed below, would seem to be more broadly efficacious since they are independent of BRAF activating mutations, but in fact they are less so.

MULTI-KINASE INHIBITORS

RAF multi-kinase inhibitor, *Sorafenib* (BAY 43-9006 or Nexavar), is an oral agent that inhibits many cellular targets including: VEGFR-2, platelet-derived growth factor receptor (PDGFR), c-KIT, FLT-3, CRAF, and BRAF. *In vitro* studies have demonstrated that Sorafenib induces cell cycle arrest and apoptosis in melanoma cell lines via MAPK activity inhibition (Gray-Schopfer et al., 2007). Sorafenib has been granted FDA approval for the treatment of advanced clear-cell renal carcinoma (Wilhelm et al., 2006), based on a randomized trial demonstrating prolonged PFS in patients (Escudier et al., 2007). However, it has demonstrated modest treatment outcomes in patients with advanced melanoma (Eisen et al., 2006; Flaherty, 2006). A recent phase II clinical trial (NCT00119249) confirmed that Sorafenib monotherapy had limited activity in patients with metastatic melanoma regardless of the $BRAF^{V600E}$ mutational status of their tumor tissue (Ott et al., 2010). By contrast, a more recent study of 28 melanoma patients, showed that after 10 months follow-up there was a 21% overall response rate with a median PFS of 3.6 months and a 1-year survival rate of 33% (Pecuchet et al., 2012). Although Sorafenib has not shown increased PFS in melanoma patients there are ongoing clinical trials (NCT01303341, NCT00565968, and NCT01078961) currently recruiting participants which are investigating the effects of Sorafenib in combination with other treatments.

Broad spectrum kinase inhibitors *RAF-265* and *XL281*, known to target ARAF, BRAF, CRAF genes, and VEGFR receptors, have greater effectiveness and modestly improved selectivity for targeting BRAF compared with Sorafenib, in preclinical models and in patients with advanced solid tumors (Venetsanakos et al., 2006; Schwartz et al., 2009). A study using orthotropic implants of metastatic melanoma in mice, showed a 41% response rate, with more than 50% reduction in tumor growth after treatment with RAF-265 (Su et al., 2012b). Since the development of more potent BRAF inhibitors, clinical evaluation of RAF-265 inhibitor as a single-agent treatment for melanoma patients is not a strong focus. There is however, an ongoing phase I/II clinical trial (NCT00304525) evaluating the maximum tolerated dose of RAF-265 as an oral agent in patients with locally advanced or metastatic melanoma. Another ongoing phase Ib study (NCT01352273) is investigating the combination of the MEK inhibitor (MEK162) with RAF-265 in patients with advanced solid tumors harboring $BRAF^{V600E}$ mutations and/or RAS mutations.

It is critical for the field of melanoma therapeutics, to enhance the longevity of the successful responses obtained with BRAF inhibitors. Therefore the focus now is on novel inhibitors designed to target other kinases within the MAPK pathway, for use individually or in combination strategies as additional treatment options.

NRAS INHIBITION

Inhibition of NRAS has proven challenging as its GTPase activity has not allowed for successful design of specific small-molecule

antagonists. RNA (siRNA)-mediated depletion of NRAS in two melanoma cell lines (224 and BL, which harbor a Q61R NRAS mutation) inhibits proliferation and renders cells more sensitive to chemotherapy (Eskandarpour et al., 2005). A single-agent, single-arm phase II trial conducted with metastatic melanoma patients investigated Farnesyltransferase inhibitors (FTI's) which block farnesylation, the key activating post-translational modification of RAS (Sebti, 2005). The outcome of this trial using the FTI *Tipifarnib* (otherwise known as R115777), showed a low response in the first 14 patients which led to early closure of the trial (Gajewski et al., 2006). However, in this trial patients were not selected based on the presence or absence of NRAS mutations.

Due to the absence of successful specific RAS inhibitors for the treatment of melanoma, there are currently no registered clinical trials for the evaluation of NRAS inhibitors. Inhibition of RAS effector pathways would appear to be a more favorable option and investigations of these are underway. The next kinase in the pathway, MEK, has proven to be a more favorable target (Flaherty et al., 2012a).

MEK INHIBITORS

Selumetinib (also known as AZD6244, ARRY-142886, or PD0325901) is a selective non-ATP-competitive inhibitor of the mitogen-activated protein/ERK kinase (MEK1/2) (**Figure 1**) (Davies et al., 2007). A successful early phase I trial with Selumetinib, opened the door for MEK inhibitors to be considered as efficacious for patients with metastatic cancer (Lorusso et al., 2005). In this phase I study, the BRAF status of most patients was unknown. However, two cases with known $BRAF^{V600E}$ and one with an NRAS (not specified) mutation, displayed a positive response to treatment (Lorusso et al., 2005; Davies et al., 2007). A later phase II single-agent trial compared Selumetinib to Temozolomide. In this study of 100 genetically tested patients, 67 were BRAF and 24 were NRAS positive patients. Only six patients (five of them BRAF positive) receiving Selumetinib showed an 11% response rate. It is unclear why this trial showed such low response rates in patients and did not show a significant PFS rate relative to Temozolomide (Dummer et al., 2008). However a currently recruiting, phase II clinical trial (NCT01519427) will be investigating the efficacy of a combination of Selumetinib and the AKT inhibitor MK2206, for BRAF positive stage III and/or IV melanoma patients who had previously relapsed whilst on Vemurafenib or Dabrafenib treatment.

MEK162 (also referred to as ARRY-162 or ARRY-438162) is a selective ATP-non-competitive inhibitor of MEK1/2 which inhibits the MEK protein as well as ERK phosphorylation in numerous cancer cell lines (Roberts and Der, 2007; Yeh et al., 2007). The phase I study of orally administered MEK162 in 28 patients with biliary tract cancer showed the drug was well tolerated and had clinical efficacy in patients. An 8% ($n = 2$ of 26 patients) overall response rate was observed in this study population. One patient was reported to have a complete response with a PFS of 8.1 months and another subject had a partial response to treatment with a PFS of 9.8 months. Overall 46% ($n = 12$) of patients had stable disease outcomes (Finn et al., 2012). Due to the overall positive response to treatment reported in this study, a phase II clinical trial (NCT01320085) investigating the safety and efficacy of MEK162 in patients with advanced or unresectable metastatic malignant melanoma, harboring $BRAF^{V600}$ or NRAS mutations, is currently underway.

Trametinib (known as GSK1120212 or JTP-74057) is a selective oral MEK1/2 inhibitor which mediates blockage of the MAPK kinase MEK protein. Trametinib has been associated with improved PFS and overall survival in patients harboring $BRAF^{V600E/K}$ mutations (Falchook et al., 2012a; Flaherty et al., 2012b). In a phase II trial (NCT01037127), patients harboring $BRAF^{V600E/K}$ mutant melanoma were given 2 mg of oral Trametinib once daily. Of the patients who were previously treated with BRAF inhibitors ($n = 40$), 3% had complete response, 25% stable disease, and the median PFS was 1.8 months. By contrast, patients who previously received chemotherapy ($n = 57$), 4% had complete responses, 30% had partial responses, and 47% stable disease. This minimal activity observed in patients previously treated with BRAF inhibitors suggests that BRAF resistant mechanisms may also confer resistance to MEK inhibitor monotherapy (Kim et al., 2011).

Following this trial, an ongoing phase III randomized trial (NCT01245062) was initiated to investigate the efficacy of Trametinib compared to chemotherapy in patients with $BRAF^{V600E/K}$ advanced or metastatic melanoma. Of the 322 enrolled patients, 214 received Trametinib while 108 received chemotherapy. At the time of analysis the confirmed overall response rate was 24% in the Trametinib patients and 7% in the chemotherapy group. A median PFS of 4.8 months for the Trametinib patients compared to 1.4 months for the chemotherapy patients was reported. The hazard ratio of overall survival was 0.53 (95% CI 0.30–0.94; $p = 0.0181$), favoring the Trametinib subjects. Frequent adverse events in the Trametinib patients included skin rash, diarrhea, edema, hypertension, and fatigue. This study found that, compared with chemotherapy, Trametinib provided a significant improvement in progression-free and overall survival for patients with metastatic melanoma (Robert et al., 2012).

COMBINATION TARGETED THERAPIES

More recently, greater improvements have been noted in metastatic melanoma patients treated with *combination targeted therapies*, particularity so the combination of BRAF (Dabrafenib) and MEK (Trametinib) inhibitors (Flaherty et al., 2012a). When used as a single-agent Dabrafenib, like Vemurafenib, has shown patients developing resistance after approximately 5–7 months (Falchook et al., 2012b; Hauschild et al., 2012; Long et al., 2012). Therefore the rationale for adding a MEK inhibitor is that it may block the escape route for the BRAF inhibitor and allow continual response and remission in patients. The phase I clinical trial NCT01072175 tested the combination of oral Dabrafenib (150 mg) and Trametinib (2 mg) compared to Dabrafenib (150 mg) alone in 162 patients with metastatic melanoma containing the $BRAF^{V600E/K}$ mutation. The Dabrafenib group ($n = 54$) had a median PFS of 5.8 months compared with a 9.4 month PFS in the combination group ($n = 54$) (Flaherty et al., 2012a). Currently the phase III trial (NCT01682083) is underway in metastatic $BRAF^{V600}$ mutated melanoma patients comparing treatment combinations of Dabrafenib and Trametinib versus Dabrafenib alone. Other clinical trials currently recruiting

patients with advanced or metastatic melanoma using the combination of Trametinib and Dabrafenib include NCT01619774, NCT01584648, NCT 01072175, and NCT01597908.

KIT INHIBITORS

Activating mutations in c-KIT result in stimulation of the MAPK and PI3K-Akt pathways causing increased proliferation and survival advantages (**Figure 1**) (Webster et al., 2006). The c-KIT inhibitor *Sunitinib* is a potent inhibitor of mutant KIT with additional inhibitory effects on VEGF receptors (Chow and Eckhardt, 2007). A recent study conducted by Minor et al. showed that Sunitinib may provide a treatment option for melanoma patients with KIT mutations. Tumor tissues from 90 patients with stage III or IV acral, mucosal or cumulative sun-damaged melanoma were collected. The tumor tissues were sequenced for KIT, BRAF, NRAS, and GNAQ mutant genes and patients with amplification or overexpression of KIT were treated with Sunitinib. Of the melanoma patients treated with Sunitinib, 11% had mutations in KIT [other patients presented with mutations in BRAF (23%), NRAS (14%), or GNAQ (0%)]. Patients positive for KIT mutations ($n = 4$; exon 11; W557G, V559G, or L576P) showed varied responses to the treatment. One patient had complete remission for 15 months, while two patients demonstrated partial responses for 1 and 7 months respectively (Minor et al., 2012). A clinical trial (NCT00859326) is now in progress investigating the efficacy of a combination of Sunitinib and Temozolomide (an oral, cytotoxic chemotherapy agent) for the treatment of metastatic and unresectable malignant melanoma patients.

Imatinib or Imatinib mesylate (also known as ST1571, Gleevec, or Glivec) is a receptor protein kinase inhibitor targeting Abl, c-KIT, and the PDGFR (Fecher et al., 2007; Stuart and Sellers, 2009). In two phase II trials in patients with metastatic melanoma, Imatinib has shown no response and poor survival outcomes in 16 and 25 patients, respectively (Ugurel et al., 2005; Wyman et al., 2006). In contrast, a case report revealed that Imatinib may be an effective treatment, since in one patient with a c-KIT mutation in exon 11, a positive outcome to the treatment was observed (Hodi et al., 2008).

More recently the Imatinib inhibitor has been evaluated as a treatment option in melanoma patients presenting c-KIT mutations (Carvajal et al., 2011; Guo et al., 2011). The phase II trial, in 46 metastatic melanoma patients with c-KIT mutations or amplifications, demonstrated an overall response rate of 23.3%. All patients received a continuous dose of 400 mg of Imatinib, unless toxicities or disease progression occurred. Fifteen patients who experienced reoccurrence were given an increased dose of 800 mg per day. The median PFS for the 46 patients was 3.5 months, with a 6-month PFS rate of 36.6%, and an overall 1-year survival rate of 51%. The overall rate of disease control was 53.5%. This study found that Imatinib increased the overall PFS rate, response rate, and overall survival rate in patients presenting c-KIT mutations in exon 11 and 13. However, patients who had increased doses of Imatinib did not show improvements in disease control (Guo et al., 2011). Ongoing, is the phase II clinical trial (NCT00470470), investigating Imatinib in patients with unresectable stage III or IV melanoma harboring somatic alterations of c-KIT.

Nilotinib (also known as AMN107) is a second generation tyrosine kinase inhibitor known to inhibit KIT, PDGFR, and Bcr-Abl. It was approved by the FDA in 2010 for the treatment of chronic myeloid leukemia (CML) and has a similar target profile to Imatinib (Manley et al., 2010). A phase I clinical trial demonstrated that Nilotinib activity is safe and effective in CML resistant to treatment with Imatinib (Kantarjian et al., 2006) and a major clinical response was observed to Imatinib in KIT-mutated metastatic rectal melanoma (Hodi et al., 2008). A current clinical trial (NCT01168050) is examining Nilotinib as a first or second line treatment of primary melanoma, stage III unresectable, or stage IV melanomas with c-KIT mutations or amplifications (NILOMEL). Another clinical trial (NCT01099514) will also be investigating Nilotinib in metastatic melanoma with KIT aberrations.

Dasatinib (also known as Bosulif, Sprycel, or BMS-354825) is a tyrosine kinase inhibitor responsible for inhibiting src family kinases (c-src, yes, lck, and fyn), Bcr-Abl, c-KIT, PDGFβ receptor, and EPHA2 (Lombardo et al., 2004). Dasatinib was approved by the FDA for CML and gastrointestinal stromal tumors (GIST) (von Mehren, 2006; Pavlu and Marin, 2009). A recent single-arm phase II study of Dasatinib recruited 17 patients with advanced melanoma. The objective response rate was 5% with evidence of tumor regression after only four cycles of therapy ($n = 5$). The median PFS was 8 weeks. This study revealed that Dasatinib had limited activity in patients with advanced or unresectable melanoma and did not meet the pre-specified response rate (30%) or the 6-month PFS (Kluger et al., 2011). However a clinical trial (NCT01092728) is currently recruiting participants to investigate Dasatinib monotherapy in patients with acral lentiginous mucosal or chronic sun-damaged cutaneous melanoma.

mTOR INHIBITORS

The therapeutic value of targeting the PI3K/AKT pathway in melanoma has not been as clearly elucidated as it has been for the MAPK pathway. However, it is clear that an active cross-talk between these two pathways supports the development of melanoma and leads to resistance to BRAF inhibitors. Due to the lack of PI3K and AKT inhibitors currently available for clinical trial evaluations in melanoma, attention has turned to mTOR for which several inhibitors are under development.

Temsirolimus, an mTOR inhibitor (also known as CCI-779), is an analog of Sirolimus (rapamycin) that has demonstrated immunosuppressive activity against melanoma in preclinical models and revealed benefits in patients with breast and renal carcinoma (Hidalgo and Rowinsky, 2000; Huang and Houghton, 2003; Lu et al., 2003). By contrast, an early study demonstrated that Temsirolimus activity resulted in poor clinical responses and limited disease PFS rates in metastatic melanoma patients (Margolin et al., 2005).

While this mTOR inhibitor study diminishes the therapeutic value of targeting the PI3K pathway in melanoma, preclinical evidence has shown, however, that co-targeting this pathway along with the MAPK pathway remains an important therapeutic option (Meier et al., 2007). For example, both PI3K and mTOR inhibitors have revealed synergistic responses when used in combination therapies with Sorafenib or MEK inhibitors (Molhoek et al.,

2005; Meier et al., 2007; Lasithiotakis et al., 2008; Chappell et al., 2011). Interestingly the same response has not been generated with BRAF inhibitors (Meier et al., 2005; Molhoek et al., 2005). Current phase I/II clinical trials (NCT00281957, NCT01614301, and NCT01565837) investigating combination treatments which include Sorafenib, MEK inhibitors, chemotherapy agents, and stereotactic ablative radiation therapy along with Temsirolimus in patients with metastatic melanoma or advanced cancers are underway.

Another mTOR inhibitor *Everolimus* (also known as RAD001) is currently being investigated in patients with metastatic melanoma in the clinical trial NCT00976573, in which the chemotherapeutic agents (Carboplatin and Paclitaxel) and Bevacizumab are used with Everolimus. Another phase II study (NCT00521001) investigated the combination of Everolimus (10 mg daily, for 5 of 7 days) and Temozolomide (200 mg/m^2 1–5 days, every 28 days) in patients ($n = 48$) with stage IV metastatic melanoma. From the 48 patients, 8% ($n = 4$) achieved a partial response, the median PFS was 2.4 months and the overall survival was 8.6 months. The combination of Everolimus and Temozolomide did not offer a therapeutic advantage over Temozolomide alone (Dronca et al., 2010). However, a recent phase I study investigating the combination of Everolimus with Capecitabine in patients with advanced solid malignancies demonstrated a prolonged clinical benefit for 39% of patients (Deenen et al., 2012). Currently two clinical trials (NCT01252251 and NCT00976573) are investigating the therapeutic benefit of Everolimus treatment plus chemotherapy in patients with melanoma.

From the studies detailed above, it is clear that current and future clinical trials will focus on implementing several combination targeted therapies for melanoma patients in the hope of increasing survival rates and minimizing tumor regression. Since improved survival rates have been demonstrated in patients with advanced melanoma, particularly for Vemurafenib and Dabrafenib, trials are underway to develop novel inhibitors that target several genes within the MAPK pathways, as these can be used in combination targeted therapies with the hope of prolonging PFS. However this strategy is for patients with BRAF/NRAS/MEK mutations only. For patients with mutations in alternate pathways (PI3K and AKT) alternate therapies are required. The lack of efficacy to date, when alternate pathways are targeted may imply that combination treatments that also target the MAPK pathways, such as BRAF or MEK inhibitors together with an mTOR inhibitor, are required to prolong PFS and to prevent escape mutations. An alternate therapeutic option is immunotherapy, which is proving to be efficacious (Wilmott et al., 2012).

IMMUNOTHERAPY THERAPIES

Ipilimumab (also known as Yervoy, MDX-010, or BMS-734016) a monoclonal antibody to the T-lymphocyte associated antigen 4 (CTLA-4) was approved by the US FDA in March 2011 and it is currently implemented as a treatment option for patients with stage III and IV metastatic melanoma. CTLA-4 is member of the immunoglobulin receptor family essential for the development of regulatory T-cells. Signaling through this molecule induces an inhibitory response that abrogates the cytotoxic response of the T-cells. Blocking this inhibitory signaling allows the tumor infiltrating lymphocytes to attack the tumors cells.

A phase III clinical trial (NCT00094653) reported by Hodi et al. (2010) demonstrated an improved survival rate in patients with unresectable stage III and IV melanoma. These patients received Ipilimumab either alone ($n = 102$) or in combination with the glycoprotein 100 peptide vaccine (gp100) ($n = 403$) (Hodi et al., 2010). In another phase III trial (NCT00324155) investigating Ipilimumab in combination with Dacarbazine for patients with previously untreated metastatic melanoma overall survival rates were 47.3% for 1-year, 28.5% for 2-years, and 20.8% for 3-years (Robert et al., 2011). This study demonstrated a slight improvement in the overall survival responses for patients who received Ipilimumab-plus Dacarbazine compared with patients who had received Ipilimumab-plus the gp100 vaccine. Di Giacomo et al. (2012) have reported on a more recent phase II clinical trial (NCT01654692) which assessed the combination of Ipilimumab and Fotemustine in patients with advanced, unresectable stage III or IV melanoma. A total of 46.5% ($n = 40$) of the study population maintained a stable disease within 12 months and a median PFS of 5 months (Di Giacomo et al., 2012). More than 50% ($n = 10$) of patients with brain metastases survived longer than 12 months, compared to approximately 20% survival reported for patients undergoing radiotherapy or surgery (Eigentler et al., 2011). Currently a phase I/II clinical trial (NCT01400451) combining BRAF targeted therapy (Vemurafenib) with immunotherapy (Ipilimumab) is underway in subjects with BRAF$^{V600E/K}$ metastatic melanoma as a strategy to prolong PFS.

Other immunotherapeutic agents currently being tested are antibodies that interfere with the PD-1 (programed death-1) and PD-L1 (PD-1 ligand). PD-1 is a key immune co-inhibitory receptor expressed by activated T-cells which mediate immunosuppression. The primary function of PD-1 is in peripheral tissue where T-cells encounter immunosuppressive ligands PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also referred to as B7-DC or CD273) which are expressed by tumor and/or stromal cells (Dong et al., 1999; Menzies et al., 2012; Topalian et al., 2012). Anti-PD-1 antibodies interfere with the interactions between PD-1 and PD-L1 allowing the T-cells to attack the tumor cells (Iwai et al., 2002; Dong et al., 2003). The anti-PD-1 inhibitor monoclonal antibody *MDX-1106* (also referred to as BMS-936558 and ONO-4538) showed favorable preliminary evidence when administrated as a single-agent in a pilot study involving 39 patients with advanced solid tumors (Brahmer et al., 2010). In another study amongst participants with melanoma ($n = 94$), 28% ($n = 26$) had objective responses, lasting for 1 year or more (Topalian et al., 2012). Various trials are underway comparing the clinical benefit and overall survival after treatment with this anti-PD-1 antibody (NCT01621490, NCT01176474, and NCT01721772). *MK-3475* is another anti-PD-1 inhibitor which is being investigated in a phase I clinical trial (NCT01295827). Encouraging anti-tumor activity was reported at the recent Society for Melanoma Research Congress in November 2012 (Hamid, 2012). Objective anti-tumor responses were recorded in 51% ($n = 43$) of 85 patients analyzed to date. Of those 9% ($n = 8$) of patients demonstrated a complete response to MK-3475. Furthermore, a study conducted by Brahmer et al. (2012) has shown that the anti-PD-L1 antibody

BMS-936559 provides durable tumor responses in patients with advanced cancer including melanoma. These results validate the interaction between PD-1 and PD-L1 as an important target for therapeutic intervention in melanoma patients.

In general the anti-PD-1 and anti-PD-L1 treatments have achieved the highest rate of anti-tumor activity reported for an immunotherapeutic agent in the past 30 years (Ribas, 2012). Together with Ipilimumab, these immunotherapeutic agents have demonstrated an increased durability of the tumor response (Hodi et al., 2010; Brahmer et al., 2012; Topalian et al., 2012). Their low response rate compared to targeted therapies such as the $BRAF^{V600E}$ inhibitors support their use in combination therapies. With two different modes of action, combination therapies that together target both cellular proliferation and immune response might provide enhanced inhibition of the spread of melanoma, and may overcome the development of drug resistance.

RESISTANCE TO BRAF INHIBITORS AND COMBINATION THERAPIES

Although there have been encouraging results with targeted BRAF inhibitors, such as Vemurafenib and Dabrafenib (Hauschild et al., 2012; Sosman et al., 2012), almost all patients on these therapies develop drug resistance after the initial response, leading to clinical relapse. The underlying reasons for the development of drug resistance can be found in the redundancy of molecular and cellular processes that mediate the development of melanoma (**Figure 1**). Significant efforts have been dedicated to the study of acquired resistance to BRAF inhibitors. Results from various groups indicate that resistance to BRAF inhibition can be attributed to a series of heterogeneous mechanisms that lead to the reactivation of the MAPK pathway. These mechanisms of reactivation include upregulation of NRAS through activating mutations (Q61K/R) (Nazarian et al., 2010), overexpression of COT/Tp12 by increased copy number of the *MAP3K8* locus (Johannessen et al., 2010), activation of MEK1 by mutation C121S (Wagle et al., 2011), alternative BRAF splicing (Poulikakos et al., 2011), or $BRAF^{V600E}$ gene amplification (Shi et al., 2012b). Alternative, resistance is achieved by the activation of PI3K-AKT and RAS-CRAF-MEK pathways through receptor tyrosine kinase (RTK) signaling. Such activation includes, overexpression of platelet-derived growth factor β (PDGFβ) (Nazarian et al., 2010; Shi et al., 2012b) and activation of IGF1R (Villanueva et al., 2010). Interestingly, all these escape mechanisms are largely mutually exclusive and differ between patients and in some cases between tumors within a patient (Nazarian et al., 2010; Shi et al., 2012b).

MEK MUTATION

Wagle et al. (2011) profiled tumors sensitive and resistant to BRAF inhibitors by massive parallel sequencing and identified the reactivation of the MAPK pathway by a newly identified mutation, $MEK1^{C121S}$. $MEK1^{C121S}$ also confers cross-resistance to the MEK inhibitor Selumetinib. However, this mutation has not been observed in any other studies of Vemurafenib-resistant tumors since then (Shi et al., 2012b). On the other hand, commonly found MEK exon 3 activating mutations such as MEK^{P124S} and MEK^{I111S} are shown to not confer Vemurafenib resistance (Shi et al., 2012a). Escape through a MEK activating mutation is therefore unusual and in contrast to most other mechanisms of acquired drug resistance, where the activation emerges downstream of the targeted kinase (Wagle et al., 2011).

NRAS MUTATIONS

Nazarian et al. demonstrated that acquired resistance to Vemurafenib developed in melanoma cell lines and patient tumors by the acquisition of NRAS mutations. Interestingly, two biopsies from the same patient had two different activating NRAS mutations ($NRAS^{Q61R}$ and $NRAS^{Q61K}$) (Nazarian et al., 2010). More recently Shi et al. (2012b) reported that 5 of 15 patients with disease progression after responding to Vemurafenib, carried NRAS mutations. The NRAS mutated cells were sensitive to the MEK inhibitor, Selumetinib, in the presence or absence of Vemurafenib, suggesting that reactivation of the MAPK pathway might have occurred via CRAF bypassing the BRAF inhibition. This was later confirmed by re-sensitization of a cell line ($NRAS^{Q61K}$/$BRAF^{V600E}$) to Vemurafenib by knocking down CRAF expression (Shi et al., 2012b).

COT OVEREXPRESSION

Through the screening of an "open reading frame" expression library encoding approximately 75% of the human kinases, Johannessen et al. (2010) identified that overexpression of COT/Tpl2 and CRAF reduced sensitivity to BRAF inhibitor PLX4720 (a preclinical version of Vemurafenib). Moreover, increased COT transcript levels were observed in two biopsies collected during Vemurafenib treatment and compared to lesion-matched pretreatment biopsies. Furthermore, high levels of COT expression were related to an increased copy number of the *MAP3K8* locus in two cell lines resistant to PLX4720. Over-activation of MEK in the melanoma cell line A375 through COT signaling resulted in resistance to the MEK inhibitors Selumetinib and CI-1040. Nevertheless, the authors found that co-inhibition of both BRAF and MEK can overcome resistance to BRAF inhibitors caused by increased COT levels.

REACTIVATION OF BRAF

Contrary to intuition, no compensatory BRAF mutations have been found as a mechanism of resistance to BRAF inhibitors. However, reactivation of tumor progression after response to BRAF inhibitors can be achieved by tumor cells with an increased copy number of $BRAF^{V600E}$. Indeed, Shi et al. (2012b) demonstrated that 20% of melanoma patients treated with BRAF inhibitors (Vemurafenib and Dabrafenib) showed an increase in genomic copy number of $BRAF^{V600E}$ and $BRAF^{V600E}$ amplification resulted in $BRAF^{V600E}$ overexpression in tumors of melanoma patients whose cancer had progressed after initial response. Cell lines with $BRAF^{V600E}$ gene amplification, thus resistant to BRAF inhibitors, remained sensitive to Selumetinib, with Vemurafenib and Selumetinib combination therapy producing a synergistic effect.

Poulikakos et al. identified $BRAF^{V600E}$ splicing variants which lack a RAS-binding domain (RBD) in two cell lines. These cell lines displayed acquired resistance to Vemurafenib, that could not be explained by mechanisms previously described (Poulikakos et al., 2011). The observed truncated form of BRAF (p61BRAF) was

the result of an in-frame deletion of exons 4–8. While the mechanism underlying this exon skipping phenomena remains to be identified, exons 4–8 encode domains essential for RAF activation, including the RBD and the cysteine-rich domain (CRD) (Wellbrock et al., 2004). The truncated BRAF lacking the RBD is able to dimerize independently of RAS signaling. Introduction of a mutation that abolishes p61BRAF dimerization restored sensitivity to Vemurafenib. Confirming this as a mechanism of resistance, BRAF variants lacking the RBDs were found in 6 of the 19 patients undergoing Vemurafenib treatment (Poulikakos et al., 2011), while Shi et al. (2012b) reported the same mechanism in another two patients. P61BRAF^{V600E} expressing cells remained sensitive to the MEK inhibitor Selumetinib. It is possible that these mechanisms of resistance may benefit from dose-escalation of the BRAF inhibitor, such as Dabrafenib, for which, the maximum tolerated dose has not yet been determined.

RTK ACTIVATION

In addition to the above mechanisms of acquired resistance to BRAF inhibitors, RTK overexpression or activation has been shown to bypass mutant BRAF and reactivate ERK through CRAF-MEK or via ERK independent mechanisms by activating the PI3K/AKT pathways. Upregulation of PDGFRβ and EGFR were demonstrated to mediate resistance to Vemurafenib developed in melanoma cell lines by Nazarian et al. (2010). In particular PDGFRβ displayed increased activation associated with tyrosine phosphorylation. Moreover the authors found that 4 of 11 post-relapse biopsies from melanoma patients treated with Vemurafenib showed increased expression of PDGFRβ in comparison to pre-treatment biopsies. The same increase was also observed in three relapse tumor biopsies from a patient treated with Dabrafenib (Shi et al., 2012b).

Platelet-derived growth factor receptor β knockdown by RNAi in resistant cell lines led to re-sensitization of the growth inhibition by Vemurafenib, but did not activate the apoptotic response (Nazarian et al., 2010). Thus, PDGFRβ overexpression might not be the only mechanism of resistance in these cells. Moreover, the PDGFRβ inhibitor Imatinib or the MEK inhibitor Selumetinib did not restore sensitivity to Vemurafenib (Shi et al., 2011). It is possible that resistance may involve the activation of more than one RTK.

BRAF inhibitor resistance also has been demonstrated to occur via phospho-activation of the RTK, IGF1R, with subsequent downstream activation of the PI3K/AKT pathways (Villanueva et al., 2010). Inhibition of IGF1R led to slower cell survival, but little improvement was observed when added in combination with the BRAF inhibitor. IGFR inhibition diminished pAKT activation, but did not suppress pMEK/pERK activation. Combination IGF1R inhibitor, PPP, with a MEK inhibitor, Trametinib, led to increased apoptosis and decreased cell viability (Villanueva et al., 2010).

Two recent reports showed RTK-mediated resistance to BRAF inhibition in colorectal carcinoma (Corcoran et al., 2012; Prahallad et al., 2012). Both studies showed activation of EGFR and downstream pathways (PI3K/AKT and MEK/ERK). All these studies underscore the role of RTK expression and activation in BRAF inhibitor acquired resistance. Given the redundancy and promiscuity of the RTKs signaling in melanoma cells, RTK reprograming might not effectively halt tumor growth. This leads to a proposition that co-targeting MEK, and the PIK/AKT/mTOR pathway would be a more effective strategy in response to this type of BRAF inhibitor induced resistance (Lo, 2012).

MOLECULAR CHARACTERIZATION OF CIRCULATING TUMOR CELLS FOR PERSONALIZED TREATMENT MONITORING

Targeted cancer therapies are effective in only a proportion of patients. For effective therapy accurate molecular analysis of a patient's tumors is required, as incorrect administration can negatively impact on patient survival. Molecular tools are required that determine which patients are likely to benefit from the therapy and reveal, early during treatment, whether the therapy is effective. The quantification and molecular profiling of circulating tumor cells (CTCs) has been proposed as an aiding methodology for tumor genotyping and for early detection of therapy efficacy.

Several studies have investigated the value of detecting CTCs in melanoma patients by multimarker RT-PCR to predict response to therapeutic regimens with mixed outcomes. Reynolds et al. (2003) observed that therapy with a polyvalent melanoma vaccine was associated with clearance of melanoma cell markers (tyrosinase, gp100, MART-1, and MAGE-3) from the circulation and improved prognosis. Monitoring of CTCs by expression of five melanoma-associated biomarkers (*MART-1*, *GalNAc-T*, *PAX-3*, *MAGE-A3*, and *MITF*) in patients receiving biochemotherapy and maintenance biotherapy for stage IV melanoma suggests that CTCs detection may be useful for predicting therapeutic efficacy and disease outcome (Koyanagi et al., 2010; Reid et al., 2013). In a multivariate analysis, pre-treatment and serial CTC positivity (*MART-1*, *MAGE-A3*, and *PAX-3 RT-PCR*) was significantly associated with disease-free survival and overall survival (Hoshimoto et al., 2012) (NCT00052156). However, Fusi et al. (2012) reported that although CTCs positivity (*Mart-1 and tyrosinase*) was time dependant prognostic factor, it was not predictive of treatment outcome. Overall, CTC quantification using RT-PCR has been deemed prone to false positive results and the lack of validated and standardized methodologies has preclude its use as a biomarker in clinical trials (Nezos et al., 2009).

Several methodologies have been developed for cytometric detection of CTCs. At the fore front of these is the CellSearch system. Using this platform CTCs have been detected in cancer patients at both early and late stages, with the number of tumor cells in peripheral blood showing significant utility for prognosis in breast, colorectal, prostate, and non-small-cell lung cancers (Cristofanilli et al., 2004; Cohen et al., 2008; de Bono et al., 2008; Krebs et al., 2011). More recently, Khoja et al. demonstrated that CTCs were detectable in 40% of patients with advance cutaneous melanoma and the number of CTCs was prognostic for overall survival. They also showed preliminary evidence that changes in the number of CTCs during treatment may reflect outcome (Khoja et al., 2012). Currently additional trials are underway investigating the prognostic and predictive value of CTCs to identify responding patients treated with Ipilimumab (NCT01565837), Imatinib (NCT00470470), Everolimus (NCT00976573), and BRAF^{V600E} inhibitors (NCT01573494).

Circulating tumor cells not only constitute seeds for metastases and indicate the spread of the disease, but they also reflect the tumors within a patient, thus genetic changes in tumors could be readily detected in CTCs. Thus, CTCs could constitute an accessible sample with which to analyze the genetic profile of the tumors in a particular individual and possibly better represent the mutation status of all the tumors within a patient than a single biopsy. The detection of the $BRAF^{V600E}$ mutation in CTCs isolated from melanoma patients has been previously reported (Kitago et al., 2009; Freeman et al., 2012). A recent report by Sakaizawa et al. (2012) successfully identified BRAF and KIT activating mutations at a single cell level in CTCs from patients with melanoma. Another study also showed the detection of $BRAF^{V600E}$ in CTCs with a 91% (19/21) correspondence with the matched tumor tissue. Moreover, in one of those individuals CTCs were shown to bear the $BRAF^{V600E}$ mutation while this was not present at the tissue level, again suggesting that the CTCs reflect the heterogeneity of the tumors (Fusi et al., 2011). This is consistent with previous observations of intra- and inter-tumor heterogeneity of BRAF mutation status in melanoma (Sensi et al., 2006; Yancovitz et al., 2012). Inter- and intra-tumor heterogeneity have been identified in several tumor types and it has been shown to affect responses to targeted therapies in GIST and lung cancer (Liegl et al., 2008; Taniguchi et al., 2008). Given the diverse clinical responses of melanoma patients to BRAF inhibitors, studies on the association between tumor heterogeneity and clinical outcome are needed. In this context, CTCs could constitute an accessible sample with which to analyze the genetic profile of the tumors in a particular individual and possibly better represent the mutation status of all the tumors within a patient than a single biopsy.

Molecular characterization of CTCs for personalized treatment monitoring has been demonstrated in other tumors besides melanoma. For example Maheswaran et al. (2008) described a successful molecular analysis of CTCs from patients with metastatic non-small-cell lung cancer. The drug resistance mutation T790M was detected in CTCs collected from patients with EGFR mutations that had received tyrosine kinase inhibitors Gefitinib (Iressa) or Erlotinib (Tarceva). The presence of the mutation correlated with reduced PFS from 16.5 to 7.7 months ($p < 0.001$). This result supports the idea of monitoring changes in tumor genotypes during the course of treatment, by genotyping CTCs. Similarly, the presence of KRAS mutations in EGFR-positive colorectal cancer partially explains why these tumors do not respond to anti-EGFR mAb Cetuximab (Erbitux). Molecular analysis of the primary tumor determines the suitability of this targeted therapy, however discordances in the KRAS mutational status between the primary and metastatic tumors have been reported in a small subset of patients with metastatic colorectal cancer (Artale et al., 2008; Italiano et al., 2010). This could explain the observed resistance in some patients despite having a wild-type KRAS primary tumor. Yang et al. (2010) detected the KRAS mutation in blood CTCs and suggested that the blood might be a better sample to assess the tumor genotype for treatment decisions.

Chromosomal amplification of androgen receptor (AR), rearrangement of ERG gene, PTEN deletion, and MYC amplification were detected in CTCs from patients with metastatic prostate cancer by FISH (Attard et al., 2009; Leversha et al., 2009). Moreover, Attard and colleagues demonstrated that CTCs, metastases and prostate tissue invariably had the same ERG gene status in therapy-naive prostate cancer patients. However, significant heterogeneity of AR copy number gain and PTEN loss were observed in CTCs, illustrating the heterogeneity of the tumors and the representation of this diversity in CTCs.

Altogether these observations support CTCs as a superior sample with which to examine the genetic profile of the sum of the patient's tumors and may therefore be useful for monitoring the development of escape mutations during treatment. Nevertheless, prior studies that isolate and analyze CTCs are limited in that they concentrate on methodologies that utilize only one or two surface proteins, gene deletions, amplifications, or point mutations. More comprehensive studies are required that determine the extent to which CTCs represent the parental tumors. The rapid progress in next generation sequencing and onco-proteomics will enable in the near future, better characterization of CTCs. Hopefully this will uncover more informative biomarkers with which to select CTCs and thus provide more specific information about patients who will benefit from targeted treatments as well as improve evaluation of therapeutic responses.

In parallel, improvements in the methodologies used to isolate and quantify CTCs are needed. Different methodologies that bias toward different tumor cell subsets might not reflect the overall tumor(s) heterogeneity. Issues such as collective migration (microemboli), epithelial-mesenchymal transition (EMT), and metastatic potential of the CTCs still need to be addressed in the context of well designed clinical trials with highly sensitive molecular analyses to determine which procedures provide the best prediction of clinical treatment outcomes. It is likely that this will be different for different cancer types and therapeutic interventions. The use of CTCs as a companion to treatments is a valuable tool that should be evaluated as part of therapy clinical trials to facilitate a swift implementation into clinical practice.

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Comparison of responses of human melanoma cell lines to MEK and BRAF inhibitors

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The *NRAS* and *BRAF* genes are frequently mutated in melanoma, suggesting that the NRAS-BRAF-MEK-ERK signaling pathway is an important target for therapy. Two classes of drugs, one targeting activated BRAF and one targeting MEK, are currently undergoing clinical evaluation. We have analysed the *NRAS* and *BRAF* mutational status of a series of 44 early passage lines developed from New Zealand patients with metastatic melanoma. 41% of the lines analysed had *BRAF* mutations, 23% had *NRAS* mutations, and 36% had neither. We then determined IC_{50} values (drug concentrations for 50% growth inhibition) for CI-1040, a commonly used inhibitor of MEK kinase; trametinib, a clinical agent targeting MEK kinase; and vemurafenib, an inhibitor of mutant BRAF kinase. Cell lines with activating *BRAF* mutations were significantly more sensitive to vemurafenib than lines with *NRAS* mutations or lines lacking either mutation ($p < 0.001$). IC_{50} values for CI-1040 and trametinib were strongly correlated ($r = 0.98$) with trametinib showing ~100-fold greater potency. Cell lines sensitive to vemurafenib were also sensitive to CI-1040 and trametinib, but there was no relationship between IC_{50} values and *NRAS* mutation status. A small number of lines lacking a *BRAF* mutation were sensitive to CI-1040 but resistant to vemurafenib. We used western blotting to investigate the effect on ERK phosphorylation of CI-1040 in four lines, of vemurafenib in two lines and of trametinib in two lines. The results support the view that MEK inhibitors might be combined with BRAF inhibitors in the treatment of melanomas with activated *BRAF*. The high sensitivity to trametinib of some lines with wildtype *BRAF* status also suggests that MEK inhibitors could have a therapeutic effect against some melanomas as single agents.

Keywords: mitogen-activated protein kinase pathway, melanoma treatment, NRAS, BRAF, MEK, ERK, vemurafenib, trametinib

INTRODUCTION

Malignant melanoma is an important public health issue, particularly in Australia and New Zealand where the incidence rates for melanoma are very high (Coory et al., 2006; Liang et al., 2010). While early stage melanoma can usually be treated successfully by surgery, metastatic melanoma has a poor survival rate and is highly resistant to conventional cytotoxic chemotherapy. Activating mutations in the *BRAF* gene have been reported in 40–70% of melanomas and activating mutations in the *NRAS* gene in another 10–30% (Davies et al., 2002). There is considerable interest in developing therapies targeting this pathway, and clinical trials of drugs such as vemurafenib (PLX4032), which target mutant BRAF protein, have provided very promising results with 81% of patients with *BRAF* mutant melanoma having clinical responses in a Phase I trial (Flaherty et al., 2010). Since preclinical studies indicate that BRAF inhibitors are ineffective in melanomas lacking *BRAF* mutations and may even enhance growth (Hatzivassiliou et al., 2012), advanced clinical trials of vemurafenib and other BRAF inhibitors are being carried out specifically in patients whose melanomas contain *BRAF* mutations (Solit et al., 2006; Flaherty et al., 2010).

Resistance to BRAF inhibitors develops relatively rapidly because of BRAF-independent activation of MEK and ERK (Johannessen et al., 2010) and other chemotherapeutic approaches will be necessary, both for melanomas lacking mutant *BRAF* and for melanomas that have developed resistance. The MEK protein, which functions downstream from BRAF, is thus a further potential target (Johannessen et al., 2010). Preclinical studies with MEK inhibitors reported that *BRAF* mutant melanoma cells growing both *in vitro* and *in vivo* as xenografts were more responsive to MEK inhibition than cell lines with wild type *BRAF* status (Davies et al., 2002; Solit et al., 2006). Furthermore, the new MEK inhibitor trametinib (GSK1120212) has shown evidence of clinical efficacy against melanoma (Falchook et al., 2012), and has shown survival benefits in phase III trial (Flaherty et al., 2012).

In this study, we have characterized the *BRAF* and *NRAS* mutation status of a series of melanoma cell lines developed from New Zealand patients with metastatic melanoma (Marshall et al., 1994; Charters et al., 2011; Kim et al., 2012). We determined the IC_{50} values of these cell lines to CI-1040, a MEK inhibitor that has been utilized extensively in preclinical studies (Sebolt-Leopold,

2004) and compared these values to those for the mutant BRAF inhibitor vemurafenib. For a subset of cell lines we determined IC_{50} values for trametinib. Since rapid development of resistance (within hours) through up-regulation of MEK pathway signaling in the absence of *BRAF* mutations has been reported in melanoma cell lines (Friday et al., 2008), we have also measured in some cell lines the time-dependent effects of CI-1040 and vemurafenib on ERK phosphorylation.

MATERIALS AND METHODS

CELL LINES AND TISSUE CULTURE

New Zealand Melanoma (NZM) cell lines were derived from metastatic tumors and developed at the Auckland Cancer Society Research Centre, New Zealand. The cell lines were maintained in α-MEM medium (Invitrogen), supplemented with 5% foetal calf serum (Invitrogen), penicillin-streptomycin sulfate, and insulin-transferrin-selenite, in a 37°C incubator at 5% CO_2 and O_2. The final concentrations of the supplements in media were 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite.

GENOMIC PROFILING OF CELL LINES

DNA from cell lines was sequenced for activating mutations in *NRAS* exon 2 and 3 and *BRAF* exon 11 and 15. DNA was extracted using phenol-chloroform-isoamyl alcohol. Exons of interest were amplified by PCR using Taq polymerase from Qiagen. The primer sequences for *BRAF* exon 15 and *NRAS* exon 2 and 3 were designed using DNA Star; the sequences are provided in **Table 1**. The primers for *BRAF* exon 11 are from a published source (Davies et al., 2002). The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 30 cycles (*BRAF* exon 11) or 40 cycles (*BRAF* exon 15, *NRAS* exon 2 and 3) consisting of denaturation at 95°C for 1 min, annealing at the appropriate temperature for 1 min, extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The annealing temperatures for the PCR reactions were as follows: 60°C for *BRAF* exon 11, 56°C for *BRAF* exon 15, 58°C for *NRAS* exon 2, and 60°C for *NRAS* exon 3. Polyethylene glycol precipitation (Lis and Schleif, 1975) was used to purify the *NRAS* exon 2 and 3 and *BRAF* exon 15 PCR products. Enzymatic digestion of unused PCR reaction ingredients by exonuclease 1 Affymetrix USB and shrimp alkaline phosphatase Affymetrix USB was used to purify *BRAF* exon 11.

The PCR products were sequenced using thermal cycle sequencing, with Big Dye Terminator 3.1 chemistry (Applied Biosystems). The sequencing cycle conditions were as follows: an initial denaturation step at 95°C for 5 min followed by 25 amplification cycles of 1 min each of denaturation at 95°C, annealing at 50°C for 5 min, and primer extension at 60°C for 4 min. The sequencing products were purified by ethanol precipitation and the sequences run on an Applied Biosystems 3130XL capillary sequencing machine at the Centre for Genomics and Proteomics, University of Auckland. Mutations were confirmed by sequencing in the opposite direction using separately amplified DNA.

DETERMINATION OF IC_{50} VALUES

The sensitivity of the cell lines to inhibitors was measured using a ^{3}H-thymidine incorporation method (Marshall et al., 1992). Melanoma cells were plated in 96 well plates at 1000 cells per well and incubated overnight at 37°C at 5% CO_2 and O_2. Drugs were added and plates incubated for 5 days at 37°C at 5% CO_2 and O_2. ^{3}H-thymidine (0.04 μCi/well), 5-fluorodeoxyuridine (0.1 μM), and thymidine (0.1 μM) were added 6 h before harvesting the cultures. To harvest, Pronase (2 mg/mL in 4 mM EDTA in PBS) was added per well for 1 h and the plates incubated at 37°C at 5% CO_2 and O_2, to detach the cells. The cells were transferred onto Wallac glass fiber filter mats using a Tomtec cell harvester, and the beta emission counted using a Wallac Trilux Microbeta scintillation counter. IC_{50} values (mean and SEM) were calculated using SigmaPlot.

WESTERN BLOT ANALYSIS

Cells were plated in 6 well tissue culture plates (Falcon) at 2.5×10^5 cells per well and incubated overnight at 37°C at 5% O_2 to allow the cells to attach. Drugs were added to the wells on the following day and the cells were harvested at the indicated time points using a lysis buffer containing phosphatase and protease inhibitors (Cheng et al., 2004). The protein concentration of cell lysates was determined using the bicinchoninic acid (BCA) assay and the lysates (50 μg of protein per well) were subjected to western blotting. The proteins were transferred to PVDF membranes and probed with antibodies for

Table 1 | BRAF and NRAS sequencing primers.

Gene and exon	Primers	Primer sequence	Amplicon size	Location on reference sequence
BRAF exon 11	Forward	Davies et al. (2002)	271 bp	140481587-140481567
	Reverse	Davies et al. (2002)		140481275-140481298
BRAF exon 15	Forward	CACCTCATCCTAACACATTTCAAG	765 bp	140453433-140453410
	Reverse	TTTCAACAGGGTACACAGAACAT		140452668-140452690
NRAS exon 2	Forward	ATTAATCCGGTGTTTTTGCGTTCT	633 bp	115258944-115258921
	Reverse	CATCTCTGAATCCTTTATCTCCAT		115258311-115258334
NRAS exon 3	Forward	AACAGCACAAATAAAACAGTCCAG	799 bp	115256971-115256948
	Reverse	GGTTCCAAGTCATTCCCAGTA		115256172-115256192

The reference sequences cited are NC_000007.13 (BRAF) and NC_000001.10 (NRAS).

Table 2 | Genetic and IC_{50} data for NZM cell lines.

Cell line	BRAF status	BRAF DNA	NRAS status	NRAS DNA	CI-1040 IC_{50} (nM)	Trametinib IC_{50} (nM)	Vemurafenib IC_{50} (nM)
NZM1	wildtype	WT	wildtype	WT	<7.8		1600
NZM2	wildtype	WT	wildtype	WT	8.7	0.48	150
NZM3	V600E	GTG to GAG 600	wildtype	WT	36		29
NZM4	V600E	GTG to GAG 600	wildtype	WT	33	0.36	17
NZM5	wildtype	WT	wildtype	WT	16	0.84	255
NZM6	V600E	GTG to GAG 600	wildtype	WT	65		59
NZM7	V600E	GTG to GAG 600	wildtype	WT	38	0.85	33
NZM9	wildtype	WT	wildtype	WT	72		1600
NZM10	wildtype	WT	Q61K	CAA to AAA 61	23	0.63	2500
NZM11	V600E	GTG to GAG 600	wildtype	WT	120		15
NZM13	wildtype	WT	wildtype	WT	1070		
NZM14	V600K	GTG to AAG 600	wildtype	WT	10	0.33	85
NZM15	wildtype	WT	Q61K	CAA to AAA 61	<7.8		1050
NZM17	wildtype	WT	Q61K	CAA to AAA 61	430		2000
NZM19	wildtype	WT	wildtype	WT	102		1600
NZM20	V600E	GTG to GAG 600	wildtype	WT	9.1	0.30	13
NZM21	wildtype	WT	wildtype	WT	101	0.75	
NZM22	wildtype	WT	wildtype	WT	1410	10	1030
NZM23	wildtype	WT	wildtype	WT	740		1040
NZM24	wildtype	WT	G12D	GGT to GAT 12	21		760
NZM28	G469A L584F	GGA to GCA 469 CTT to TTT 584	wildtype	WT	8.6		3.3
NZM29	wildtype	WT	wildtype	WT	710		900
NZM30	V600E	GTG to GAG 600	wildtype	WT	22	0.35	66
NZM31	V600E	GTG to GAG 600	wildtype	WT	17		47
NZM33	wildtype	WT	Q61R	CAA to CGA 61	<7.8	0.36	2300
NZM34	V600E	GTG to GAG 600	wildtype	WT	64		72
NZM35	wildtype	WT	wildtype	WT	520	2.3	1040
NZM36	wildtype	WT	wildtype	WT	8.5		2000
NZM37	Ins T600	Ins ACA 600	wildtype	WT	19		400
NZM38	V600E	GTG to GAG 600	wildtype	WT	99		55
NZM39	wildtype	WT	wildtype	WT	<7.8	0.35	1300
NZM40	wildtype	WT	Q61H	CAA to CAT 61	790	5.5	590
NZM41	D594N	TGA to TAA 594	wildtype	WT	200		660
NZM43	V600K	GTG to AAG 600	wildtype	WT	<7.8		170
NZM44	wildtype	WT	wildtype	WT	140		2000
NZM45	wildtype	WT	Q61L	CAA to CTA 61	170		510
NZM46	wildtype	WT	Q61H	CAA to CAT 61	10		140
NZM48	wildtype	WT	Q61K	CAA to AAA 61	34		550
NZM49	V600E	GTG to GAG 600	wildtype	WT	70	0.40	70
NZM55	V600E	GTG to GAG 600	wildtype	WT	28		3.8
NZM56	wildtype	WT	wildtype	WT	90	1.0	590
NZM58	V600E	GTG to GAG 600	wildtype	WT	67	0.33	25
NZM61	wildtype	WT	wildtype	WT	90	0.75	560
NZM63	wildtype	WT	G13L	GGT to CGT 13	<7.8	0.31	920

p-ERK, total ERK, p-MEK, total MEK, p-AKT, total AKT, cyclin D1 (all from Cell Signaling Technology), tubulin (Sigma) and β-actin (Millipore). The western blots were photographed using a LAS3000 Luminescent Image Analyzer (Fuji), and quantified using Image J software.

RESULTS

BRAF AND *NRAS* MUTATIONS IN MELANOMA CELL LINES

Screening results for the 44 melanoma cell lines are shown in **Table 2**. Thirteen lines (30%) had activating V600E and another 2 lines (5%) had activating V600K mutations. The NZM28 line

contained a L584F amino acid substitution as well as a G469A substitution, the NZM41 line contained a D594N mutation, and the NZM37 had a Thr600ins mutation. The cell lines were also evaluated for mutations of the *NRAS* gene; four lines (9%) had a Q61K mutation, one a G12D mutation, one a G13L mutation, two a Q61H mutation, and one a Q61R mutation. All the identified mutations are described in the Welcome Trust COSMIC DNA mutation database.

SENSITIVITY OF MELANOMA LINES TO CI-1040, VEMURAFENIB AND TRAMETINIB

The response of the melanoma cell lines to the MEK and BRAF inhibitors was tested using IC_{50} assays and the results are shown in **Table 2** and **Figure 1**. The main study, with CI-1040 (**Figure 1A**), showed a clustering of low IC_{50} values for CI-1040 and vemurafenib for cell lines with activating *BRAF* mutations (V600E and V600K). The NZM28 cell line, which contained both G469A and L584F substitutions was very sensitive to both inhibitors and thus fell into this cluster. On the other hand NZM37, with a Thr600 insertion, and NZM41, with a D594N substitution, were relatively insensitive to vemurafenib (**Table 2**). Lines with *NRAS* mutations (Q61K, G12D, Q61H, and Q61R) were all resistant to vemurafenib and there was no correlation between the presence of mutation and sensitivity to CI-1040. A smaller study (**Figure 1B**) compared cell line sensitivity to trametinib. IC_{50} values for trametinib were highly correlated with those for CI-1040 ($r = 0.985$) but trametinib was, on average, more than 100-fold more potent. Clustering of IC_{50} values was again observed, with all vemurafenib sensitive lines also showing sensitivity to trametinib.

MODULATION OF ERK PHOSPHORYLATION IN RESPONSE TO MEK AND BRAF INHIBITORS

In order to compare signaling changes in the ERK pathway to inhibition of proliferation, we measured changes to ERK phosphorylation induced by CI-1040, trametinib, and vemurafenib in NZM22, which is *NRAS* and *BRAF* wildtype and relatively resistant to all three inhibitors (**Table 2**), and in NZM4, which is BRAF mutant and relatively sensitive to the three inhibitors tested. ERK phosphorylation was more sensitive in NZM4 cells than in NZM22 cells in response to both CI-1040 and vemurafenib at both the 1-h and 24-h time points (**Figure 2**). Comparison of sensitivity to trametinib was also carried out but both cell lines were sensitive to the lowest drug concentration used.

ERK phosphorylation in response to CI-1040 was measured for NZM41, which is moderately resistant ($IC_{50} = 200$ nM). The phosphorylation status of MEK, which phosphorylates and

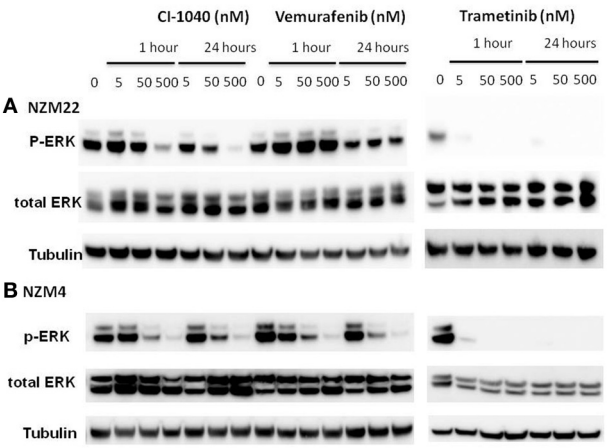

FIGURE 2 | Western blots showing changes in ERK phosphorylation 1 and 24 h after addition of different concentrations of the MEK inhibitors CI-1040 and trametinib, and the mutant BRAF inhibitor vemurafenib. (A) NZM22 line (BRAF wild type). **(B)** NZM4 line (V600E BRAF).

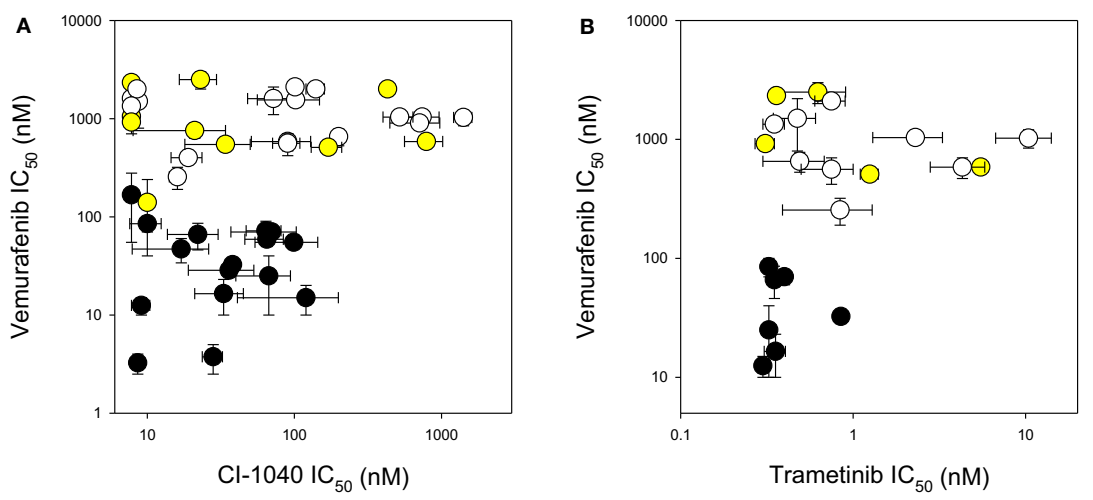

FIGURE 1 | Comparison of IC_{50} values for (A) CI-1040 vs. vemurafenib and (B) trametinib vs. vemurafenib using a panel of melanoma cell lines. Black circles: mutant BRAF. Yellow circles: mutant NRAS. White circles: wildtype for BRAF and NRAS. Vertical and horizontal bars indicate the standard errors of the means where available; IC_{50} values of <7.8 nM are shown as 7.8 nM.

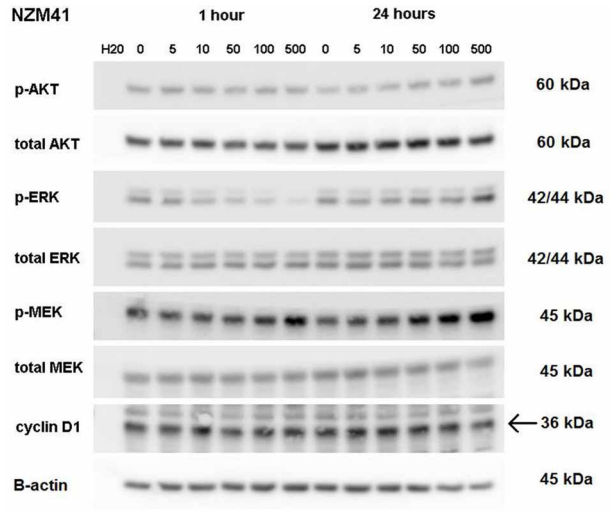

FIGURE 3 | Western blots showing pathway signaling in response to CI-1040 (nanomolar concentrations) for the NZM41 line (BRAF D549N mutation) at 1 and 24 h. The arrow indicates the protein of interest in blots where non-specific bands are also present.

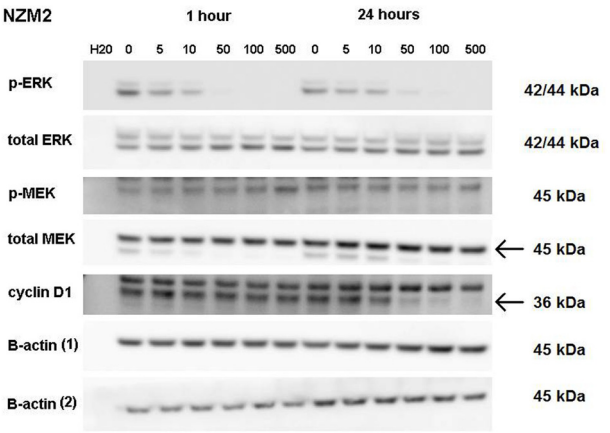

FIGURE 4 | Western blots showing pathway signaling in response to CI-1040 (nanomolar concentrations) for the NZM2 line (BRAF wildtype) at 1 and 24 h. The arrows indicate the protein of interest in blots where non-specific bands are also present.

activates ERK, was measured for comparison. Since expression of cyclin D1 has been reported to be down-regulated following MEK inhibition in cells with BRAF V600E mutations (Pritchard et al., 2007), expression of cyclin D1 was also measured, but there was no change in expression. ERK phosphorylation was inhibited at a CI-1040 concentration of 10 nM after 1 h but was comparatively unaffected after 24 h, even at 500 nM (**Figure 3**). This is in agreement with a report that sensitivity to a MEK inhibitor may decrease with exposure time (Friday et al., 2008). Interestingly, NZM41 showed evidence of CI-1040 resistance since MEK phosphorylation was increased following exposure to CI-1040 at 500 nM after 1 h and even at 50 nM after 24 h (**Figure 3**). The experiment was repeated with the NZM2 line, which is sensitive to CI-1040 (IC_{50} = 8.7 nM) and wildtype for *BRAF* and *NRAS*. ERK phosphorylation was highly sensitive to CI-1040 at both the 1-h and 24-h time points (**Figure 4**). No changes in MEK phosphorylation was observed but a decrease in cyclin D1 expression was apparent after 24 h.

DISCUSSION

New Zealand has a high incidence of melanoma and it was therefore of interest to compare the frequencies of activating BRAF and NRAS mutations in New Zealand-derived melanoma lines with published values. The BRAF V600E mutation frequently observed was found in this study at 30% (**Table 2**), lower than that reported by other groups (Davies et al., 2002; Houben et al., 2004; Edlundh-Rose et al., 2006; Thomas et al., 2007) while that for V600K was 5%. The mutation frequency for NRAS was 23% (**Table 2**), within the range reported by other groups (Davies et al., 2002; Houben et al., 2004; Edlundh-Rose et al., 2006; Thomas et al., 2007). The data in **Figure 1** and **Table 2** clearly show that cell lines with activating V600E and V600K mutations were generally sensitive to CI-1040, trametinib and vemurafenib inhibition. As shown in **Table 2** the NZM28 cell line, which contained both G469A and L584F substitutions, was very sensitive to both inhibitors. SIFT algorithm analysis (Kumar et al., 2009) was undertaken to provide an indication of the effect of mutation, and predicted that the L584F mutation alters protein function, consistent with this effect. On the other hand NZM37, with a Thr600 insertion and NZM41, with a D594N substitution, were relatively insensitive to vemurafenib (**Table 2**), raising the question of why they might be selected for during melanoma development. The G469A mutation has been reported to have no enhancing effect on BRAF (Smalley and Flaherty, 2009) but it has been reported that kinase-dead BRAF mutations of D594 can have an indirect effect on tumor progression by enhancing CRAF activity (Heidorn et al., 2010). Several other studies have explored the relationship between mutation status and sensitivity to MEK inhibition for a variety of tumor types including melanoma, breast, ovarian, and lung cancers (Davies et al., 2002; Solit et al., 2006). In these studies, cell lines with *BRAF* mutations were very sensitive to MEK inhibition of cell growth while cell lines with *NRAS* mutations showed a range of sensitivities, in agreement with the present results.

It has been reported that either PI3K oncogenic mutations or deletion of PTEN reduces sensitivity of cells to MEK inhibitors (Wee et al., 2009). In this study, the NZM40 and NZM46 lines were found to have an activated mutated PI3K enzyme and the NZM6, NZM30, NZM34, and NZM43 lines were found to lack PTEN expression (Kim et al., 2012). However, there was no clear indication of altered sensitivity to CI-1040 among these cell lines. There are also reports that up-regulation of MEK can lead to reduced sensitivity of cells to MEK inhibitors (Friday et al., 2008). We investigated ERK phosphorylation in a number of melanoma lines (**Figures 2–4**). Although some evidence of loss of initial sensitivity in resistant lines was found (**Figure 3**) the pattern of phosphorylation results broadly followed that of the IC_{50} results.

In conclusion, we have assessed the responses of a series of 44 melanoma lines, generally of low passage number, to CI-1040,

a prototypic MEK inhibitor, as well as to trametinib, a clinical MEK inhibitor and vemurafenib, a clinical BRAF inhibitor. We identified a sub-set of 16 lines (36%) with activating BRAF mutations (**Figure 1**) that showed sensitivity to both clinical inhibitors, supporting the hypothesis that a combination of both BRAF and MEK inhibitors might have advantages over either drug alone because of potentially synergistic inhibitory effects on signal transduction. We also identified a second sub-set of 10 cell lines (23%) that were resistant to vemurafenib but sensitive to a MEK inhibitor. Some but not all of these cell lines exhibited NRAS mutations, suggesting that some melanomas that are wild-type for both BRAF and NRAS may respond to trametinib, a MEK inhibitor. If this applies *in vivo*, then a proportion of melanoma patients whose disease is resistant to BRAF inhibitor therapy may respond to therapy with a MEK inhibitor.

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Lack of *GNAQ* and *GNA11* germ-line mutations in familial melanoma pedigrees with uveal melanoma or blue nevi

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Approximately 10% of melanoma cases are familial, but only 25–40% of familial melanoma cases can be attributed to germ-line mutations in the *CDKN2A* – the most significant high-risk melanoma susceptibility locus identified to date. The pathogenic mutation(s) in most of the remaining familial melanoma pedigrees have not yet been identified. The most common mutations in nevi and sporadic melanoma are found in *BRAF* and *NRAS*, both of which result in constitutive activation of the MAPK pathway. However, these mutations are not found in uveal melanomas or the intradermal melanocytic proliferations known as blue nevi. Rather, multiple studies report a strong association between these lesions and somatic mutations in *Guanine nucleotide-binding protein G(q) subunit alpha* (*GNAQ*), *Guanine nucleotide-binding protein G(q) subunit alpha-11* (*GNA11*), and *BRCA1-associated protein-1* (*BAP1*). Recently, germ-line mutations in BAP1, the gene encoding a tumor suppressing deubiquitinating enzyme, have been associated with predisposition to a variety of cancers including uveal melanoma, but no studies have examined the association of germ-line mutations in *GNAQ* and *GNA11* with uveal melanoma and blue nevi. We have now done so by sequencing exon 5 of both of these genes in 13 unique familial melanoma pedigrees, members of which have had either uveal or cutaneous melanoma and/or blue nevi. Germ-line DNA from a total of 22 individuals was used for sequencing; however no deleterious mutations were detected. Nevertheless, such candidate gene studies and the discovery of novel germ-line mutations associated with an increased MM susceptibility can lead to a better understanding of the pathways involved in melanocyte transformation, formulation of risk assessment, and the development of specific drug therapies.

Keywords: *GNAQ, GNA11*, familial melanoma, germ-line, blue nevi, uveal melanoma

INTRODUCTION

Approximately 10% of melanoma cases are familial (Goldstein and Tucker, 2001). However, only 25–40% of familial melanoma cases can be specifically attributed to pathogenic germ-line mutations in *cyclin-dependant kinase inhibitor 2A* (*CDKN2A/p16*) – the most significant high-risk melanoma susceptibility gene identified to date (Goldstein and Tucker, 2001; Eliason et al., 2006; Leachman et al., 2009). Two other genes, *cyclin-dependant kinase 4* (*CDK4*) and *alternate reading frame* (*ARF*) have been confirmed as additional high penetrance melanoma predisposition genes, but account for less than 5% of hereditary melanoma families worldwide (Leachman et al., 2009). GWAS analyses have identified several additional moderate and low-penetrance melanoma predisposition genes but these contribute a small percentage to the overall genetic risk (Amos et al., 2011). Therefore, the majority of melanoma cases do not carry a known genetic mutation that accounts for their increased risk of melanoma (Hayward, 2003).

The most common mutations in sporadic melanoma are those of *v-Raf murine sarcoma viral oncogene homolog B1* (*BRAF*) and *neuroblastoma RAS viral oncogene homolog* (*NRAS*), both of which result in constitutive activation of the MAPK pathway and subsequent activation of pro-proliferative genes such as *cyclin-D1* (*CCND1*) (Onken et al., 2008). However, these mutations do not characterize all melanocytic neoplasms or intradermal melanocytic proliferations such as uveal melanoma and blue nevi, respectively (Saldanha et al., 2004). Rather, multiple studies have reported a strong association between these melanocytic lesions and somatic *guanine nucleotide-binding protein G(q) subunit alpha* (*GNAQ*), *guanine nucleotide-binding protein G(q) subunit alpha-11* (*GNA11*), and *BRCA1-associated protein-1* (*BAP1*) mutations in the absence of *BRAF*, *NRAS*, and *KIT* mutations (Harbour et al., 2010; Van Raamsdonk et al., 2010). Recently, germ-line mutations in *BAP1*, the gene encoding a tumor suppressing deubiquitinating enzyme, have been associated with predisposition to a variety of cancers including uveal and cutaneous melanoma as well as mesothelioma (Abdel-Rahman et al., 2011; Testa et al., 2011; Harbour, 2012; Wadt et al., 2012), but no studies have examined the association of germ-line mutations in *GNAQ* and *GNA11* with uveal melanoma and blue nevi.

GNAQ (OMIM ID 600998), found on chromosome 9q21, and *GNA11* (OMIM ID 139313), found on chromosome 19p13.3, encode the G-protein α subunit of heterotrimeric GTP-binding proteins and couple to the endothelin B receptor in melanocytes – a required signaling pathway for melanocyte development (Dong et al., 1995; Shin et al., 1999). The *GNAQ* and *GNA11* mutations associated with uveal melanoma and blue nevi occur almost exclusively in exon 5 (most commonly Q209L; **Figure 1**) and involve the glutamine residue within the *ras*-like domain, which plays an essential role in the GTP hydrolysis activity of this gene's protein products (Van Raamsdonk et al., 2008, 2010). Activating *GNAQ* and *GNA11* mutations, such as those at codon 209, lock the GTP-binding protein in their active, GTP-bound state resulting in constitutive activation of the MAPK pathway in the absence of *BRAF* and *NRAS* mutations (Van Raamsdonk et al., 2008). In mice, these activating mutations ultimately function as oncogenes resulting in proliferation of intradermal and transformed melanocytes (Van Raamsdonk et al., 2004, 2008). These mouse studies provide a genetic basis to help explain why intradermal melanocytic proliferations affecting the conjunctiva and periorbital skin (nevi of Ota) are a risk factor for uveal melanoma (Van Raamsdonk et al., 2008). The work of Van Raamsdonk et al. and others suggest that mutations in *GNAQ* and *GNA11* represent an early event in the development of melanocytic tumors and may contribute directly to the increased melanoma risk in hereditary melanoma families that also have an increased incidence of uveal melanoma and/or blue nevi.

We hypothesized that an increased melanoma risk in familial melanoma families with uveal melanoma and/or blue nevi is due to *GNAQ* and *GNA11* germ-line mutations in exon 5 which result in constitutive activation of the MAPK pathway. To test this hypothesis, we investigated the frequency of *GNAQ* and *GNA11* exon 5 germ-line mutations in 22 patients who had a personal history of uveal melanoma and/or blue nevi from a total of 13 unique familial melanoma pedigrees previously identified as being high-risk for the development of melanoma.

MATERIALS AND METHODS

STUDY SUBJECTS

Through the Familial Melanoma Research Clinic at the Huntsman Cancer Institute, we identified 22 study subjects who had a personal history of uveal melanoma and/or blue nevi and were also members of a pedigree with familial melanoma (defined as ≥2 first-degree relatives with a history of melanoma or pancreatic cancer or ≥3 family member with a history of melanoma of any relationship) (Supplementary Material). This study was approved by the University of Utah's Institutional Review Board (IRB# 7616), which also acts as the University's Ethical Review Board.

NUCLEIC ACID ISOLATION AND PCR AMPLIFICATION

From each of the 22 study subjects, archived DNA for genetic analysis was obtained using peripheral whole blood collected in Acid Citrate Dextrose (ACD) Venous Blood Vacuum Collection Tubes. Genomic DNA was isolated using Gentra Puregene Kit (Qiagen Inc.). DNA purity and concentration was determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). PCR amplification of exon 5 of *GNAQ* and *GNA11* was performed using HotStarTaq DNA Polymerase (Qiagen Inc.) with the primers listed in **Table 1**. All PCR primers were designed and purchased from the University of Utah's DNA Sequencing and Genomics Core Facility. For all PCR reactions, 1 μL of genomic DNA [50 ng/μL] was used as a PCR template in 20 μL total reaction volume containing 2 μL 10× PCR Buffer (Denville Scientific, Inc.), 1.6 μL 2.5 mM dNTP Mix (Invitrogen), 1 μL of each forward and reverse primer [10 mM], 13.2 μL H2O, and 0.2 μL Hot-Start Taq (5 U/mL) (Denville Scientific, Inc.). The conditions for PCR amplification were 95°C for 5 min followed by 35 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. Following amplification, 3 μL of product and 1 μL of 1 kb Plus DNA Ladder (Invitrogen) were loaded and run on 1% agarose gels at 100 V for 30 min and DNA bands were visualized on a UV transilluminator after ethidium bromide staining. All PCR products where then purified using the ExoSAP-IT PCR Cleanup Protocol (Affymetrix/USB). PCR products where then purified using the ExoSAP-IT PCR Cleanup Protocol (Affymetrix/USB).

GENETIC ANALYSIS OF STUDY SUBJECTS

The University of Utah's DNA Sequencing and Genomics Core Facility performed sequencing reactions in both directions using Big Dye Terminator chemistry on an ABI Prism 3700 DNA analyzer. Sequences were aligned and analyzed for single

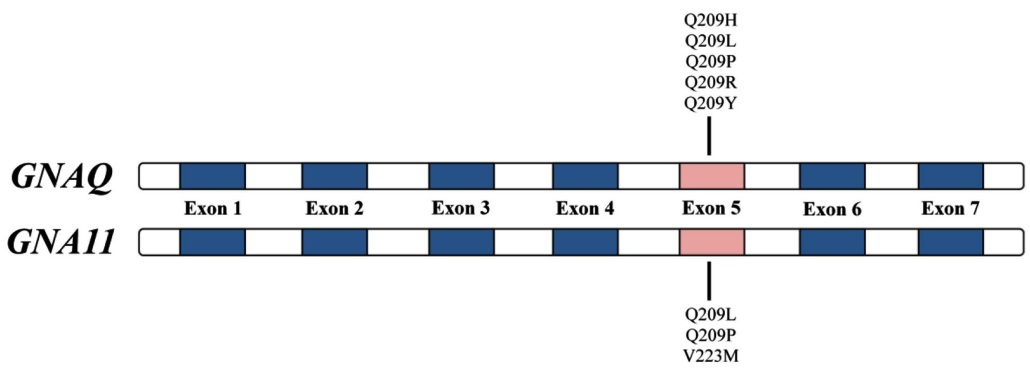

FIGURE 1 | Most common activating exon 5 *GNAQ* and *GNA11* gene mutations.

Table 1 | PCR primers used for the mutation profiling of *GNAQ*, *GNA11*, and *BAP1*.

Gene	Exon	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′
GNAQ	5	TTTCCCTAAGTTTGTAAGTAGTGCT	AAGTTCACTCCATT CCCCAC
GNA11	5	AGCCGATGTCAGTCTGGTGT	AAGGCAGAGGGAAT CAGAGG
BAP1	4	AGTGATGACGCAGTGCAAAG	CTCCATTTCCACTT CCCAAG
	5	TGTCCAGATATGACTGACCTG	ATGTGGTAGCATTCC CAGTG
	6–7	TCTGAAGCTTTGCCTTCCAC	GCCACTGGGTACCA CATACC
	8	TGTCTTCCTTCCCACTCCTG	TGGATACTCTCTGT CCCTCCC
	9	CTCAACCTGATGGCGGG	AATGCAGGGAGGG TTGG
	10	TTCCTTTAGGTCCTCAGCCC	AAAAGACTTTCCCT GTTTAGG
	11	TCTCTGGGAAGTGCTGGTTC	CATGGGAAAATTGC CTGTTG
	12	CCGAGCAGCACTTGTTTG	GATCCGAAGCACCT AGAACC
	13	AGCCATTCTGGGTACTGCTG	GAGTGCAGGACAC TTTGTGG
	15–16	CTGCCTATTGCTCGTGGG	CAAGGTCTGCTCA AGCCTC
	17	ACAGGGAGGGCCATGAG	TACTGGGAAAAGG GGAAGTG

nucleotide polymorphisms and/or mutations with respect to published reference sequences found in the UCSC Genome Browser using Sequencher 4.5 software (Ann Arbor, MI, USA). Analysis of *CDKN2A* was performed by sequencing the 3 exons plus 95 non-coding base pairs of *p16* (Myriad Genetics, Salt Lake City, UT, USA), as well as exon 1-beta which codes for a portion of *p14 ARF* (Gene Dx, Gaithersburg, MD, USA). *CDK4* analysis was carried out by sequencing exon 2 and flanking splice sites (Gene Dx, Gaithersburg, MD, USA).

RESULTS

Of these 22 study subjects (**Table 2**), 14 had a personal history of cutaneous melanoma, 3 had a personal history of uveal melanoma (although there are four pedigrees that have individuals with uveal melanoma), 13 had a personal history of blue nevi, and 5 had a personal history of cutaneous melanoma as well as blue nevi. The 22 study subjects were from a total of 13 unique familial melanoma pedigrees (Supplementary Material). Of the 22 samples studied, all were wild-type at exon 5 for both *GNAQ* and *GNA11*. The results collected from this subset of high-risk melanoma families indicate that the inherited risk observed in these hereditary melanoma families is not due to activating germ-line mutations in exon 5 of *GNAQ* and *GNA11*.

The studied pedigrees were previously determined to lack germ-line mutations in *CDKN2A*, *p16*, *ARF*, and *CDK4*, with the exception of three pedigrees for which sequencing data could not be obtained. These sequencing results are listed in **Table 2**. Additionally, screening for *BAP1* mutations in exons 9 and 12 was performed on all 22 study subjects. Sequencing of exons 4–13 and 15–17 of *BAP1* was performed on Study Subjects 3 (pedigree C) and 7 (pedigree G), both of whom had a personal history of uveal and cutaneous melanoma. In all instances, no *BAP1* mutations were found.

DISCUSSION

Malignant melanoma is a devastating malignancy for which few effective targeted treatments (e.g., *BRAF* inhibitors) are available. The major aim of the current investigation was to determine whether or not germ-line mutations in exon 5 of *GNAQ* and *GNA11* represent an early event in the development of melanocytic tumors and/or potential genetic biomarkers associated with the increased melanoma risk observed in hereditary melanoma families that lack other known pathogenic germ-line mutations. The lack of *GNAQ* and *GNA11* germ-line mutations in familial melanoma pedigrees with an increased incidence of uveal melanoma and blue nevi further is supportive of the importance of sporadic mutations in these genes in blue nevi and uveal melanoma as previously published. Nevertheless, the functional consequence of activating *GNAQ* and *GNA11* mutations on the MAPK pathway highlights an important concept: that specific gene mutations may result in an alternate route of MAPK pathway activation and subsequent melanocyte proliferation in the absence of more common gene mutations such as those in *BRAF*, *NRAS*, and *KIT*.

The major limitation of this study is the small sample size and limited number of familial melanoma pedigrees ($n = 13$) and uveal melanoma cases ($n = 4$). Therefore, it is necessary that further studies be done and our hypothesis be considered in a larger sample size before any final conclusion can be drawn. However, to our knowledge, this is one of the largest studies to date looking specifically at germ-line mutations in familial melanoma pedigrees with uveal melanoma and/or blue nevi. This study is also a retrospective study and is not designed to elucidate the complex interaction between specific gene mutations, phenotype characteristics, and MM susceptibility. Additionally, this study is not a complete survey of all of the genes thought to confer an increased familial melanoma risk and the screening for germ-line mutations in *CDKN2A*, *p16*, *ARF*, *CDK4*, and *BAP1* was incomplete. Subsequent studies are therefore necessary to determine the genetic basis for the increased risk of MM seen in the families included in this study. Finally, our study was limited to exon 5 of *GNAQ* and *GNA11*. It is, however, possible that an activating mutation outside of the *ras*-like domain may be present in the families we studied though this is unlikely given that activating mutations are found almost exclusively in exon 5 as mentioned above (Van Raamsdonk et al., 2008, 2010).

In summary, we report the absence of germ-line mutations in exon 5 of *GNAQ* and *GNA11* in familial melanoma pedigrees with an increased incidence of uveal melanoma and/or blue

Table 2 | Study subject characteristics and summary of sequencing results for *CDKN2A*, *CDK4*, and exon 5 of *GNAQ* and *GNA11*.

Study Subject ID	Pedigree ID	Individual	Individual	Individual	Individual	Individual	Pedigree	Pedigree	Pedigree	Pedigree	Pedigree
		History of cutaneous melanoma	History of uveal melanoma	History of blue nevi	*GNAQ* exon 5 genotyping results	*GNA11* exon 5 genotyping results	Number of cutaneous melanomas	Number of blue nevi	Number of uveal melanoma	*CDKN2A* genotyping results	*CDK4* genotyping results
1	A	Yes	No	Yes	WT	WT	7	1	0	WT	WT
2	B	Yes	No	Yes	WT	WT	17	1	0	WT	WT
3	C	Yes	Yes	No	WT	WT	3	0	1	WT	WT
4	D	Yes	No	Yes	WT	WT	9	1	0	WT	WT
5	E	Yes	No	Yes	WT	WT	42	1	0	WT	WT
6	F	No	No	Yes	WT	WT	4	1	0	WT	WT
7	G	Yes	Yes	No	WT	WT	8	0	1	WT	WT
8	H	No	No	Yes	WT	WT	4	3	1	A148T G > A (p16)	WT
9	H	No	No	Yes	WT	WT	–	–	–	A148T G > A (p16)	WT
10	H	Yes	No	No	WT	WT	–	–	–	A148T G > A (p16)	WT
11	H	No	No	Yes	WT	WT	–	–	–	A148T G > A (p16)	WT
12	H	Yes	No	No	WT	WT	–	–	–	A148T G > A (p16)	WT
13	I	No	No	Yes	WT	WT	3	2	0	Not determined	Not determined
14	J	Yes	No	Yes	WT	WT	6	2	0	Not determined	Not determined
15	J	No	No	Yes	WT	WT	–	–	–	Not determined	Not determined
16	K	No	No	Yes	WT	WT	3	1	0	WT	WT
17	L	No	No	Yes	WT	WT	2	1	0	Not determined	Not determined
18	M	Yes	No	No	WT	WT	5	0	1	WT	WT
19	M	Yes	No	No	WT	WT	–	–	–	WT	WT
20	M	Yes	Yes	No	WT	WT	–	–	–	WT	WT
21	M	Yes	No	No	WT	WT	–	–	–	WT	WT
22	M	Yes	No	No	WT	WT	–	–	–	WT	WT

WT, wild-type.

nevi. Melanoma's high incidence and poor treatment outcomes as well as the high number of familial melanoma cases lacking known pathogenic germ-line mutations, underscores the importance of future studies using a candidate gene approach when phenotypic annotation is available. Such candidate gene studies and the discovery of novel germ-line mutations associated with an increased MM susceptibility can lead to a better understanding of the pathways involved in melanocyte transformation, formulation of risk assessment, and the development of specific drug therapies. Additionally, our study not only shows that our families don't have known genetic mutations accounting for their increased melanoma risk, but also suggests that the genetic cause of familial ocular melanoma and blue nevi is yet to be discovered and that further investigation of these families could lead to identification of new targets for ocular melanoma. Further, a better understanding of the genetic basis observed in the inherited risk associated with familial melanoma may yield insights into the molecular pathogenesis of sporadic melanoma and, ultimately, improved methods of detection and treatment.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Cancer_Genetics/10.3389/fonc.2013.00160/abstract

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Targeted therapy; from advanced melanoma to the adjuvant setting

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Targeted therapy has revolutionized treatment for advanced melanoma. Clinical trials have demonstrated unprecedented survival benefits in advanced melanoma patients treated with Vemurafenib (1, 2). Vemurafenib is a targeted inhibitor that specifically binds to mutant BRAF proteins containing V600E or V600K amino acid substitutions, preventing constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, and resulting in antitumor effects of inhibition of cell proliferation and apoptosis induction (3).

New treatments are evolving rapidly in this area. The FDA has approved two other monotherapeutic drugs, Dabrafenib and Trametinib, which are also inhibitors of growth stimulatory effects of mutant BRAF, or its downstream effector pathways, and these have proven to improve survival rates (4, 5). Moreover, clinical trials are demonstrating further prolonged survival in addition to reduced treatment related toxic side-effects through combinatorial use of several of these targeted drugs (6). However, there is a great downside to targeted therapy in advanced melanoma: in practically all cases, drug resistance inevitably develops, and patient death inexorably follows (2, 6).

Stage IIB-C and stage III melanomas have a lower disease burden than stage IV melanoma, and yet these melanomas are at a significant risk of tumor recurrence following surgical resection (7). Currently there is a high demand for new and effective adjuvant treatments to mitigate the risk of recurrence, and there are a number of adjuvant therapies under investigation for stage IIB-C and stage III patients. The only FDA-approved adjuvant drugs for melanoma are interferon-alpha and pegylated interferon, which marginally improve overall survival (OS) for high-risk recurrent tumors (8). With the progress of targeted therapies in advanced melanoma and the need for better adjuvant drugs, many are now asking whether precision treatment could be used at an earlier stage of melanoma diagnosis in the adjuvant setting, which accounts for the majority of melanoma diagnoses. Indeed, a number of adjuvant clinical trials using targeted therapies for the treatment of stage IIC and stage III melanomas have now been initiated.

Several targeted monotherapies and combination therapies are currently being evaluated for melanoma treatment in the adjuvant setting in both stage IIC and stage III melanomas (clinical trials NCT-01667419, NCT01682083, NCT00553618, NCT01782508, and NCT01682213). However, because the risk of recurrence is less than 100% for these patients, multiple patients would need to be treated for every one patient who would receive benefit from the adjuvant therapy (9). Prognostic biomarkers are therefore needed to predict melanoma recurrence, but to date good prognostic biomarkers that accurately predict the outcome of stage IIB-C or stage III melanomas are lacking.

Prognostic markers determine the risk of tumor recurrence as a result of growth of cancerous cells that have escaped surgical resection, most likely due to metastasis, and as such these cancer cells are undetectable at the time of diagnosis. Indeed, initial presentation of recurrence was local in 10.9%, in transit in 9.9%, involving a regional lymph node in 34.4%, and at a distant site in 44.9% of patients with metastasis (9). Several studies have demonstrated the presence of *BRAF* mutations as a marker of poor prognosis in both the metastatic and locally advanced settings. This is important because targeted therapy could help to eliminate metastasized cells that harbor the *BRAF* mutation.

BRAF mutation predicts shorter OS in stage IV melanoma (10), which is consistent with clinical outcomes of tumor regression upon Vemurafenib administration in advanced melanoma patients; inhibition of a marker that is directly associated with poor prognosis results in prolonged survival. In stage III resected tumors *BRAF* mutations are associated with a significantly shorter DFS and OS (11, 12), and they have been shown to promote metastasis through mechanistic studies, albeit presumably associated with the progression and growth of the metastatic disease rather than the initiation of metastasis (13). Thus, for *BRAF* mutation-positive stage III patients, adjuvant targeted therapy may be of benefit. In contrast, *BRAF* mutation does not appear to have significant impact on prognosis in stage I or stage II melanomas. Numerous studies have shown the *BRAF* mutation does not affect the Disease Free Interval (DFI) or OS after surgical resection of melanomas at these stages (14–16), and thus it does not influence tumor recurrence.

In deciding whether to use a targeted treatment for melanoma in the adjuvant setting, either for stage III or stage IIB-C, it is important to consider whether the tumor cells that avoid surgical resection, presumably due to early metastasis, would continue to harbor the mutation being targeted (e.g., *BRAF*). This can be guided by the observations that most primary melanomas with a *BRAF* mutation have paired secondary lesions also harboring the mutation (17). This may be explained by the fact that *BRAF* mutations are acquired during the early stages of tumor progression, for example during radial to vertical growth phase (18), resulting in a larger portion of the primary tumor with the mutation. Clones that acquire metastatic capability

will most likely therefore possess the *BRAF* mutation and so *BRAF* mutant targeted therapy should work.

As *BRAF* mutations influence tumor growth, it is unlikely that they would confer metastatic capability. For instance, primary tumors with a *BRAF* V600E mutation may frequently be paired with secondary lesions without the mutation (17, 19). Colombino and colleagues found 6 of 44 *BRAF* mutant primary melanoma patients whose primary melanomas were positive for the *BRAF* V600E mutation, yet had a *BRAF* wild-type secondary lesion (17). To explain this, Yancovitz and colleagues showed intratumoral heterogeneity of clones with respect to *BRAF* V600E mutation status, and concluded *BRAF* mutations were not necessary for metastasis (19). Importantly, administration of targeted therapy inhibiting mutant BRAF to *BRAF* wild-type patients has been shown not only to have absence of benefit, but can also cause a growth advantage in those tumor cells by paradoxically stimulating the MAPK pathway (20).

In addition, *BRAF* wild-type primary tumors may be paired with mutant *BRAF* secondary tumors, due to the acquisition of the *BRAF* mutation at the secondary site. These patients would be likely to benefit from adjuvant targeted therapy, with the degree of benefit depending on how early the *BRAF* mutation had occurred during the cellular evolution of the secondary tumor. Mutant *BRAF* might therefore be a useful therapeutic target in the metastatic lesions of stage III melanoma patients for this reason, as compared to those patients with a localized melanoma of stage IIB-C. Mann et al. (12) for example, have shown that *BRAF* mutation status may also be combined with an expression signature to enhance the ability to predict melanoma recurrence.

Although it might be questioned whether patients with stage IIB melanoma should also be included in the adjuvant therapy clinical trials, despite the promising leads mentioned above there is currently not a lot of prognostic information that supports the use of mutant *BRAF* targeted therapy to treat stage III melanomas in the adjuvant setting, and even less information to support the use of *BRAF* targeted therapy to treat stage IIB-C melanomas. While randomized phase III clinical trials are currently recruiting to evaluate the use of *BRAF* targeted therapy for stage IIC and III melanomas in the adjuvant setting as an alternative to interferon drugs, these treatments are not without the potential to develop some or all of the adverse side-effects of the *BRAF* targeted therapies (2). In addition, *BRAF* mutations activate the MAPK pathway, which is associated with increased MITF expression (21). Therefore inhibiting *BRAF* activity in stage II melanomas could lead to repression of both MITF and miR-211 expression in those tumors (see He et al., submitted), and if the environmental signals are conducive, then this could subsequently cause upregulated expression of BRN2, a factor that is thought to be associated with phenotype switching (22), and so induce metastasis.

Based on the points we have outlined above, it is our opinion that treatment of stage II melanoma with BRAF inhibitors in the absence of data from suitable prognostic biomarkers that adequately predict the outcome of stage II melanomas, could lead to adverse outcomes for these patients. It is hoped that these trials will provide insight as to how targeted therapies perform in the adjuvant setting in patients with tumor recurrence. The identification of which patients have a high-risk of recurrence of melanoma requires better biomarkers of tumor progression and prognosis. In addition, new biomarkers of melanoma metastasis are needed, together with the concurrent development of new or existing drugs for use in the adjuvant setting.

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MelanomaDB: a web tool for integrative analysis of melanoma genomic information to identify disease-associated molecular pathways

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Despite on-going research, metastatic melanoma survival rates remain low and treatment options are limited. Researchers can now access a rapidly growing amount of molecular and clinical information about melanoma. This information is becoming difficult to assemble and interpret due to its dispersed nature, yet as it grows it becomes increasingly valuable for understanding melanoma. Integration of this information into a comprehensive resource to aid rational experimental design and patient stratification is needed. As an initial step in this direction, we have assembled a web-accessible melanoma database, MelanomaDB, which incorporates clinical and molecular data from publically available sources, which will be regularly updated as new information becomes available. This database allows complex links to be drawn between many different aspects of melanoma biology: genetic changes (e.g., mutations) in individual melanomas revealed by DNA sequencing, associations between gene expression and patient survival, data concerning drug targets, biomarkers, druggability, and clinical trials, as well as our own statistical analysis of relationships between molecular pathways and clinical parameters that have been produced using these data sets. The database is freely available at http://genesetdb.auckland.ac.nz/melanomadb/about.html. A subset of the information in the database can also be accessed through a freely available web application in the Illumina genomic cloud computing platform BaseSpace at http://www.biomatters.com/apps/melanoma-profiler-for-research. The MelanomaDB database illustrates dysregulation of specific signaling pathways across 310 exome-sequenced melanomas and in individual tumors and identifies the distribution of somatic variants in melanoma. We suggest that MelanomaDB can provide a context in which to interpret the tumor molecular profiles of individual melanoma patients relative to biological information and available drug therapies.

Keywords: melanoma, mutation, molecular pathway, MelanomaDB, gene set analysis, BaseSpace

INTRODUCTION

THE GROWTH AND COMPLEXITY OF MELANOMA GENOMIC DATA

Melanoma researchers are faced with a rapidly growing amount of useful molecular and clinical data, particularly gene expression information. This rapid growth can be illustrated by surveying the Gene Expression Omnibus (GEO) (1), an international repository that contains a large subset of the published gene expression data (**Figure 1**). Largely based on genomic data, our understanding of the genes involved in melanoma progression has advanced from focused investigations of candidate genes to studies on a whole-genome scale (2). The advent of next-generation sequencing (NGS) in particular has opened up a floodgate of data, from the published sequence of the first melanoma genome in the beginning of 2010 (3), to more recent whole-exome studies sequencing more than one hundred tumors (4, 5). Melanoma genomic data is poised to grow rapidly with the advent of large-scale initiatives such as Australia's Melanoma Genome Project[1], melanoma analysis in The Cancer Genome Atlas (TCGA) project[2] as well as the melanoma sequencing projects underway at several individual institutions.

LIMITATIONS OF CURRENT TECHNIQUES

Unfortunately, information pertinent to melanoma exists in a diverse range of formats and locations. For example, relevant data

[1] http://www.melanoma.org.au/research/melanoma-genome-project.html
[2] http://cancergenome.nih.gov

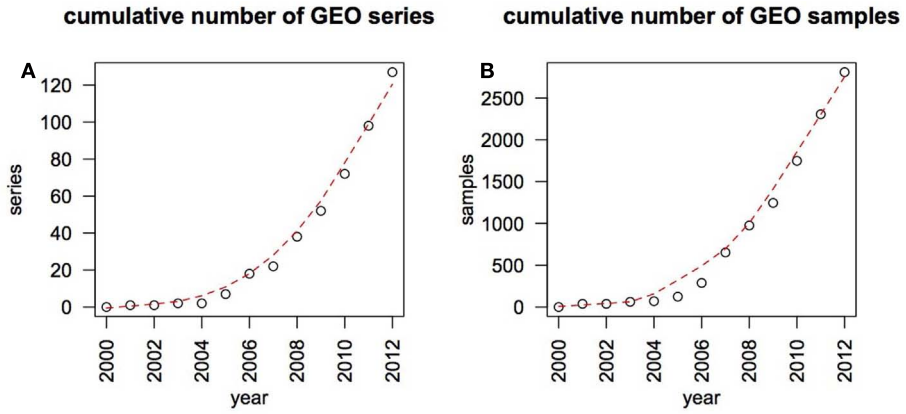

FIGURE 1 | Growth of melanoma genomic data in the GEO database. The GEO database was searched on a year by year basis, using the MESH term "melanoma" and excluding records containing the phrase "cell line." By the end of January 2013 GEO contained 128 data series made up of 2819 samples that match these search criteria. The cumulative number of data series (submitted experiments) **(A)** and individual samples **(B)** are plotted as black circles, overlaid by a red trend line fitted over this data using the loess method.

about a single gene of interest may include information about the encoded protein's structure, cellular location, and function, contribution to molecular pathways, drugs that target the protein, the gene, or protein's utility as a biomarker, genome-wide association studies, mutation frequency, chromosomal aberrations, as well as RNA expression associations with metastasis, treatment response and patient survival, clinical SNP associations, and the results of literature mining. Even within the single data type of tumor DNA sequencing, a variety of methods have been used to implicate genes in melanoma initiation and progression, and these different methods produce data in differing formats. Ideally, all these diverse forms of data could be used by researchers in an integrated fashion to triangulate in on clinically important genes.

As a further challenge, genomic information in melanoma is particularly dense due to the high mutation rate found in melanomas of sun-exposed skin (6). This is likely to be due to both ultraviolet radiation-induced DNA damage and defects in DNA repair mechanisms (3). In addition, sequencing studies suggest that malignant melanoma is a relatively heterogeneous neoplasia with a range of driver mutations (5). Despite its potential value, coherent analysis of melanoma genomic information remains difficult for individual researchers. Data repositories such as Oncomine (7), Ingenuity Pathways Analysis[3], the Catalogue of Somatic Mutations in Cancer (COSMIC) (8, 9), and the Broad Institute's Melanoma Genomics Portal (10) bring together a massive amount of useful melanoma data. However, these disparate resources do not yet enable the full potential of integrated analysis of molecular pathways across different types of data associated with melanoma.

POTENTIAL CLINICAL USE OF MOLECULAR PATHWAY DATA ABOUT INDIVIDUAL TUMORS

Tumor development involves multiple genes encoding proteins and non-coding RNAs operating in molecular pathways. Therefore, inference of molecular pathway activity from tumor genomic data using methods such as gene set analysis (GSA) (11) is useful in oncology (12, 13). Gene sets used for analysis may consist of co-expressed genes downstream of a specific molecular pathway (14) or genes that share common transcription factor binding sites (15). Statistical summaries of these gene sets have been used to infer molecular pathway activity, and these gene sets are frequently conserved across species (16). GSA has identified several molecular pathways associated with melanoma (17, 18), and can be used to identify the putative functional changes caused by the mutation, DNA gain or loss, and/or altered expression of genes in a particular patient's tumor. Popular GSA tools include GATHER (19), DAVID (20), GSEA (21), and GeneSetDB (22).

The number of clinically available targeted therapies for melanoma remains limited compared to the diverse genetic drivers of this tumor. Nevertheless, identification of drugs targeting a small number of melanoma drivers has been a major advance. For example, Vemurafenib targets the Mitogen Activated Protein Kinase (MAPK) pathway molecule BRAF (23). However, Vemurafenib is only indicated in *BRAF* V600E or V600K containing tumors and the majority of treated patients show relatively short term remission, with their relapse almost certainly caused by reactivation of the MAPK pathway, commonly through mutations in *NRAS* or *PDGFRB* (24). We propose that integration of molecular pathway data at both the patient population scale and individual tumor scale could help researchers better understand phenomena such as Vemurafenib resistance, and permit identification of rationally selected combinatorial therapies based on molecular stratification of patients.

EXPERIMENTAL OBJECTIVES

In the work described here, we have amalgamated a diverse range of genomic and clinical melanoma data, on the scales of both patient population and individual tumor into a single resource. This resource is provided as a downloadable file that can be searched and filtered using any spread sheet application. To facilitate use

[3]http://ingenuity.com/products/pathways_analysis.html

of this resource in the context of molecular pathways, we also provide a web-accessible SQL database named MelanomaDB, through which researchers can perform GSA using integrated melanoma data of several types. A subset of the information in the database can also be accessed through a freely available web application in the Illumina genomic cloud computing platform BaseSpace. While other disease-specific databases exist for other cancers such as lung (25) and ovarian (26) cancer, we know of no other database similar to ours dealing with melanoma. Furthermore, we believe that MelanomaDB's breadth across sequence and microarray data, biological and pharmacological gene sets, and pathway information, in addition to its usability and its melanoma focus, make it unique. In this paper, we use information assembled in MelanomaDB in several downstream analyses to demonstrate the utility of this resource for finding relationships between molecular pathways and clinical parameters, including the mutational patterns of members of molecular pathways (27) in individual tumors. We hope this tool will prove increasingly useful as it expands when new tumor data becomes available. In particular, we hope that it will provide a context in which to interpret the tumor molecular profiles of individual melanoma patients.

MATERIALS AND METHODS

OVERVIEW OF THE CONSTRUCTION OF MELANOMA GENE SETS

To facilitate an integrative analysis of melanoma information we combined a variety of melanoma data in the form of gene sets, attempting to collect information for all genes in the genome. These melanoma gene sets were groups of genes that shared biological or clinical relevance for melanoma, derived from five types of publically available information: drug and biomarker information, druggability, literature relationship strength, disease-specific survival, and somatic mutation data. Drug information includes information on compounds and the proteins they target, while Druggability information comprises of estimations of the degree to which proteins are amenable to targeting by drugs, and protein characteristics relevant to this. A detailed description of this information is available in Data Sheet 1 in Supplementary Material.

SOURCES OF SPECIFIC INFORMATION

Further explanations of the gene sets used are in the MelanomaDB help page at http://genesetdb.auckland.ac.nz/melanomadb/help.html

Drug and biomarker information

Drug information was taken from online databases DrugBank version 3 (28), KEGG DRUG (27), Therapeutic Targets Database (29), and ClinicalTrials.gov. Biomarker information was taken from published papers by Gould Rothberg et al. (30), Schramm and Mann (31), Utikal et al. (85), Mehta et al. (32), and from the database KEGG BRITE (27). It should be noted that gene sets such as those derived from DrugBank include all genes encoding proteins to which each drug binds, including both intended and unintended targets. However, metabolising enzymes, transporters and carrier proteins are excluded. For example, targets of the drug Cetuximab include the intended target (the human epidermal growth factor receptor) but also compliment components and Fc receptors, as is expected due to the nature of this drug as an antibody[4]. For further explanations of the gene sets used see the MelanomaDB help page at http://genesetdb.auckland.ac.nz/melanomadb/help.html

Druggability information

Druggability data was sourced from the Sophic Integrated Druggable Genome Database (33), EBI's DrugEBIlity database (34), and published papers by Li and Lai (35) and Tiedemann et al. (36). Data on protein characteristics relevant to druggability were taken from Affymetrix annotations[5], and online databases UniProt Consortium (37), Secreted Protein Database (38), and KinBase (39).

Literature and genomic data relationship strength information

Information on Literature Relationship strength was derived from the IRIDESCENT (40) and GAMMA (41) software packages. IRIDESCENT searches every published MEDLINE abstract for associations between objects, and creates a network of tentative relationships between these objects. Objects encompass genes, diseases, phenotypes, chemical compounds, drugs, and ontology categories. The relative strength of association between two objects is determined by the frequency in which they appear in the same abstract or sentence. Here, this network is used to score the strength of association between genes and the terms "melanoma" or "metastatic melanoma."

GAMMA conducts a meta-analysis of gene expression behavior across 16,000 wide-ranging microarray experiments to identify genes that are consistently and specifically co-expressed across heterogeneous experimental conditions. In this way GAMMA extends the connections in IRIDESCENT's association network to genes without any published associations to melanoma by identifying which of these genes are consistently co-expressed with multiple known melanoma genes. To date, GAMMA has been used successfully to identify phenotypes and/or disease relevance for several previously uncharacterized genes (42–45).

Disease-specific survival data

Strength of statistical associations between RNA abundance and melanoma-specific survival were gathered from several published studies, and from our additional statistical analysis of two published sets of linked microarray and clinical data. Associations between gene expression in melanomas and patient survival were taken directly from John et al. (46), Mandruzzato et al. (47), and Journe et al. (48), and associations between gene expression and metastasis were taken directly from Timar et al. (49). We performed our own analyses on the microarray data of Bogunovic et al. (50) and Jönsson et al. (51) based on patient survival data and Affymetrix CEL files retrieved from GEO. The Bogunovic study's raw Affymetrix data was normalized using RMA normalization performed using the `affy` package in the R statistical software (52). The Illumina data from the Jonsson et al. study was obtained in a normalized format, however, we removed three patients for whom patient survival data was missing, and adjusted all microarray values by adding the minimum value in order to eliminate negative values. R was used to split the patients into two groups,

[4]http://www.drugbank.ca/drugs/DB00002

[5]http://www.affymetrix.com/support/technical/annotationfilesmain.affx

create a survival object for each group and then compare these two survival objects using a Log Rank test. For each probe set this splitting was performed nine times, once at each RNA abundance decile across the patient population. R was also used to fit a Cox proportional hazards regression model for each probe set.

To facilitate the use of these data in exploratory analyses for hypothesis generation, we also generated additional gene sets in which we aggregated several different RNA associations with patient survival to allow broader surveys. For example, four gene sets were identified from the expression and survival data of Bogunovic et al. (50) using different statistical criteria.

Somatic variant data

Multiple studies reporting melanoma variants were collated for use with MelanomaDB. A literature review identified 11 exome sequencing studies suitable for inclusion (4–6, 53–60). In addition, the Cancer Cell Line Encyclopedia (61), and the Sanger Institute's COSMIC (8, 9), and Matched Pair Cancer Cell Lines (3) were searched for mutations detected in melanoma cell lines. In total, we collected data on 58 established melanoma cell lines, 119 primary "short-passage" cell lines, 38 primary tumors, and 96 metastatic melanoma tumors. Non-silent variants were reported in 16,488 genes. With the exception of the 10 samples from the 2010 study of Berger et al. (53), and some of the samples from COSMIC, these samples have all been paired with matched normal samples to ensure that the variants reported are somatic. In the current iteration of this database only non-synonymous coding mutations, indels, splice-site mutations, and structural rearrangements (including gene fusions and read-through transcripts) are included. Synonymous coding mutations are not included. Presently, this somatic variant data includes more than 35,000 non-synonymous coding mutations, and more than 3,500 structural rearrangements and indels. We have not provided this somatic variant data as a supplementary file but instead invite readers to contact us to obtain the links to this data. We do this so we can ensure that access permission and ethical issues associated with this individual patient data are adhered to.

AMALGAMATION OF ALL DATA INTO GENE SETS

To facilitate the construction of gene sets, all data described above was combined into a single matrix, which is available as Data Sheet 2 in Supplementary Material. This matrix is gene-based and uses Entrez Gene ID as a unique index for each gene[6]. Every gene is represented by one row, and each column contains data from a single source. Columns annotating genes with references to other databases were derived from NCBI's Gene database FTP directory[7] and supplemented by Affymetrix annotations (see text footnote 5).

From this data matrix, a number of gene sets were derived. In most cases, columns of the matrix were converted directly into gene sets by including in that set every gene with an entry in that column. In some cases, such as statistical associations between RNA expression and patient survival, a cut-off was required for defining gene set membership. For example, only genes encoding proteins with positive DrugEBIlity ensemble scores were included in the gene set "DrugEBIlity: Positive ensemble scores." A further description of the melanoma gene sets is available in Data Sheet 1 in Supplementary Material.

SQL DATABASE GENERATION

To facilitate access, combination, and filtering of different types of genomic data related to melanoma, and interpretation of this data in terms of molecular pathways and functional categories, the data matrix described above was used to generate a web-accessible SQL database named MelanomaDB. The web interface is implemented using Apache, PHP, Javascript, and HTML. The meta-gene set database GeneSetDB (22) was accessed from within MelanomaDB to identify the intersection between melanoma-specific gene sets and gene sets related to biological functions and molecular pathways. The R framework was used for statistical calculations. GSA was performed using the hyper-geometric distribution to calculate the probability of overrepresentation, followed by multiple testing correction using the Benjamini and Hochberg method (62).

BaseSpace APPLICATION PREPARATION

A subset of the information in MelanomaDB is also included in a freely available Illumina BaseSpace application. This BaseSpace application retrieves a tumor and corresponding normal germ line sequence pair from the BaseSpace archive or the user's own BaseSpace account as vcf files. Then, variants present in the tumor but the not normal germ line tissue of the patient are identified using the Genome Analysis Tool Kit's SelectVariants java tool (63). This list of tumor variant genes is identified. Then, the molecular pathways these genes correspond to, along with any statistically significant pathway enrichment within the list of variant genes and targeting drugs, are retrieved from the GeneSetDB pathway analysis web tool (22). A diagram showing tumor variant genes in the context of molecular pathways is generated using the KEGG, Reactome, and Biocarta pathways included in the R graphite package (64), and a clustered heatmap showing how the genetic variants in the sample tumor compare to variants in the 310 tumors cataloged in MelanomaDB is generated. This clustered heatmap is generated: (i) using a modification of the heatmap.2 function from the R gtools package (see Data Sheet 5 in Supplementary Material) (65), using the "binary" method for distance calculation and the "single" method for clustering and (ii) as a reverse-orientation waterfall plot to illustrate patterns of somatic variant co-occurrence in melanoma.

ASSEMBLY OF INFORMATION FOR INDIVIDUAL TUMORS

From the exome and whole-genome sequencing information assembled above, we constructed a tumor-based matrix in which each row was a gene, each column was an individual tumor and each cell described any somatic variants present in a certain gene for a certain tumor. After duplicated tumors were removed, this somatic variant data included 310 samples, 183, and 72 of which had somatic alterations in the *BRAF* and *NRAS* genes, respectively. When multiple sequenced tumors or cell lines from the same patient were available, the union of somatic variants found in these samples was used. Links to the papers and their supplementary web sites used to construct this tumor-specific somatic variant data is available in Data Sheet 3 in Supplementary Material. The authors

[6]http://www.ncbi.nlm.nih.gov/gene, accessed on 30/7/12
[7]ftp://ftp.ncbi.nih.gov/gene/DATA/

can assist researchers with the precise sources of information used to construct this resource.

VISUALIZATION

The statistical software R was used to construct a clustered heat map of tumor variants for genes included in the KEGG "Melanoma" signalling pathway with a modified heatmap.2 function of the R package "gplots,"[8] using the "binary" method for distance and the "single" method for clustering. R was also used to draw gene network diagrams. Molecular pathways were obtained from the pathways included in the graphite R package[9] and were plotted using the graphite (see text footnote 9) R package.

The R scripts used to generate **Figures 2A–C** as well as the pathway diagrams and heatmaps in **Figures 4–7** are given in Data Sheet 5 in Supplementary Material.

RESULTS AND DISCUSSION

Here we describe the assembly and use of the MelanomaDB database.

ASSEMBLY OF MELANOMA GENOMIC INFORMATION FROM DIVERSE SOURCES INTO A MELANOMA DATA MATRIX

Firstly, a melanoma data matrix (Data Sheet 2 in Supplementary Material) was constructed, with genes (or genomic loci in some cases) as rows. The columns of this matrix represent diverse features of biological functions related to melanoma and are described in Data Sheet 1 in Supplementary Material. This melanoma data matrix can be utilized in a variety of ways. Most simply, researchers can access a variety of data pertaining to their particular gene of interest. The melanoma data matrix can also be manipulated with spread sheet software to sort, find, and filter information in order to generate gene lists useful for hypothesis generation.

ASSEMBLY OF SOMATIC VARIANT INFORMATION FOR MELANOMAS OF INDIVIDUAL PATIENTS

Next, we assembled as much information about somatic variation in individual exome-sequenced and genome-wide-sequenced melanomas as possible. We gathered information about somatic variations in 58 established melanoma cell lines, 119 primary "fresh" cell lines, 38 primary tumors, and 96 metastatic melanoma tumors, which was appended to the information matrix described above (Data Sheet 3 in Supplementary Material, Tab "Tables Used"). Information about non-synonymous coding mutations, structural rearrangements, and indels was included (intronic and synonymous coding mutations were excluded from the current iteration of this data resource). The information contained in Data Sheet 2 in Supplementary Material was read into the statistical environment R and visualized, as described in the Section "Materials and Methods" and Data Sheet 5 in Supplementary Material. Firstly, the distribution of somatic variations for individual genes is shown in **Figure 2A**. The majority of genes showed somatic variations in only small numbers of tumors. Comparison of each gene's total exon length versus the number of tumors with a mutation in that gene using R (**Figure 2B**), revealed a statistically significant but weak correlation between somatic variation frequency and total exon length (Pearson's correlation coefficient = 0.47, $p \leq 0.001$). Although variations in large genes such as *Titan* (*TTN*) have been

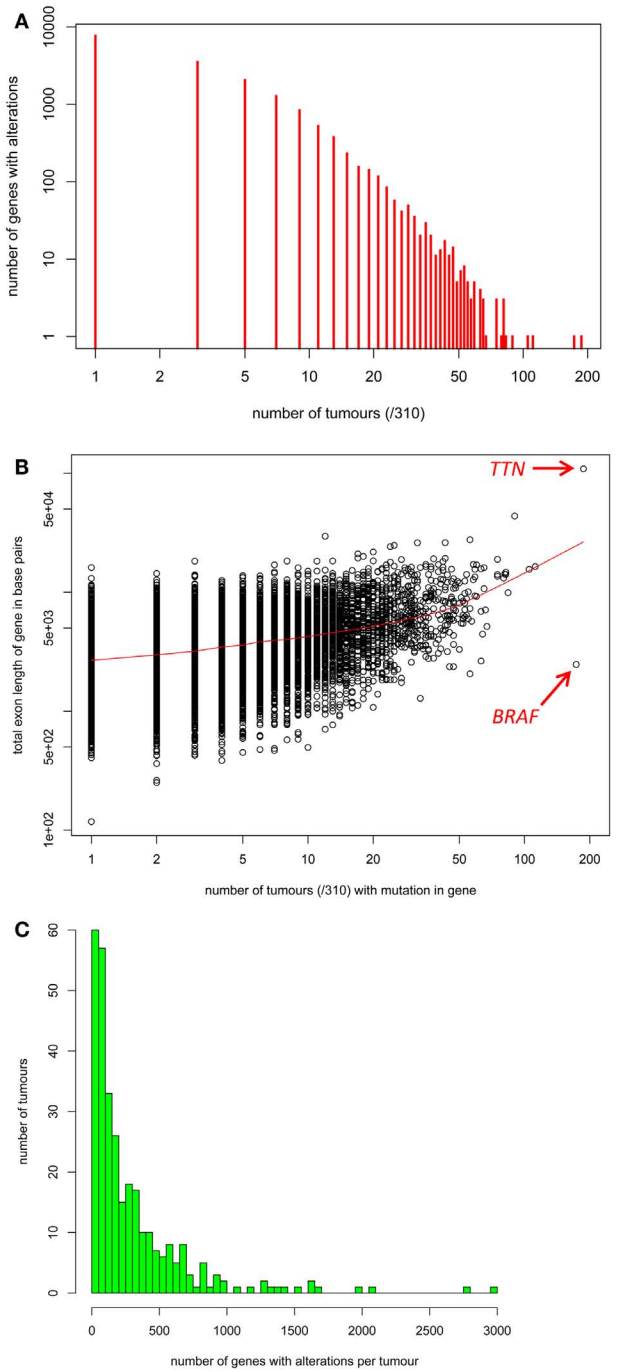

FIGURE 2 | (A) The distribution of the number of tumors with somatic alterations in each individual gene. **(B)** Each gene's total exon length in base pairs (*y*-axis) versus the number of the 310 tumors with a mutation in that gene (*x*-axis). **(C)** The distribution of the number of genes with somatic alterations in each individual tumor.

[8]http://cran.r-project.org/web/packages/gplots/index.html
[9]http://www.bioconductor.org/packages/release/bioc/html/graphite.html

implicated as cancer drivers, these may also occur in so many melanomas due to large gene size increasing the likelihood of passenger mutations. However, the *BRAF* gene clearly stands out as frequently mutated in melanomas despite its moderate length. The distribution of the number of genes with somatic alterations in each individual tumor was performed using R and is shown in **Figure 2C**.

USE OF THE COMBINED MELANOMA INFORMATION

As an example of using the information assembled above, an approach to identifying novel candidate novel drug targets for melanoma using this melanoma data matrix (Data Sheet 2 in Supplementary Material) can be performed by filtering and sorting Data Sheet 2 in Supplementary Material in a spreadsheet application and is described in **Figure 3**.

This process generates a short list of 129 genes that can be examined more closely in order to select a final list of genes that may warrant investigation in the laboratory. A variant on this approach may be to place more weight on particular data, for example, on selected druggability measures. By using a spreadsheet application to take the 987 genes in Data Sheet 2 in Supplementary Material encoding proteins that have scored greater than 0.5 on either DrugEBIlity's Ensemble score or Li and Lai's druggability measure, and eliminating proteins already targeted by existing drugs, we have a list of 803 genes that are predicted to be probably druggable. Of these, 21 also have high RNA expression significantly associated with reduced disease-free survival in melanoma patients, making them possible new drug targets. These genes are *AKR7A2*, *AKR7A3*, *ARIH1*, *ARPC1A*, *CD163*, *DCT*, *DHRS11*, *DUS4L*, *FAH*, *FSCN1*, *HS3ST3A1*, *NRAS*, *NUP155*, *PANK2*, *PRMT3*, *QTRT1*, *RAD1*, *RAE1*, *SUV39H2*, *UPP1*, *USP13*. It is interesting to see *NRAS* on this list, which is a potential melanoma drug candidate but has proved remarkably resistant to drug development efforts to date (66). *CD163* expression on melanoma-infiltrating macrophages has been suggested as a prognostic marker in melanoma (67).

Similarly, a list of putative melanoma tumor suppressor genes or melanoma oncogenes can be generated using a spreadsheet application from this melanoma data matrix (Data Sheet 2 in Supplementary Material). For example, a list consisting of genes that are mutated in more than 10% of melanoma metastases and have shorter melanoma-free patient survival associated with their low (putative tumor suppressor) or high (oncogene) RNA expression. Known tumor suppressors and oncogenes that were identified by this strategy (*NRAS*, *KIT*, and *WNT* family members) were removed. This list of putative melanoma tumor suppressors and oncogenes that remains is shown in **Table 1**.

Combined melanoma information with gene set analysis

Combining this assembled melanoma information with statistical GSA can potentially provide additional insights. For example, with a spreadsheet application we could generate a list of 245 genes from Data Sheet 2 in Supplementary Material that have coding region mutations in more than 10% of melanoma metastases, and subject this list to gene set enrichment analysis in order to identify biological functions that may be commonly disrupted in melanoma. When submitted to the web tool GeneSetDB (a meta-database of biologically relevant sets of genes) for enrichment analysis (with false discovery rate set to 0.01), this list of 245 genes was found to be significantly enriched for several gene sets including sets associated with the extracellular matrix (ECM), cell adhesion, and collagen fibril organization. We encourage users to use a spreadsheet application and simple web tools such as GeneSetDB to perform their own exploration of Data Sheet 2 in Supplementary Material.

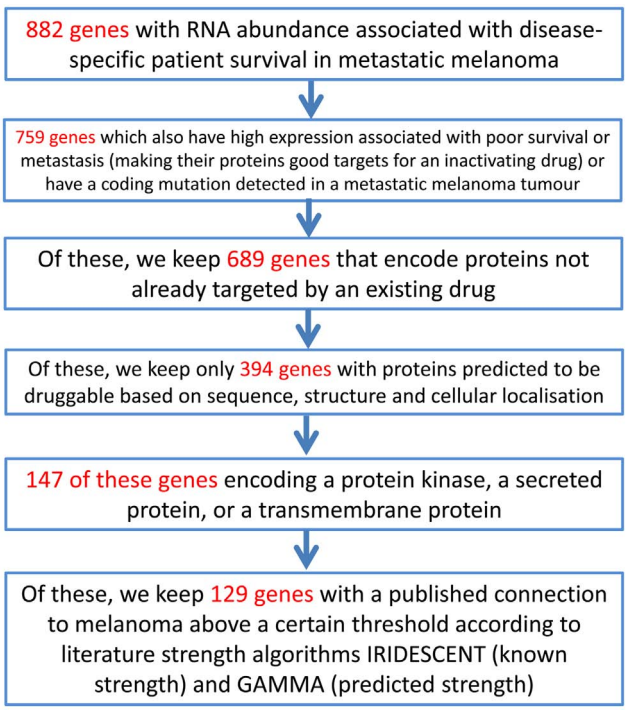

FIGURE 3 | An example of a process through which the melanoma data matrix (Data Sheet 2 in Supplementary Material) can be used to generate a short list of putative drug targets. The initial gene list consists of those genes in the melanoma data matrix (Data Sheet 2 in Supplementary Material) that have an entry in any of the columns describing the data of the studies of Jönsson et al. (51), John et al. (46), Mandruzzato et al. (47), Journe et al. (48), or Bogunovic et al. (50). (Please note that this example is for use with the data matrix in Data Sheet 2 in Supplementary Material, rather than for the MelanomaDB web tool).

ASSEMBLY OF MelanomaDB – A WEB-ACCESSIBLE GENOMIC MELANOMA SQL DATABASE, AND OF A CORRESPONDING BaseSpace APP

In order to make use of this assembly of melanoma information and its regular updating easier, we converted this melanoma data matrix (Data Sheet 2 in Supplementary Material) into a web-accessible SQL database. This database, named MelanomaDB, features melanoma gene sets derived from Data Sheet 2 in Supplementary Material and directly links into a molecular pathway/GSA meta-database previously generated by our research group named GeneSetDB (22). Using MelanomaDB, a user can easily find the union or intersection between any number of melanoma gene sets (taken from the columns of Data Sheet 2 in Supplementary Material) and also their own user-submitted gene lists (copied and pasted, or uploaded from a file, using any of over 50 types of commonly used gene identifier), then interrogate the molecular

Table 1 | Four putative melanoma oncogenes and two putative tumor suppressor genes derived from the amalgamated data.

Entrez gene	Gene symbol	Gene title	Chromosomal location	Putative tumor suppressor or oncogene?
7373	*COL14A1*	Collagen, type XIV, alpha 1	8q23	Tumor suppressor
387357	*THEMIS*	Thymocyte selection associated	6q22.33	Tumor suppressor
6299	*SALL1*	Sal-like 1 (*Drosophila*)	16q12.1	Oncogene
5069	*PAPPA*	Pregnancy-associated plasma protein A, pappalysin 1	9q33.2	Oncogene
26278	*SACS*	Spastic ataxia of Charlevoix-Saguenay (sacsin)	13q12	Oncogene
81832	*NETO1*	Neuropilin (NRP) and tolloid (TLL)-like 1	18q22.2	Oncogene

pathways for which the genes in these lists are enriched. Multiple iterations are possible, so that a user might find the union of some melanoma-associated gene sets and then find the intersection of this union with other gene sets, which can finally be directly piped into the gene set meta-database GeneSetDB to identify enriched molecular pathways. MelanomaDB is available at http://genesetdb.auckland.ac.nz/melanomadb/about.html

A subset of the information in MelanomaDB was also included in a freely available Illumina BaseSpace application, which can be accessed at http://www.biomatters.com/apps/melanoma-profiler-for-research (click on "sample project" and navigate using green tabs at top of screen). This BaseSpace application performs variant calling against reference sequences for a user-defined tumor, then uses information from MelanomaDB to identify molecular pathways that genes which contain non-synonymous variants constitute. These pathways are visualized relative to targeting drugs and other clinically related information using pathway diagrams, heatmaps, and waterfall plots, in comparison to the 310 melanomas described above. We hope that this app may be of particular use to researchers involved in generating new melanoma tumor sequences.

MelanomaDB FACILITATES ASSESSMENT OF FUNCTIONAL RELATIONSHIPS INHERENT IN TUMOR SOMATIC VARIANTS

The tumor gene sequence information included in MelanomaDB allows calculation of the proportion of melanomas that carry somatic variations in each gene/loci on a genome-wide scale. For example, by selecting gene sets using the MelanomaDB web tool, we identified those genes in which over 10% of the 96 sequenced metastatic melanomas currently in the database carried non-synonymous somatic variations. This list of 245 genes included genes that have been the focus of recent publications describing mutations in melanoma, such as *PREX2* (6), *GRM3* (57), and *ERBB4* (56) [other melanoma-associated genes such as *MAP3K5/9* (58), *MAP2K1/2* (54), and *RAC1* (4–6) are included as mutated genes in human tumors in MelanomaDB but fall outside this list of 245 genes]. As would be expected, this composite list featured genes also indicated as frequently mutated in melanoma by the larger sequencing studies (4, 5) that were used in its construction, for example, half of the genes identified by Berger et al. (6) as "significantly mutated" appear on our composite list. By selecting the option in MelanomaDB to pipe these 245 genes to the GeneSetDB web tool, we identified that these genes were significantly enriched for a small group of biological functions including cell adhesion, collagen fibril organization, and ECM. Cell adhesion is briefly mentioned in some of the sequencing studies' discussions (4, 54), and the ECM is a focus for one study (55). However, other pathways emphasized by these sequencing studies, such as the glutamate pathway (60) or chromatin remodeling pathways (5), did not feature in the results of our analysis.

ANALYSIS OF SPECIFIC SIGNALING PATHWAYS RELEVANT TO MELANOMA

The information in MelanomaDB can be used to annotate the signalling pathways contained within the R graphite package (27). This can be done either as a function of the MelanomaDB web tool, or using R scripts supplied in Data Sheet 5 in Supplementary Material. For example, **Figure 4** shows the KEGG pathway named "Melanoma" with nodes colored in shades of red according to the frequency of non-synonymous somatic variations. Thirteen nodes were plotted as boxes rather than circles to indicate that the abundance of their encoded mRNA in melanoma metastases was significantly associated with patient survival in our analysis of the data of Bogunovic et al. (50) (Cox proportional hazards model, $p \leq 0.05$, no multiple testing correction applied). Significantly more of the genes in the KEGG pathway named "Melanoma" carried more somatic variants than expected due to chance alone (Fisher's exact test with right-tailed hyper-geometric distribution, $p \leq 0.002$), in agreement with the known importance of the signaling events represented in this pathway to melanoma formation and progression.

ANALYSIS OF MELANOMA SIGNALING PATHWAYS IN INDIVIDUAL TUMORS

As an example of how this pathway-specific information can be used to place the tumors of individual patients into the context of tumors from the patient population, as well as into the context of other information within MelanomaDB, we used the information assembled here to draw a clustered heat map for genes encoding molecules of the KEGG "Melanoma" signaling pathway (**Figure 5**). This clustered heatmap is annotated with gene-survival associations, druggability indices, current drug targets, COSMIC census genes, known melanoma driver mutations and somatic variant frequency in melanoma. This can be done either as a function of the MelanomaDB web tool, or using R scripts supplied in Data Sheet 5 in Supplementary Material. In this analysis, somatic variants in genes drive the tumor clustering and potentially stratify patients into those with common biological changes, which may be susceptible to particular pathway-targeted therapies. For instance, there is a cluster of tumors with *BRAF* as the

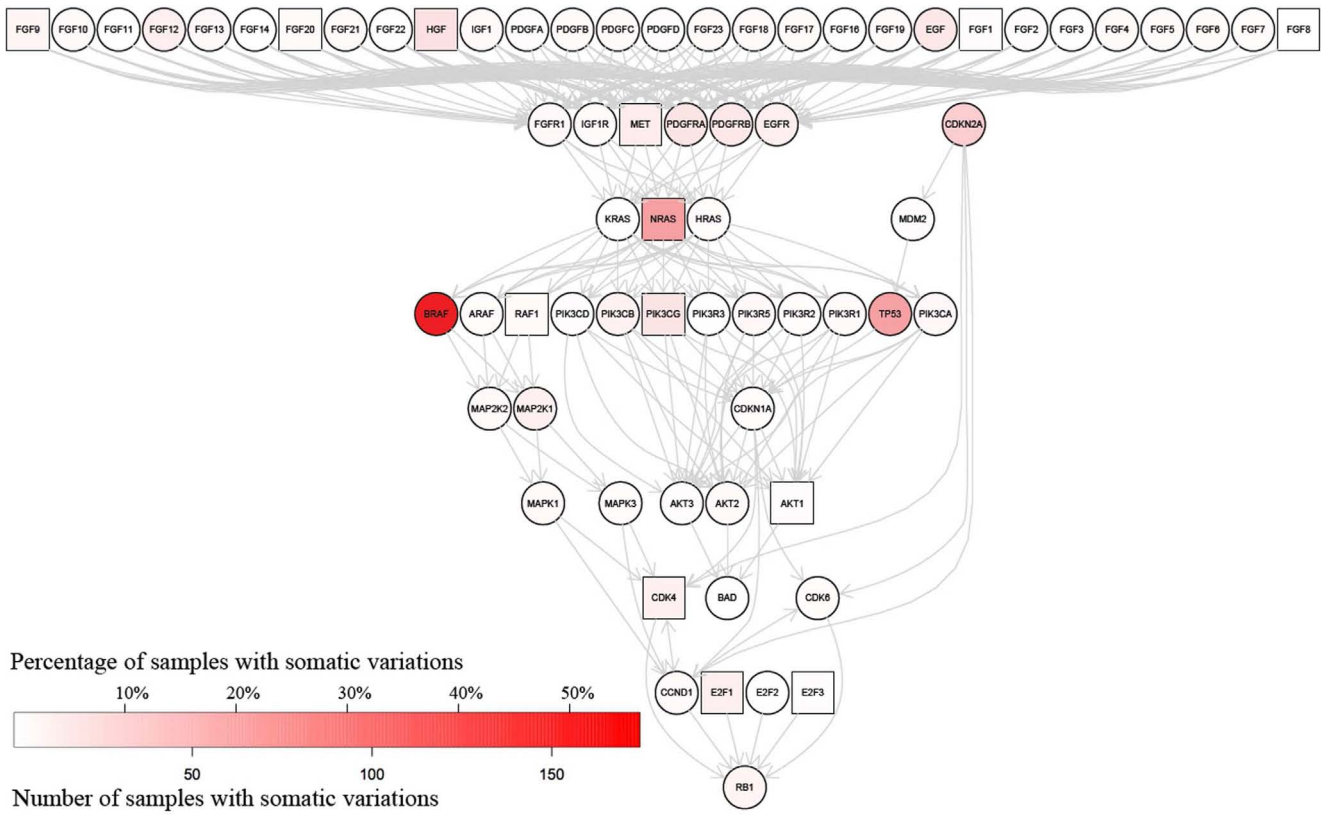

FIGURE 4 | Somatic variations in genes encoding proteins of the KEGG "Melanoma" signaling pathway. The color of each gene's node indicates the number of melanomas in which at least one non-synonymous somatic variation has been identified; white indicates no melanomas with reported somatic variation in the gene, while the degree of red saturation indicates the number of melanomas containing somatic variations in that gene (refer to color key in lower left). Square nodes indicate RNA expression in melanoma metastases significantly associated [$p \leq 0.05$ no multiple testing correction applied, Cox proportion hazards model, Bogunovic et al. (50) data], with patient disease-free survival, while circular nodes indicate the absence of any significant association between RNA abundance and patient survival. This graph was generated using the pathwayGraph function to access the KEGG pathway information contained within the R graphite package.

only somatic variant in this pathway (middle horizontal block in **Figure 5**). Of these 51 *BRAF*-variant only melanomas, 42 carry the *BRAF* V600E mutation and may putatively be tumors for stratification to Vemurafenib therapy, given their lack of somatic variants in genes encoding other proteins in this signaling pathway that could potentially contribute to Vemurafenib resistance. Some tumors carry only *NRAS* mutations, while others have either more complex mutational patterns, or no somatic mutations in this pathway. This is in accordance with previous studies reporting that mutations in *NRAS* and *BRAF* tend to be mutually exclusive but collectively occur in approximately 90% of melanomas (68). To assist interpretation of the different mutations seen in each tumor and in clusters of genetically similar tumors, the heatmap has been annotated with information about inferred melanoma driver mutations, known drug targets, and potentially druggable proteins. This type of heat map can be generated for any molecular pathway or combination of pathways. Extending this analysis, a new patient's mutation profile could be added to an established clustering analysis of large numbers of melanomas in order to identify which previously studied tumors were similar in mutation complement, which may assist prognostication and treatment stratification. In the future it will be interesting to use MelanomaDB to investigate the genomes of multiple samples from single melanomas to assess the intra-tumoral heterogeneity seen in this disease (69).

In addition, using a function in the MelanomaDB web tool of the R scripts supplied in Data Sheet 5 in Supplementary Material, somatic alteration of genes in specific molecular pathways can be drawn on a patient-by-patient basis (**Figure 6**). This allows visualization of protein-altering gene sequence variants in the context of the encoded protein's position in molecular pathways relevant to specific targeted therapies. For instance, using a well-known example from other tumor types, the position in pathway diagrams of a genetic variant known to be activating (e.g., mutant *KRAS*), downstream of a drug (e.g., cetuximab) target (e.g., EGFR) may indicate potential for resistance to the drug.

We then used an R script (Data Sheet 5 in Supplementary Material) to perform gene set enrichment analysis using the GATHER

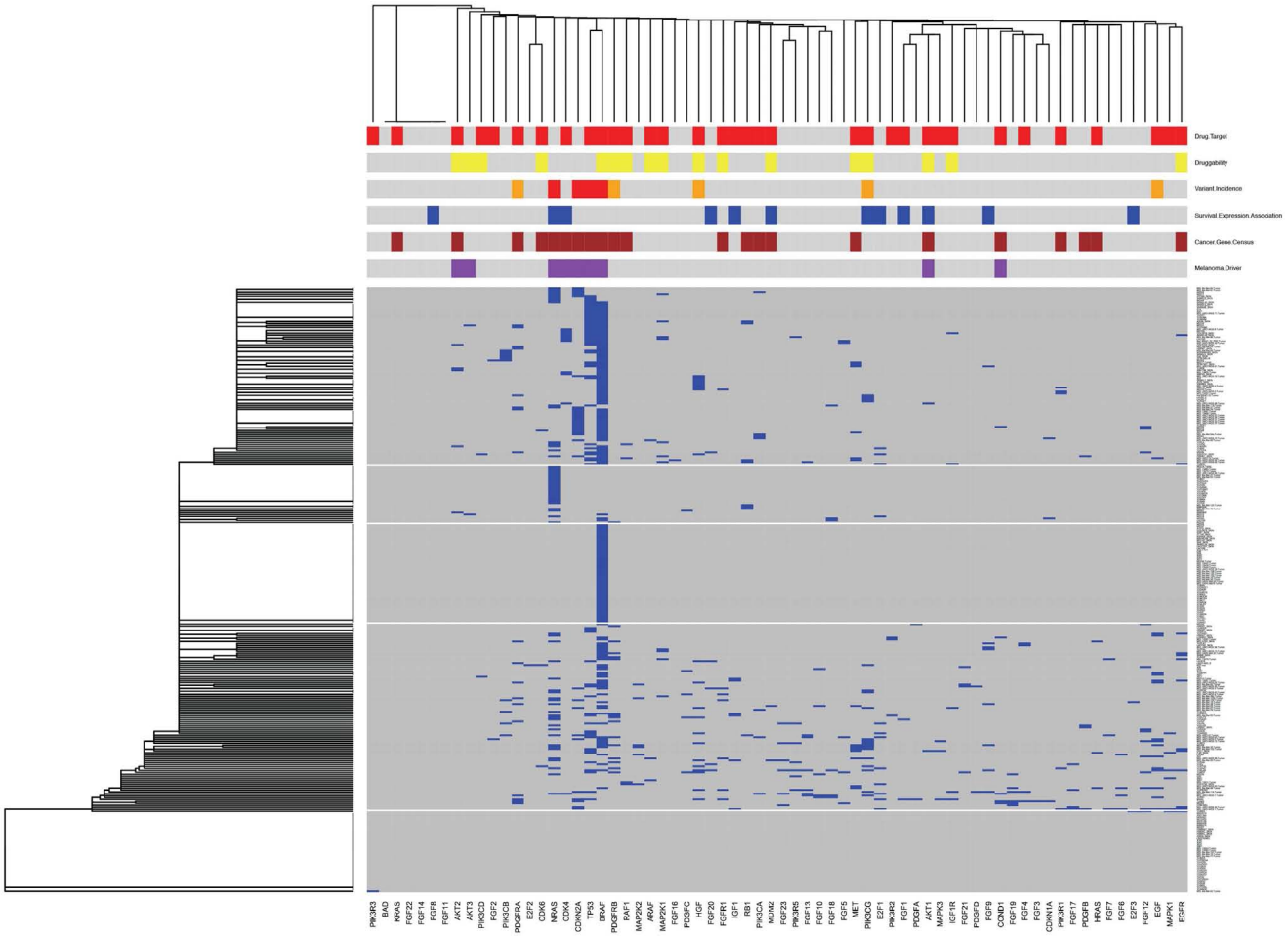

FIGURE 5 | Clustered heatmap for genes encoding proteins of the KEGG "Melanoma" signaling pathway. Gene names are on the horizontal axis, individual melanoma tumor names are on the vertical axis. Blue blocks at the intersection of a gene and a tumor indicates the presence of a protein-altering somatic variant in that gene in that tumor. Clustering of genes and tumors using single linkage clustering with binary distance was performed based on this variant information. The clustered figure was then annotated with additional information above the heatmap. In the first row above the heatmap red blocks mark genes encoding known drug targets according to version 3 of the DrugBank database. In the second row yellow blocks mark genes encoding potentially druggable proteins, as indicated by the MelanomaDB gene set "Druggability: Sophic ENSEMBL list" (33). In the third orange and red blocks indicate genes mutated in $\geq$1 or $\geq$5% of the 310 melanomas in our database, respectively. In the fourth row blue blocks mark genes that encode RNAs with a significant association between expression and patient survival [$p \leq 0.05$ no multiple testing correction applied, Cox proportion hazards model, Bogunovic et al. (50) data]. In the fifth row brown blocks indicate genes that are members of the Wellcome Trust Cosmic "Cancer Gene Census" gene set, as on 1st March 2013 (http://cancer.sanger.ac.uk/cancergenome/projects/census/). In the sixth row, purple blocks mark genes thought to be melanoma drivers when mutated [MelanomaDB gene set "Melanomagenesis Drivers" (84)]. This graph was generated using a modification of the heatmap.2 function of the gplots package in R.

web tool[10] (19) to identify any KEGG pathways for which genes somatically altered in each tumor were significantly enriched (Data Sheet 4 in Supplementary Material). KEGG pathways that appeared as significantly enriched in individual tumors included the "ECM receptor interaction" and "Neuroactive ligand-receptor interaction" KEGG pathways. To illustrate this, we selected one sequenced metastatic melanoma, ME029 from the Berger et al. (6) cohort, and drew these two pathways along with the KEGG "Melamoma" pathway for this single tumor (**Figure 7**). Two of these pathways are drawn for all 310 tumors included in this study in: Presentation 1 ("Melanoma") and Presentation 2 ("Neuroactive ligand-receptor interaction").

LIMITATIONS OF OUR APPROACH

The approach we have described, while already functioning in a useful way as a melanoma-focused integrated genomic database, provides a template for further development to address the limitations below: (i) It will be important to identify the likely effects of specific somatic variations in the sequenced tumors (e.g., loss of function, altered function, or activation of the encoded protein). In future iterations of MelanomaDB, based on larger

[10]http://gather.genome.duke.edu

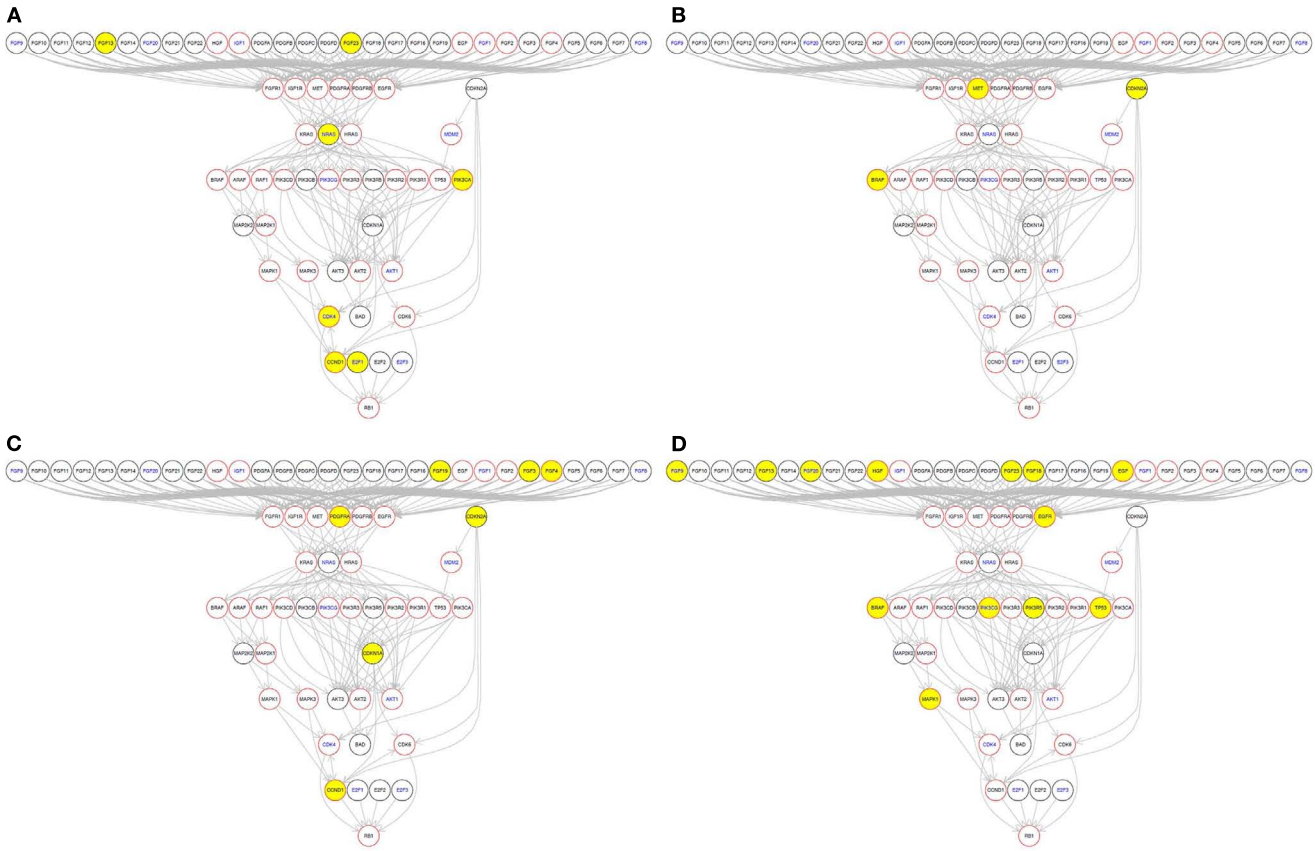

FIGURE 6 | Somatic variations in individual tumors of molecules in the KEGG "Melanoma" signaling pathway. Yellow nodes indicate that the gene has a somatic variation in that particular tumor. Red node borders indicate that there is a drug available to target that gene's encoded protein. Blue node text indicates that this gene's RNA abundance is associated with patient survival in metastatic melanoma, in the data of Bogunovic et al. (50). Four individual tumors with different mutation profiles are shown as examples: **(A)** ME049 from Berger et al. (6); **(B)** 01T from Wei et al. (60); **(C)** melanoma reported by Turajlic et al. (59); **(D)** YUKLAB from Krauthammer et al. (5). This graph was generated using the pathwayGraph function and KEGG information contained within the R graphite package.

numbers of tumors, we will include capacity to dissect the type of genetic alteration such as deletions, coding region mutations, promoter mutations, etc. The database may also be expanded to include the results of analyses from software that predict the effects of coding variants on protein function, such as SIFT (70), PolyPhen (71), or PROVEAN (72), as well as the known effects of specific mutations using resources such as COSMIC (8). (ii) Data on naevi and synonymous mutations can also be added. (iii) Information from model organisms such as mouse could also be added. (iv) Results from the ENCODE project (73) could be added along with whole genome sequencing of melanomas will allow inclusion of numerous additional functional genetic loci [e.g., ncRNAs, both general (74) and melanoma specific (75)] in the database. The ENCODE project suggests that mutations in regulatory regions such as distal enhancers can affect the expression of genes located hundreds of kilobases away (76); a way to include this in MelanomaDB could be to take a gene network approach to identify distant genes that have expression correlated with these mutations, as well as methods such as chromatin conformation capture (77). (v) Future additions to the database will also aim to incorporate data concerning the role of epigenetics, including methylation, in melanoma (78–80). (vi) There is also room to expand upon melanoma drivers, such as those highlighted in GISTIC (81), JISTIC (82), and CONEXIC (83). (vii) There is an inherent risk in any assembly or meta-analysis of data from several sources that errors in the original data are perpetuated. While it is possible that the intersection of multiple independent sources of similar types of information may reduce the change of propagating random errors, systematic errors co-occur in independent data sources. This risk affects any project of this sort and is difficult to control. Here we have attempted to minimize this risk by selecting constituent databases that are extensively used and have been peer reviewed, and on which we could perform spot checks. We consider these data sources to be the best possible choices, within our ability to assess them. (viii) The final limitation is that the molecular pathways used when assembling this database are limited by current knowledge, and overlap with one another. The database will be updated with new pathway information as it becomes available. Identifying the pathways that are not affected can be as useful as identifying those that are. The data we have

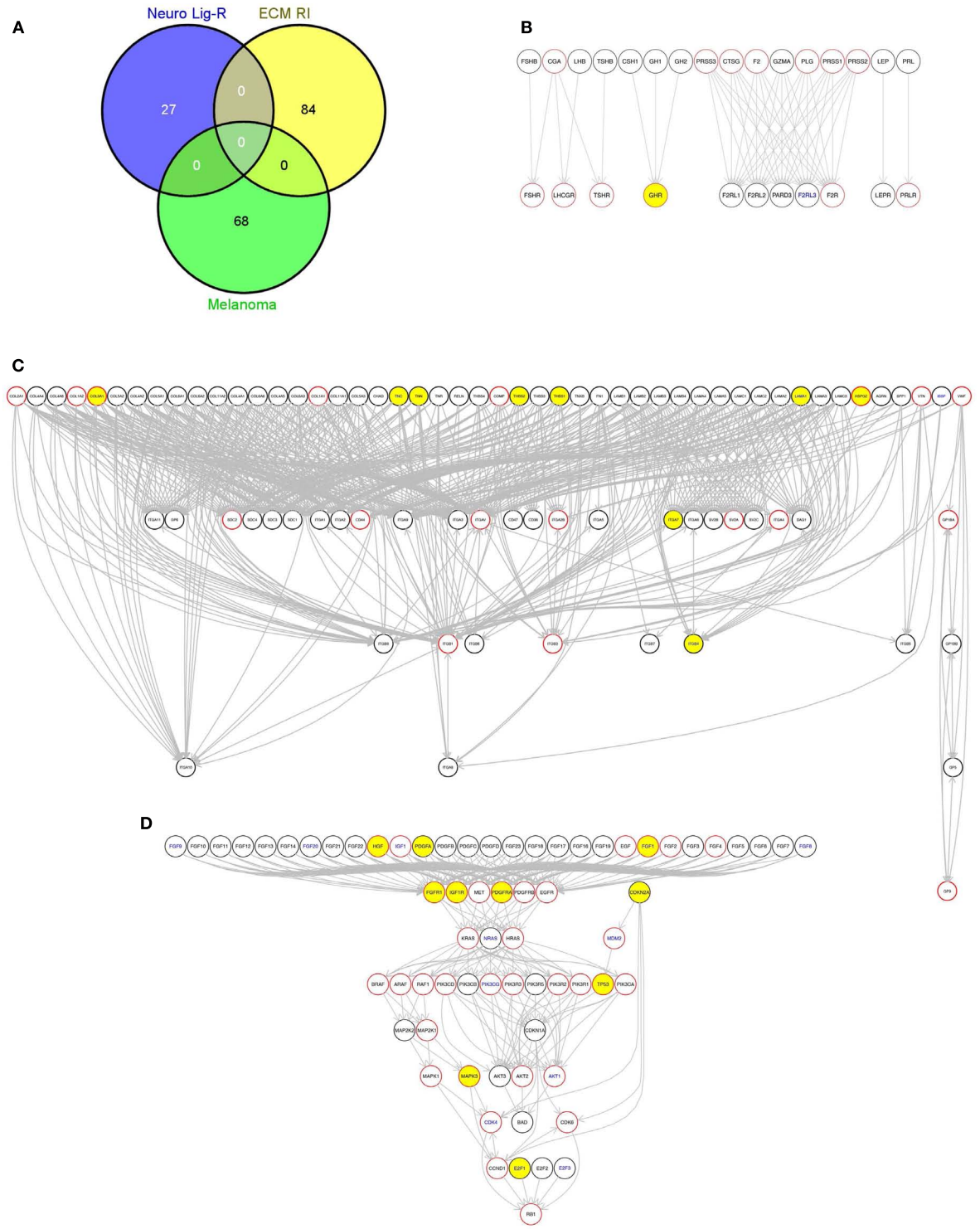

FIGURE 7 | (A) Venn diagram showing the overlap of genes between these three pathways used in this figure, generated using the Venny web tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html). "Melanoma," "Neuro Lig-RI," and "ECM RI" indicate members of the "Melanoma," "Neuroactive ligand-receptor interaction," and "Extracellular matrix (ECM) receptor interaction" KEGG pathways, respectively, contained in the R graphite package; **(B)** The KEGG "Neuroactive ligand-receptor interaction" pathway; **(C)** The KEGG "Extracellular matrix (ECM) receptor interaction pathway"; **(D)** The KEGG "Melanoma" pathway. Yellow fill color in nodes indicate genes with protein-altering somatic variations in this sample. Nodes with red borders represent genes that encode targets of existing drugs according to version 3 of the DrugBank database. Nodes with blue text indicate genes that encode RNAs with a significant association between expression and patient survival [$p \leq 0.05$ no multiple testing correction applied, Cox proportion hazards model, Bogunovic et al. (50) data, see Materials and Methods]. This graph was generated using the pathwayGraph function and KEGG information contained within the R graphite package. Similar graphs can also be generated using the MelanomaDB web tool.

generated using literature relationships with the IRREDESCENT and GAMMA methods has not been experimentally verified and is intended primarily for hypothesis generating.

CONCLUSION

We have brought together a large collection of melanoma genomic data of several types from published studies and publicly available datasets into an easily utilized data matrix that can be analyzed using a spread sheet application. We also assembled data on tumors from individual patients. We then incorporated this information into a web-accessible SQL database, MelanomaDB, which researchers can use to perform molecular pathway and GSA of melanoma genomic data, and into a BaseSpace application. By way of illustration, we used this information to analyze the mutational and expression patterns of genes encoding proteins in specific directional signaling pathways within individual tumors, and annotated these visualizations with information about existing drugs, druggability, associations between RNA expression and survival, and driver mutations. We hope that this resource will prove increasingly useful when it expands as new tumor data becomes available. In particular, we hope it may provide a context in which to interpret the melanoma molecular profiles of new patients as well as patient-specific molecular pathway disruption. We have demonstrated possible uses of this integrated information, and encourage melanoma researchers to employ these resources.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cancer_Genetics/10.3389/fonc.2013.00184/abstract

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Commentary on "MelanomaDB: a web tool for integrative analysis of melanoma genomic information to identify disease-associated molecular pathways"

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In the recent manuscript "MelanomaDB: a web tool for integrative analysis of melanoma genomic information to identify disease-associated molecular pathways" (Trevarton et al., 2013) an interesting dichotomy presents itself, which is in fact more broadly applicable to the field in general. In this work, the authors introduce an integrative tool designed to facilitate and organize disparate forms of data relevant to melanoma, including sequence, microarray, biological, drug target, drug-ability, biomarker, pharmacological, clinical trial, survival, and pathway information. It combines this data into a single matrix, for the purpose of facilitating gene set analysis interpretation, rational experimental design, interpretation of molecular profiles of tumors for individual patients, and aiding in patient stratification.

Included in their tool currently or prospectively are data from the DrugBank[1], KEGG Drug[2], the Therapeutic Targets Database[3], ClinicalTrials.gov[4], KEGG BRITE[5], DrugEBIlity[6], UniProt[7], the Secreted Protein Database[8], KinBase[9], Gene Expression Omnibus (GEO)[10], Cancer Cell Line Encyclopedia[11], the Catalogue Of Somatic Mutations In Cancer (COSMIC)[12], Matched Pair Cancer Cell Lines[13], Australia's Melanoma Genome Project[14], The Cancer Genome Atlas (TCGA) project[15], Oncomine[16], the Broad Institute's Melanoma Genomics Portal[17], as well as data from multiple publications.

Certainly this may be seen as an asset. No one group has the ability to generate all the data needed for true systems biology or pharmacology, and so, as a field we are all dependent on data generated by others. The MelanomaDB tool brings together multiple forms of data that, while available from their individual sources, would be challenging, time consuming, and require specific knowledge of those multiple data sources for the user to compile. Especially of interest is the integration of the molecular forms of gene data with those genes commonly mutated in metastatic melanomas, and drug-ability information. Thus, the authors aim to facilitate the fluent integration of disease-relevant information, a huge problem in the field in general.

Unfortunately, there are also inherent dangers for this type of approach. An obvious danger is that when compiling data from multiple sources, one will be subject to any flaws inherent in those data. That is, one is heavily reliant on the work of other groups that one has no detailed knowledge of. Assessment of the reliability of the component parts that are being assembled from multiple data sources is difficult or impossible. Nonetheless, all conclusions are completely reliant on these data. Websites that integrate data from other websites clearly are susceptible to perpetuating data problems or inaccuracies as well as potentially amplifying their influence in the field.

Some forms of data will be more problematic than others. DNA sequence and copy number should be relatively consistent, due to DNAs stability, reproducibility, and ease of verification. The drug databases will give an accurate picture of the incomplete knowledge of the day, realizing that target and interacting pathway information remains incomplete. mRNA and microRNA expression is and will remain subjective due to the technique and reagents used during growth and/or harvest of either cell lines or patient samples. Inclusion of gene set analysis approaches clearly introduces an additional layer of study-specific considerations.

For the DNA sequence data, the ability to repeat analysis provides a way to catch potential errors, however, once erroneous data is entered into a database it will likely remain there. The drug knowledge databases are constantly being updated as new information is obtained. mRNA (or microRNA) expression may be the most difficult to assess, as there is really no way to exactly reproduce another group results, and so there is no clear way to recognize or filter out poorly done studies.

Certainly the MelanomaDB site is not the first to be affected by these considerations, as they are endemic to the field. Careful consideration of one's sources of data, its reliability, and compatibility with other forms of data seems requisite. While recognizing that a detailed assessment of multiple data sources is outside of the scope for this (or any other) group, some

[1] DrugBank. http://www.drugbank.ca
[2] KEGGDrug. http://www.genome.jp/kegg/drug/
[3] Therapeutic Targets Database. http://bidd.nus.edu.sg/group/cjttd/
[4] ClinicalTrials.gov. http://clinicaltrials.gov
[5] KEGGBRITE. http://www.genome.jp/kegg/brite.html
[6] DrugEBIlity. (http://www.ebi.ac.uk/chembl/drugebility/)
[7] UniProt. http://www.uniprot.org
[8] Secreted Protein Database. http://spd.cbi.pku.edu.cn
[9] KinBase. http://kinase.com/kinbase/
[10] Gene Expression Omnibus. http://www.ncbi.nlm.nih.gov/geo/
[11] Cancer Cell Line Encyclopedia. http://www.broadinstitute.org/software/cprg/?q=node/11
[12] Catalogue Of Somatic Mutations In Cancer. http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/
[13] Matched Pair Cancer Cell Lines. http://www.sanger.ac.uk/genetics/CGP/Studies/Matched/
[14] Australia's Melanoma Genome Project. http://www.melanoma.org.au/research/melanoma-genome-project.html
[15] The Cancer Genome Atlas. http://cancergenome.nih.gov
[16] Oncomine. http://www.oncomine.com/resource/login.html
[17] Broad Institute's Melanoma Genomics Portal. http://www.broadinstitute.org/software/cprg/?q=node/46

consideration of what data to use and its reliability are important if the field is to make accurate and scientifically relevant conclusions. Only by inclusion of high-quality input data may one expect to draw meaningful conclusions.

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The role of the Hippo pathway in melanocytes and melanoma

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The Hippo signaling pathway comprises a series of cytoplasmic tumor suppressor proteins including Merlin and the Lats1/2 and MST1/2 kinases, and is thought to play a critical role in determining the sizes of organs and tissues. The Hippo pathway is regulated upstream by extracellular mechanosensory signaling arising from cell shape and polarity, as well as by a variety of extracellular signaling molecules. When active, the pathway maintains the transcriptional activators Yes-associated protein (YAP) and TAZ in phosphorylated forms in the cytoplasm, preventing cell proliferation. When the Hippo pathway is inactivated, YAP and TAZ are translocated to the nucleus and induce the expression of a variety of proteins concerned with entry into the cell division cycle, such as cyclin D1 and Fox M1, as well as the inhibition of apoptosis. The failure of the Hippo pathway has been implicated in the development of many different types of cancer but there is limited information available as to its involvement in melanoma. We hypothesize here firstly that the Hippo pathway is involved in maintaining density of cutaneous melanocytes on the basement membrane at the junction of the epidermis and the dermis, and secondly, that its function is disturbed in melanoma. We have analyzed a series of 23 low passage human melanoma lines as well as cultured normal melanoma, and find that melanocytes, as well as all melanoma cell lines examined express TAZ. Melanocytes and most melanoma lines also express YAP. E-cadherin, an upstream regulator of the Hippo pathway, and Axl, a receptor tyrosine kinase regulated by the Hippo pathway, are expressed in melanocytes and in several melanoma cell lines. These observations, together with published evidence for the presence of Merlin, Lats1/2, and MST1/2 in melanocytes and melanoma cells, support the hypothesis that the Hippo pathway is an important component of melanocyte and melanoma behavior.

Keywords: epidermal melanocytes, E-cadherin, cytoskeleton, merlin, cell proliferation

INTRODUCTION

The Hippo signaling pathway derives its name from the discovery of a set of four genes in *Drosophila* that together were found to control organ size. These genes specify a series of kinases and adaptor proteins including Hippo (Hpo), Warts (Wts), and Salvador (Sav), loss of function of which results in flies with enlarged, folded eyes and excess head cuticle, a "hippopotamus-like" phenotype (Wu et al., 2003). Subsequent studies have demonstrated an analogous pathway in humans, potentially providing an answer to the long-standing question in biology of how organ size is stabilized throughout life (Pan, 2012). In humans, mechanical signals mediated by cell–cell contacts and by interactions with the extracellular matrix generate signals which are integrated in space and time and form the heart of the Hippo pathway (Halder et al., 2012). Mechanical signals are complemented by those from membrane receptors including G-protein coupled receptors (GPCRs), which are known to respond to a number of ligands such as lysophosphatidic acid, sphingosine-1-phosphophate, glucagon, and epinephrine (Yu et al., 2011). Merlin, a product of the neurofibromatosis type 2 (NF2) gene (Li et al., 2012), is a key component of the pathway and associates with Kibra, a protein associated with memory performance (Xiao et al., 2011) and with Expanded, a tumor suppressor protein also associated with the Hippo pathway (Hamaratoglu et al., 2006). The Kibra–Merlin-Expanded protein complex leads to activation of the Hippo pathway by activating Mammalian Sterile 20-like kinase (Mst1/2), a homolog of Hippo in *Drosophila*, through autophosphorylation (Yu et al., 2010). Mst1/2, complexed with the scaffold protein Sav1 (the analog of Salvador), phosphorylates and activates the large tumor suppressor (Lats1/2) kinase, which is the homolog of Warts in *Drosophila* (Chan et al., 2005). Lats1/2 are also directly activated by the scaffold protein, Msp-one binder (Mob1) (Zhao et al., 2011). Kibra also contributes to the activation of Lats1/2 (Moleirinho et al., 2013).

In a number of cell types Lats1/2 kinase has been found to phosphorylate and inactivate the transcription co-activator Yes-associated protein (YAP), the homolog of Yorkie in *Drosophila*, as well as TAZ, the YAP paralog transcriptional co-activator with PDZ-binding motif (Hao et al., 2008). Phosphorylation of YAP at Ser127 by Lats1/2 generates a 14-3-3 binding site that leads to YAP cytoplasmic sequestration through 14-3-3 binding and consequent spatial separation from nuclear target transcription factors, preventing entry into the cell division cycle (Zhao et al., 2007). Furthermore, phosphorylation of YAP at Ser381, and of TAZ, leads to

further changes and to ubiquitin-mediated proteasomal degradation (Zhao et al., 2010). Loss of phosphorylation allows YAP/TAZ to enter the nucleus and initiate a complex cascade of transcription events that lead to cell proliferation, cell migration, and suppression of anoikis, a form of apoptosis (Zhao et al., 2011). Merlin may also act in a fashion similar to that of β-catenin, translocating to the nucleus and stimulating transcription (Li et al., 2012). Expanded also directly associates with Yorkie in *Drosophila* to inhibit growth of the Hippo pathway by sequestering Yorkie in the cytoplasm (Badouel et al., 2009).

Studies of the Hippo pathway suggest it has a three-dimensional "sense" that is communicated across the whole organ and controls both cell proliferation and apoptosis. A possible example of such organ size control is provided by liver; it has been known for many years that surgical reduction of liver volume leads to extensive cell division and regeneration until the liver approaches its original size. The Hippo pathway has been hypothesized to have a role in this process (Avruch et al., 2011). In this review, we explore the hypothesis that the Hippo pathway is responsible for determining the overall number of cutaneous melanocytes and that changes in this pathway contribute to the development of melanoma.

MELANOCYTES AND THEIR FUNCTIONS

Melanocytes are found in a number of locations including the eyes, ears, and brain but are particularly noted for their ability to form a two-dimensional network in skin at the junction of the dermis and the epidermis. Melanocytes are localized on the basement membrane, a layer of fibrous proteins which separates the dermis from the epidermis, and have a density of approximately 1000 cells per square millimeter (Gilchrest et al., 1979). This density is maintained throughout life. The skin comprises the epidermis, which contains melanocytes, keratinocytes, and Langerhans cells, and the dermis, which includes blood vessels, nerve cells, adipocytes, macrophages, and fibroblasts (Norris, 2011). All three epidermal cell types and many dermal cell types express toll-like receptors and contribute to recognition of pathogens in host immunity (Hari et al., 2010). Melanocytes on the basement membrane have the additional function of synthesizing melanin and transporting it in vesicles (melanosomes) to keratinocytes within the epidermis, thus protecting the epidermis from ultraviolet light (UV)-induced damage.

Epidermal melanocytes are strongly polarized and bind on one face to laminin molecules of the basement membrane via integrins including α3β1 and α6β1, and on the other face to other cells of the epidermis through long processes called dendrites (Fukunaga-Kalabis et al., 2006). Each melanocyte appears to interact with several dozen keratinocytes (Haass et al., 2005), as shown diagrammatically in **Figure 1**. Interestingly, when melanocytes are cultured on Matrigel, a mixture of proteins which approximates the basement membrane, they form a network of similar cell density, as shown in **Figure 2**. Both melanocytes and keratinocytes express E-cadherin and desmoglein, allowing the formation of adherens junctions between them and probably also between melanocytes in the network. The formation of dendrites, which may be controlled by Rac1 (Scott and Cassidy, 1998), allows contact to be made with multiple keratinocytes and the transport of melanosomes to the outer layers of the skin.

STIMULATION OF MELANOCYTE PROLIFERATION

Melanocytes, like fibroblasts, normally exist in a quiescent state but continuously preserve their ability to proliferate in response to cell loss or injury. The local production of reactive oxygen species (ROS) constitutes one of the main causes of cell injury and death (Fried and Arbiser, 2008). Melanin production is itself associated with free radical production (Arck et al., 2006) and environmental UVA and UVB are known both to generate ROS (Noonan and De Fabo, 2009) and to increase melanocyte density (Gilchrest et al., 1979). Inflammatory processes in the skin in response to pathogens

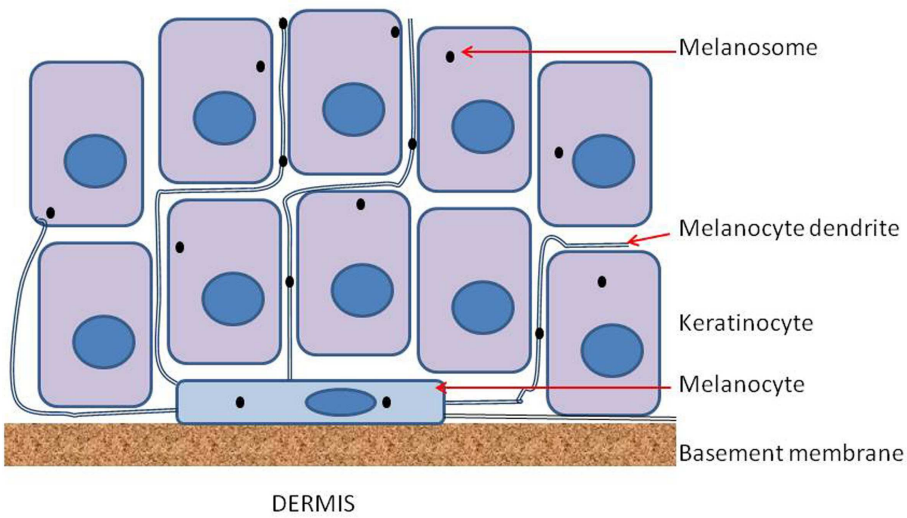

FIGURE 1 | Diagram of a melanocyte on the basement membrane and sandwiched between the epidermis and the dermis. Melanocytes extend a number of dendrites into the epidermis and these serve to transfer melanosomes from each melanocyte to a number of keratinocytes. Connections between adjacent melanocytes also involve dendrites but are not shown in the figure.

FIGURE 2 | Phase contrast photomicrograph of normal melanocytes growing on a layer of Matrigel.

also induce Langerhans cells and other epidermal cells to generate ROS. Replacement of melanocytes is an important facet of the maintenance of the epidermis and the induction of melanocyte proliferation may occur not only as a physiological response to cell injury or loss but also as a result of an oncogenic event such as an activating mutation. Examples of such events include activating mutations of the gene *BRAF* (Pollock et al., 2003), which activate the MEK/ERK pathway? and activating mutations of the gene *PIK3CA*, which activate the PI3K/AKT pathway (Hafner et al., 2007). An important cellular response to such oncogenic events is the induction of senescence. One potential cellular mechanism is provided by the p130-E2F4-DREAM complex; loss of this function leads to the activation of the protein p16, which mediates senescence (Hauser et al., 2012). This and other mechanisms are thought to contribute to the formation of moles or naevi, a collection of senescent cells within the skin epidermis.

HYPOTHESIS: THE HIPPO PATHWAY IN MELANOCYTES

We hypothesize here that the overall density of melanocytes throughout life is controlled by elements of the Hippo signaling pathway. We suggest that mechanical signals mediated by contacts between melanocytes and the basement membrane, other melanocytes and keratinocytes are integrated in space and time to activate the Hippo pathway, as has been suggested for other tissues (Halder et al., 2012). Mechanical signals are complemented by biochemical signals, particularly from keratinocytes, and relayed from surface receptors, to cellular components through protein phosphorylation. Some of the proposed upstream elements of this hypothesis are shown in the simplified diagram in **Figure 3**. Melanocytes are maintained in a physically stretched state by interaction of integrins with elements on the basement membrane, as well as by the interaction of E-cadherin, desmoglein, and other adhesion molecules on dendrites with other cells, particularly adjacent keratinocytes; F-actin polymerization and depolymerization are thought to contribute to the extension and retraction of dendrites that interact with keratinocytes.

Melanocytes express E-cadherin, along with β-catenin and α-catenin (Larue et al., 2003), which by analogy with other cell types would be expected to interact with components of the cytoskeleton to form adherens junctions (Mareel et al., 1997; Shapiro and Weis, 2009). Merlin is known to be expressed by melanoma cells and may also be expressed by melanocytes; it is recruited to nascent adherens junctions and may signal through MST1/2 (Murray et al., 2012). Glutamate metabotropic receptors are GPCRs expressed by melanocytes (Hoogduijn et al., 2006) and may provide a link between glutamate, produced by keratinocytes, and Lats1/2 in an analogous fashion to that proposed for other GPCRs (Yu et al., 2011). Another potential link is the Axl receptor tyrosine kinase, which is located on melanocytes (Sensi et al., 2011), is activated by the expression of growth arrest-specific (GAS) factors produced by keratinocytes (Manzow et al., 1996) and is regulated by the YAP pathway (Xu et al., 2011).

A scheme whereby YAP and TAZ participate in the control of proliferation of cultured melanocytes is shown in **Figure 4**. Signals for proliferation are provided by the culture substrate and the specific components of the growth medium, and it is possible *in vivo* that cell loss or injury results in loss of melanocyte contacts with the basement membrane and/or other cells, inhibiting LATS1/2 function and activating YAP/TAZ. Some of the downstream signaling pathways of activated YAP/TAZ are depicted in **Figure 4**. Dephosphorylated YAP/TAZ enters the nucleus, binding to and activating TEAD transcription factors, which in turn lead to increased transcription of target genes, increases in cell motility, invasion, anchorage-independent growth and proliferation, and resistance to apoptosis (Mizuno et al., 2012; Zhao et al., 2012). YAP-TEAD transcription induces *CCND1*, the gene encoding cyclin D1 (Cao et al., 2008) and *FOXM1*, a gene encoding a member of the Forkhead family of proteins (Mizuno et al., 2012). Cyclin D1 activates cyclin-dependent kinases 4 and 6 (cdk4 and cdk6), which in turn phosphorylate the retinoblastoma protein (Rb), allowing activation of E2F transcription factors. FOXM1 regulates the cdc25B protein phosphatase, cyclin B, polo-like kinase, aurora B kinase, and centromere proteins, controlling progression through S-phase and mitosis as well as cell cycle transitions from G1-phase to S-phase and from G2-phase to mitosis (Koo et al., 2012).

Lats1/2 also activates the dual-specificity tyrosine phosphorylation-regulated kinases (DYRK) (Tschop et al., 2011), which in turn phosphorylate and activate multi-protein complexes known as the p130-E2F4-DREAM (DP, retinoblastoma, E2F, MuvB) repressor complexes. These silence E2F target gene expression (Dick and Mymryk, 2011; Tschop et al., 2011). Dephosphorylated DYRK is no longer able to activate the p130-E2F4-DREAM complex, leading to derepression of transcription of genes under the control of activating members of the E2F family (Tschop et al., 2011), including cyclin E (Dick and Mymryk, 2011) and a number of proteins associated with DNA replication. Loss of Hippo activity might thus constitute a selective pressure for the inactivation of p16-mediated suppression of proliferation signaling and the emergence of melanoma cells. Several studies indicate the existence of cross-talk between the Hippo pathway and other signaling pathways. For instance, YAP activation has been shown to alter the function of the MAPK pathway (Kang et al., 2011). The Hippo

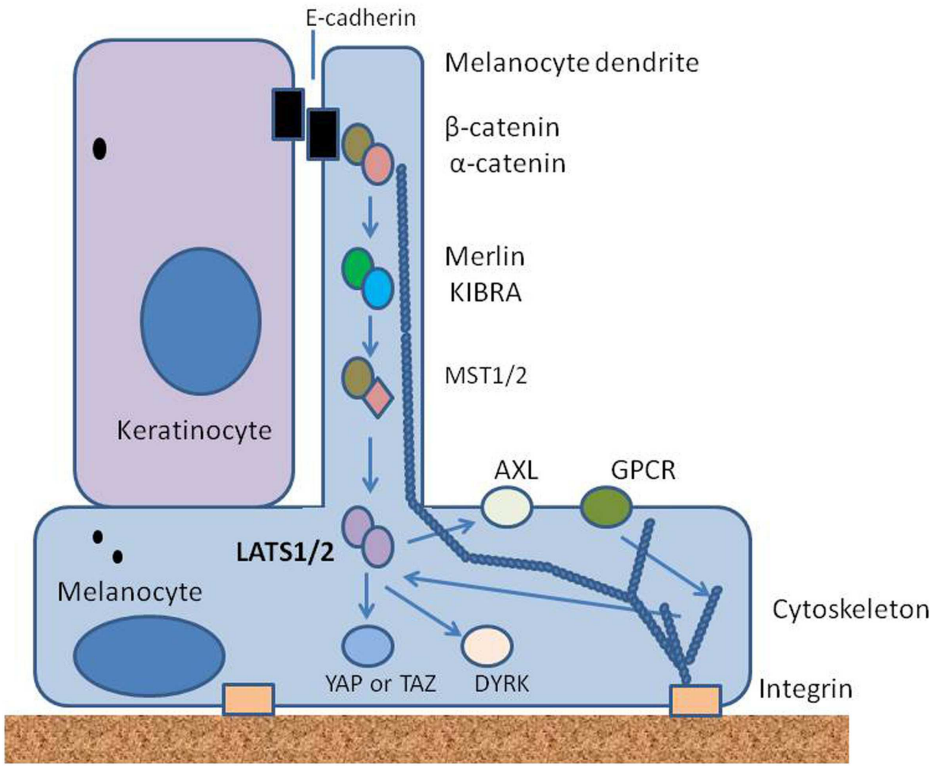

FIGURE 3 | Diagram showing some of the upstream elements of the proposed Hippo pathway. Melanocytes are strongly polarized and receive signals on the one hand from the epidermis through adherens junctions with keratinocytes, and on the other hand from the basement membrane through integrins. They also receive signals through receptor tyrosine kinases such as Axl and through G-protein coupled receptors (GPCRs). All are likely to signal through kinases to Lats1/2; arrows indicate signaling events.

pathway also inhibits the Wnt/β-catenin pathway by promoting interaction between TAZ and the Disheveled (DVL) protein of the Wnt pathway in the cytoplasm (Varelas et al., 2010; Azzolin et al., 2012), indicating a role for the Hippo pathway in morphogenetic signaling.

INVOLVEMENT OF THE HIPPO PATHWAY IN MELANOMA

To collect evidence for the involvement of the Hippo pathway in melanoma, a series of melanoma lines developed in this laboratory (Marshall et al., 1993, 1994; Kim et al., 2012) were analyzed for expression of some of the components and targets of the Hippo pathway (**Figure 5**). Many of the melanoma lines have been found to form networks on Matrigel (Zhao et al., 2005) in a manner similar to that of melanocytes (**Figure 2**), suggesting that they are capable of interaction with the extracellular matrix. Most of the lines were tested for expression of E-cadherin and N-cadherin (Kim et al., submitted), which are upstream elements of the Hippo pathway. The majority of lines had lost E-cadherin expression and replaced it with expression of N-cadherin, which is associated with a more invasive phenotype (Qi et al., 2005); a small proportion of cell lines expressed neither E-cadherin nor N-cadherin. All of the melanoma lines tested strongly expressed TAZ and many additionally expressed YAP. As shown in **Figure 5**, 35% of melanoma lines, as well as normal melanocytes, expressed Axl although this was not related to expression of cadherins. Another study reported that 38% of melanoma lines expressed Axl and postulated that expression was associated with motility and invasion (Sensi et al., 2011).

It is known that from animal models of melanoma that proliferation and invasiveness are promoted by YAP-TEAD (Lamar et al., 2012) and inhibited by Merlin (Murray et al., 2012). *CTGF* (Braig et al., 2011) and *GLI2* (Alexaki et al., 2010), two genes downstream of the YAP/TAZ-TEAD complex, have been associated with increased proliferation and invasiveness in melanoma. Taken together, these results support the involvement of YAP and TAZ in some stages of melanoma development. It is likely that the microenvironment of the melanoma may also be involved in YAP-TEAD regulation, for instance in the generation of ROS and cytokines. Further research needs to be carried out to characterize other elements of the Hippo pathway in melanoma, particularly upstream elements such as Lats1/2.

Our previous studies have suggested that two changes may be important to distinguish melanoma cell lines from cultured melanocytes: the partial loss of serum dependence of some intracellular signaling pathways (Kim et al., 2012) and the suppression of the p16 inhibitory pathway (Charters et al., 2011), which is common in melanoma (Hauser et al., 2012). Melanomas, as opposed to melanocytes, contain a number of mutated genes, raising the question of whether any of these mutations can affect the integrity of the Hippo pathway. Melanomas have a high frequency of mutant

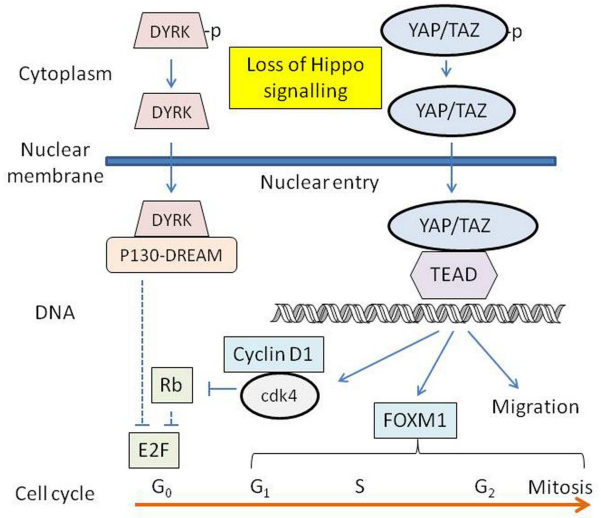

FIGURE 4 | Diagram showing some of the downstream elements of the proposed Hippo pathway. The transcription factors YAP/TAZ-TEAD are activated, and DYRK-DREAM is inactivated by loss of Hippo signalling. Resulting transcription products are involved in regulating the synthesis of a number of proteins associated with cell proliferation and migration.

BRAF mutations, and in papillary thyroid cancer, expression of mutant BRAF is associated with inhibition of MST1/2 kinases (Lee et al., 2011); it would be interesting to determine whether this is also the case in melanoma. Moreover, BRAF mutations are associated with a Rac-dependent cadherin switch in melanoma (Monaghan-Benson and Burridge, 2013), suggesting a link to the cytoskeleton. A survey of mutant genes in melanoma revealed a high frequency of Rac1 mutations (Krauthammer et al., 2012); Rac1 acts to modify the cytoskeleton and loss could potentially change Hippo pathway regulation. The Ras association domain family gene *RASSF1* is frequently inactivated by promoter hypermethylation in a variety of human tumors including melanoma (Spugnardi et al., 2003; Richter et al., 2009) and its protein product RASSF is known to be a binding partner of MST1/2 kinases (Khokhlatchev et al., 2002), again suggesting a link to the Hippo pathway. The *GRIN2* gene, which codes for a subunit of the glutamate ionotropic receptor, is mutated in approximately 25% of melanomas (Wei et al., 2012). This receptor is involved in modulation of melanocyte dendrite morphology (Song et al., 2012) and might therefore also affect the Hippo pathway. Mutations in the *NF2* gene, which encodes Merlin (**Figure 3**) have been reported in a number of cancers including approximately 30% of melanomas and could have an important role in the efficacy of the Hippo pathway. Finally, FAT4 is known to inhibit cell growth by activation of

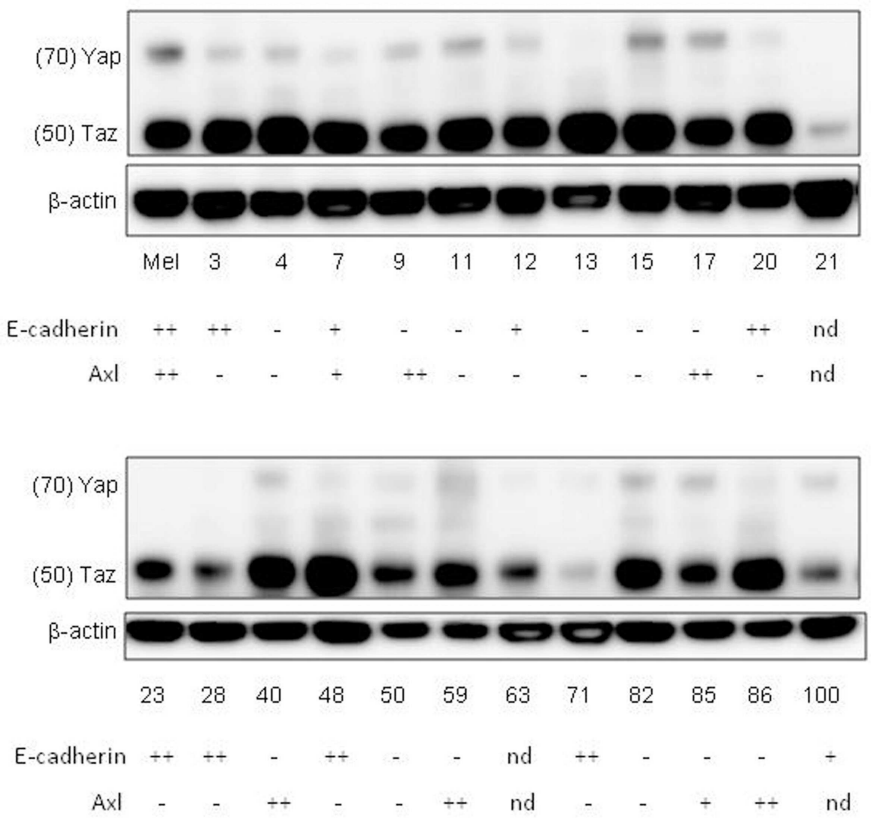

FIGURE 5 | Western blots of whole-cell extracts of cultures of normal melanocytes and of a number of melanoma lines, indicating expression of YAP and TAZ. The numbers indicate the identities of members of the New Zealand melanoma collection (e.g., 3 = NZM3). Mel indicates data for normal melanocytes. Data for expression on western blots of E-cadherin and Axl, taken from another publication (Kim et al., submitted), are shown below the NZM numbers; – expression not detected; +weak expression; ++strong expression; nd not done.

the Hippo pathway and the *FAT4* gene is recurrently mutated in several types of human cancer including melanoma (Katoh, 2012).

CONCLUSION

One of the fascinating features of the Hippo pathway is that it is able to integrate several different types of signaling, including those induced by cellular shape and adhesion changes, stress responses, and fluctuating concentrations of extracellular signaling molecules. It mediates cellular decisions on the control of proliferation, motility and cell death, and existing evidence indicates a complex and possibly redundant series of intracellular pathways (Halder et al., 2012). Melanomas are often thought of as developing mainly as the result of multiple genetic changes and a number of these may be linked to the function of the Hippo pathway. Furthermore, there is a strong possibility that extracellular signaling from the melanoma microenvironment may be important in tumor progression. The pathways underlying transduction of mechanical and cytoskeletal signals are now under intensive investigation and may provide a rich source of potential targets for the therapy of this disease.

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Heterogeneity of expression of epithelial–mesenchymal transition markers in melanocytes and melanoma cell lines

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The epithelial–mesenchymal transition (EMT) describes a reversible switch from an epithelial-like to a mesenchymal-like phenotype. It is essential for the development of the normal epithelium and also contributes to the invasive properties of carcinomas. At the molecular level, the EMT transition is characterized by a series of coordinated changes including downregulation of the junctional protein E-cadherin (*CDH1*), up-regulation of transcriptional repressors of E-cadherin such as Snail (*SNAI1*) and Slug (*SNAI2*), and up-regulation of N-cadherin. We wished to determine whether cultured normal melanocytes and melanoma cell lines, which are derived from the neural crest, showed signs of a similarly coordinated phenotypic switch. We investigated normal melanocytes and 25 cell lines derived from New Zealand patients with metastatic melanoma. Most lines had been previously genotyped for common mutations such as *BRAF*, *NRAS*, PIK3CA (phosphatidylinositol-3-kinase), *TP53* (p53), and *CDKN2A* (p16). Expression of E-cadherin, N-cadherin, microphthalmia-associated transcription factor (MITF), Snail, Slug, Axl, p53, and Hdm2 was compared by western blotting. Normal melanocytes expressed each of these proteins except for Snail, while normal melanocytes and almost every melanoma line expressed Slug. Expression of individual markers among different melanoma lines varied from high to low or undetectable. Quantitation of western blots showed that expression of MITF-M, the melanocyte-specific isoform of MITF, was positively related to that of E-cadherin but inversely related to that of N-cadherin and Axl. There was also no apparent relationship between expression of any particular marker and the presence of *BRAF*, *NRAS*, *PIK3CA*, *TP53*, or *CDKN2A* mutations. The results suggest that melanomas do not show the classical epithelial and mesenchymal phenotypes but rather display either high E-cadherin/high MITF-M expression on one hand, or high N-cadherin/high Axl expression on the other. These may correspond to differentiated and invasive phenotypes *in vivo*.

Keywords: E-cadherin, Axl, MITF, melanocyte, melanoma

INTRODUCTION

The epithelial–mesenchymal transition (EMT) describes a reversible phenotypic change in epithelial cells that is essential for embryogenesis and wound healing in normal tissues. It is characterized by the loss of functional E-cadherin containing junctions and loss of cell polarity, and is particularly associated with the expression of zinc-finger transcription factors Snail (*SNAI1*) and Slug (*SNAI2*), as well as of ZEB1 (zinc-finger E-box-binding homeobox 1), ZEB2, FoxC2 (forkhead box protein C2), and TWIST (Lim and Thiery, 2012). Expression of the intermediate filament protein vimentin appears to be upregulated by Slug in cells undergoing EMT; vimentin then up-regulates the Axl tyrosine kinase, which contributes to changes in cytoskeletal architecture and migratory potential (Ivaska, 2011). These changes in adhesion proteins cause cells to change to a morphology resembling that of mesenchymal cells and to a functional change toward migration, invasion, and resistance to apoptosis. Evidence for EMT has also been found in carcinomas, leading to the proposal that it is involved in both invasion and metastasis (Lim and Thiery, 2012).

Melanocytes differ from epithelial cells in having their origin in the neural crest, a collection of multipotent and migratory cells in the vertebrate embryo that is also important for the development of cartilage, bone, neurons, glia, and smooth muscle. Although the term EMT arose from studies in epithelial tissues, it has been applied to a variety of developmental tissues including migratory neural crest cells that are the precursors of melanocytes. Slug appears to be essential for precursor migration and melanocyte development in mammals; Slug knockout mice exhibit some features of the Waardenburg syndrome in humans, which is associated with hypopigmentation and hearing loss (Shirley et al., 2012), while loss of one Slug allele in humans is associated with piebaldism (Sanchez-Martin et al., 2003). Expression of Slug is closely related to that of microphthalmia-associated transcription factor (MITF; Sanchez-Martin et al., 2002), which in turn is essential for expression of proteins mediating the production of melanin by mature melanocytes. Such cells also express E-cadherin, presumably allowing both functional interaction with E-cadherin expressed on keratinocytes (Kuphal and Bosserhoff, 2012) and transfer of melanosomes.

Melanoma cells differ from melanocytes by acquiring invasive and/or metastatic properties, depending on the state of the melanoma (Orgaz and Sanz-Moreno, 2013). It has been suggested that the invasive and metastatic potential of melanoma cells reflects their ability to undergo EMT-like reversible phenotypic changes (Shirley et al., 2012). Histological studies of melanoma show frequent expression of Slug, E-cadherin, and MITF but also considerable heterogeneity of expression of these proteins among individual cells from the same specimen (Shirley et al., 2012). The aim of this study was to assess the degree of coordinated expression of EMT-associated markers in a series of low passage human melanoma cell lines, comparing expression with that of cultured normal melanocytes. We utilized a series of melanoma lines that were originally derived from New Zealand patients with metastatic melanoma to assess responses to radiotherapy and chemotherapy (Marshall et al., 1992, 1994; Kim et al., 2012). Many of these cell lines have been characterized for genetic mutations in *BRAF*, *NRAS*, *PIK3CA* (phosphatidylinositol-3-kinase), *TP53* (p53), and *CDKN2A* (p16) genes (Parmar et al., 2000; Charters et al., 2011). In this study, we have grown 25 of these melanoma cell lines, characterized their expression of E-cadherin, N-cadherin, Snail, Slug, Axl, p53, Hdm2, and MITF, examining the relationship between protein expression and common genetic aberrations.

MATERIALS AND METHODS

CULTURE OF MELANOMA CELLS AND MELANOCYTES

The 25 New Zealand melanoma (NZM) cell lines were generated from surgical samples of metastatic melanoma as previously described (Marshall et al., 1994; Kim et al., 2012). Written consent was obtained from all patients under Auckland Area Health Board Ethics Committee guidelines. NZM cell lines were grown under low oxygen conditions (5% O_2) in order to mimic physiologically low oxygen levels in tumors. NZM lines were grown in α-modified minimal essential medium (αMEM; Invitrogen, USA) supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), and sodium selenite (5 ng/mL; Roche Applied Sciences, Germany), 100 U/mL of penicillin, 100 μg/mL of streptomycin (PS), and 5% fetal bovine serum (FBS). Human primary melanocytes were purchased from Invitrogen and grown in light sensitive Medium 254 supplemented with human melanocyte growth supplement (HMGS-2; Invitrogen) and PS. Human melanocytes were cultured in an atmosphere of 5% CO_2 in air at 37°C. Genetic analyses of *BRAF*, *NRAS*, *TP53*, *CDKN2A*, and *PIK3CA* in NZM cell lines were carried out. Selected melanoma cell lines were sequenced for mutations in *BRAF*, *NRAS*, and *PIK3CA* as previously described (Kim et al., 2012). Sequencing for mutations in the *TP53* and *CDKN2A* genes has been previously described (Parmar et al., 2000; Charters et al., 2011).

WESTERN BLOTTING

After NZM cells were grown to about 80% confluence, they were washed in ice-cold phosphate buffered saline (PBS), lysed in radioimmunoprecipitation assay buffer and prepared for western blotting as previously described (Kim et al., 2009). Antibodies used were specific for the following epitopes: E-cadherin, N-Cadherin, Snail, Slug, and Axl were from Cell Signaling Technology; MITF was from Abcam; and p53, HDM2, and β-actin were from Santa Cruz. Western blots were quantified using Image J software and expressed as a ratio to β-actin.

STATISTICAL ANALYSIS

Spearman's rank correlation coefficient (r_s) and statistical significance (p) were calculated using standard methods (SPSS). Values of $p < 0.05$ were considered to be statistically significant. Correlation plots were also fitted with best-fit hyperbolae.

RESULTS

EXPRESSION OF E-CADHERIN, N-CADHERIN, Snail, and Slug

Since EMT is normally associated with loss of E-cadherin expression and gain of N-cadherin, we first measured cadherin expression. Normal melanocytes expressed both proteins and about half of the lines (NZM11, NZM85, NZM86, NZM9, NZM17, NZM26, NZM40, NZM50, NZM59, NZM4, and NZM82) showed moderate to strong N-cadherin expression but no E-cadherin expression. The other lines all expressed E-cadherin except for NZM22, which expressed neither (**Figure 1A**). When we quantified the western blots and normalized it to β-actin expression (**Figure 1B**), we observed an inverse correlation between E-cadherin and N-cadherin expression (**Figure 2A**). Quantification and statistical analysis showed a significant negative correlation between E-cadherin and N-cadherin expression ($r_s = -0.578$; $p = 0.002$). Slug, the putative transcriptional repressor for E-cadherin, was expressed in normal melanocytes as well as in all lines with the exception of NZM17. The relative expression of E-cadherin and Snail suggested an inverse correlation (**Figure 1A**). However, quantification (**Figure 2B**) showed this to be not statistically significant ($r_s = -0.272$; $p = 0.18$). We also tested whether expression of these markers was associated with any of the mutations shown in **Table 1**, but no clear relationship was found.

EXPRESSION OF Axl, MITF, p53, and Hdm2

It has been previously reported that EMT is associated with increased Axl expression (Gjerdrum et al., 2010) and reduced MITF expression (Sensi et al., 2011). We measured Axl expression and found it only in a proportion of cell lines (**Figure 3A**). Although it appeared from western blots that Axl expression was inversely correlated to E-cadherin expression, quantitation failed to show significance ($r_s = -0.108$). MITF has several isoforms (Yasumoto et al., 1998), and the A and M isoforms are expressed in the melanocyte lineage (Goding, 2000) with the M isoform having differentially spliced variants (Hodgkinson et al., 1993; Steingrimsson et al., 1994; Selzer et al., 2002). Both MITF-A and MITF-M were found in the cell lines (**Figure 3**), with the MITF-M isoform appearing as two differentially spliced variants. We quantified blots for MITF isoforms (**Figure 3B**) and observed a statistically significant inverse relationship ($p = 0.006$) between MITF-M expression and Axl expression (**Figure 4A**). Several cell lines (NZM49, NZM22, and NZM7), as well as melanocytes, expressed both Axl and MITF. Interestingly, NZM49 and NZM22, which express both MITF and Axl, expressed more MITF-A than other cell lines. Furthermore, there was a significant negative correlation between MITF-M and N-cadherin expression ($r_s = -0.562$; $p = 0.007$; **Figure 4B**) and a significant positive correlation between MITF-M expression and E-cadherin ($r_s = 0.514$;

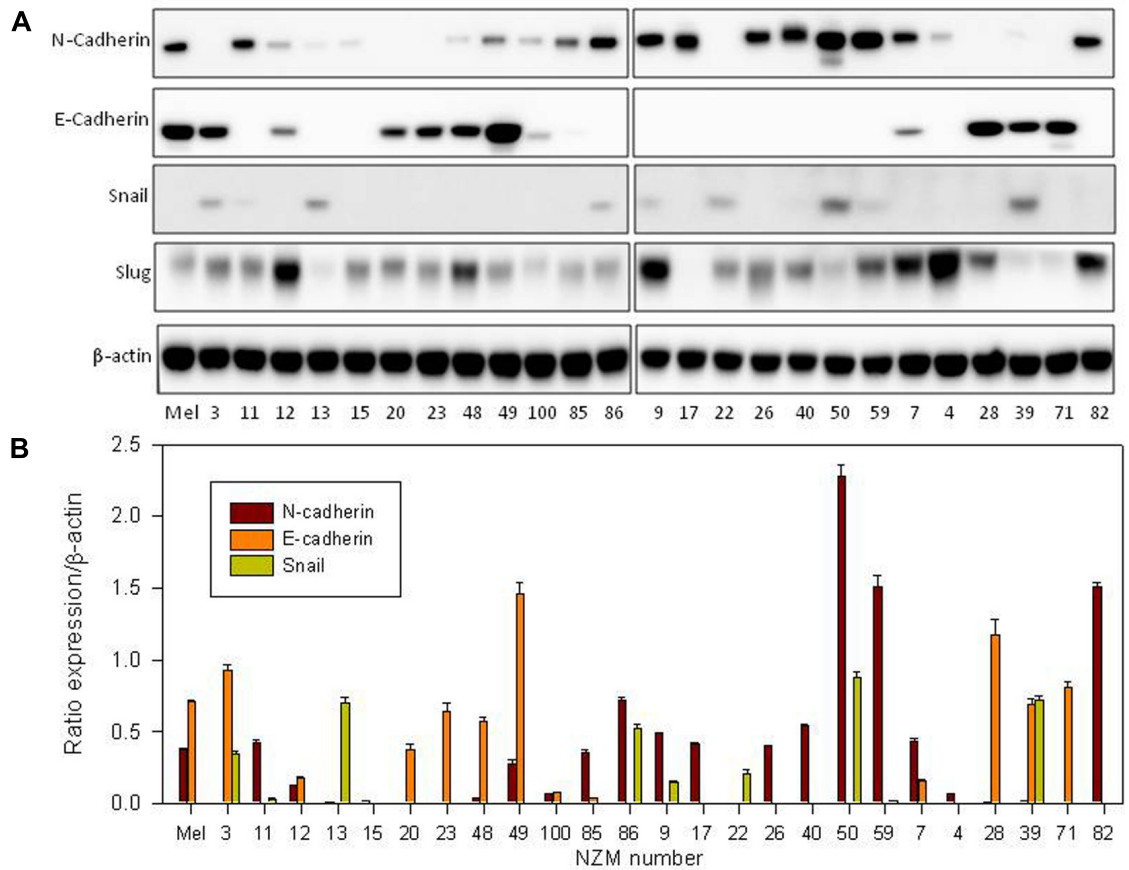

FIGURE 1 | (A) Western blots of whole-cell extracts derived from cultures of normal melanocytes and of a number of melanoma lines, indicating expression of N-cadherin, E-cadherin, Snail, and Slug. The numbers indicate the identities of members of the New Zealand melanoma collection (e.g., 3 = NZM3); Mel indicates data for normal melanocytes. The western blot shown is representative of three independent repeats. **(B)** Western blot quantification of E-cadherin, N-cadherin, and Snail as ratios to β-actin loading controls. Bars show SEM.

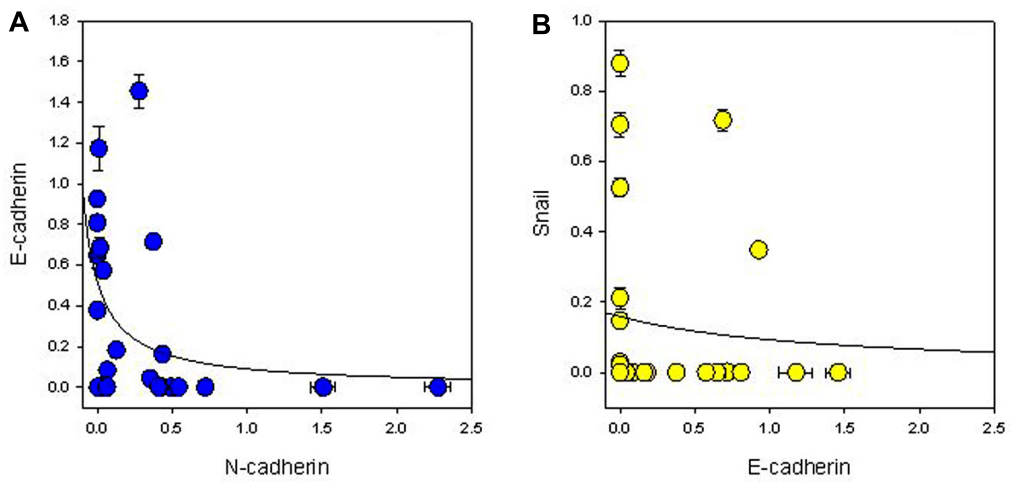

FIGURE 2 | Relationship between expression of (A) N-cadherin and E-cadherin, and (B) E-cadherin and Snail normalized to β-actin expression. The lines indicate best-fit hyperbolae.

Table 1 | Genetic status of the melanoma lines used in this study.

NZM	*BRAF*	*NRAS*	*TP53*	*CDKN2A*	*PIK3CA*
3	V600E			Deletion	
4	V600E		241S/P		
7	V600E		241S/P/WT		
9			179C/T	Deletion	
11	V600E			Deletion	
12	V600E				
13				Deletion	
15		Q61K			
17		Q61K	241S/T		
20	V600E			Deletion	
22			241S/T/W		
23					
26	V600E		136A/G		
28			241S/T/WT + 159a/v		
39			213A/G	Deletion	
40		Q61H	Del 249-253		H1047R
48		Q61K			
49	V600E			Deletion	
50			R280T		
59			Silent T/G	Deletion	
71					
82					
85					
86					
100					

$p = 0.007$; not shown). Since it has been reported that loss of p53 expression is associated with EMT (Gadea et al., 2007), we also measured expression of p53 and of Hdm2, a protein closely associated with p53 degradation (Araki et al., 2010). However, there was no obvious relationship between expression of either p53 or Hdm2 and that of other EMT markers (**Figure 3A**). As MITF has been noted to be one of the key molecular switches that determine switching of different cell progeny (Cheli et al., 2011), we also stained for MITF to observe expression in individual cells within the same cell line. Interestingly, in NZM86 and NZM40 (two cell lines that express very low MITF as determined by western blotting) we observed individual cells that expressed detectable levels of MITF (**Figure 5**) scattered amongst low MITF expressing cells.

DISCUSSION

The analysis of this series of early passage human melanoma lines has shown them to be highly heterogeneous not only with respect to expression of proteins directly associated with EMT such as E-cadherin, Snail, Slug, and Axl (**Figure 1**) but also with respect to expression of proteins that are more indirectly associated with EMT, such as MITF and p53 (**Figure 3**). Melanoma lines (with one exception) and normal melanocytes, expressed Slug. Other markers are generally strongly expressed in some lines but not others. Among the melanoma lines, we found that expression of MITF-M, the melanocyte-specific isoform of MITF, was positively related to that of E-cadherin but inversely related to that of N-cadherin and Axl (**Figures 4A,B**). A possible interpretation of the results is that melanoma lines show mesenchymal properties overall, but that individual lines vary between a high E-cadherin/high MITF-M expression and a high N-cadherin/high Axl expression phenotype. Cultured normal melanocytes show an intermediate phenotype, expressing all markers.

The results agree with an earlier study reporting that Axl-positive melanoma cells do not express MITF (Sensi et al., 2011). They also support a previous study that used a series of NZM melanoma cell lines to identify a gene expression signature that distinguished two phenotypes differing in their *in vitro* invasive potential (Jeffs et al., 2009). Although the cell lines used in that study overlap only partially with the lines used in the present study it is evident that the six lines with the "non-invasive" signature (NZM3, NZM4, NZM7, NZM12, NZM15, and NZM20) expressed MITF but little or no Axl while four with the "invasive" signature (NZM9, NZM11, NZM22, and NZM40) expressed no MITF but often expressed Axl (**Figure 3**).

One of the important questions posed in this study is whether the pattern of expression of proteins in the EMT pathway is related to genetic mutation. A detailed analysis of the mutational status of the melanoma lines will be reported elsewhere in this issue (Stones et al., 2013) but with the available data shown in **Table 1**, we have been unable to detect any significant relationship between expression of proteins shown in **Figures 1** and **3** and the mutational status of *BRAF*, *NRAS*, *TP53*, *CDKN2A*, or *PIK3CA*. These results echo those obtained from a study on the utilization of enzymes in the PI3K–PKB (phosphoinositide 3-kinase–protein kinase B), MEK–ERK (mitogen-activated protein kinase kinase–extracellular signal-regulated kinase), and mTOR–p70S6K (mammalian target of rapamycin–p70 ribosomal S6 kinase) signaling pathways. As determined by phosphorylation of signaling components, phosphorylation varied widely across a series of cell lines but did not directly reflect the *PIK3CA*, *PTEN*, *NRAS*, or *BRAF* mutational status of genes of these lines (Kim et al., 2012). A feature of the results is that individual melanoma lines vary enormously in their expression of particular proteins. This extends a previous study showing a large amount of heterogeneity in expression of MITF and the melanocyte lineage proteins PAX3 across a series of NZM lines, with cellular protein levels varying by 15-fold and more than 100-fold, respectively (He et al., 2011). Phenotypic switching has previously been suggested to explain differences in transcription signatures that correspond to different cellular phenotypes (Hoek et al., 2008; Hoek and Goding, 2010) and could account for the differences in protein expression.

Recently, MITF has been suggested to be crucial in determining whether melanoma cells proliferate (melanoma initiating cells) or change to accommodate a more invasive phenotype (Carreira et al., 2006; Hoek and Goding, 2010; Cheli et al., 2011); this has formed the basis for the hypothesis discussed separately in this

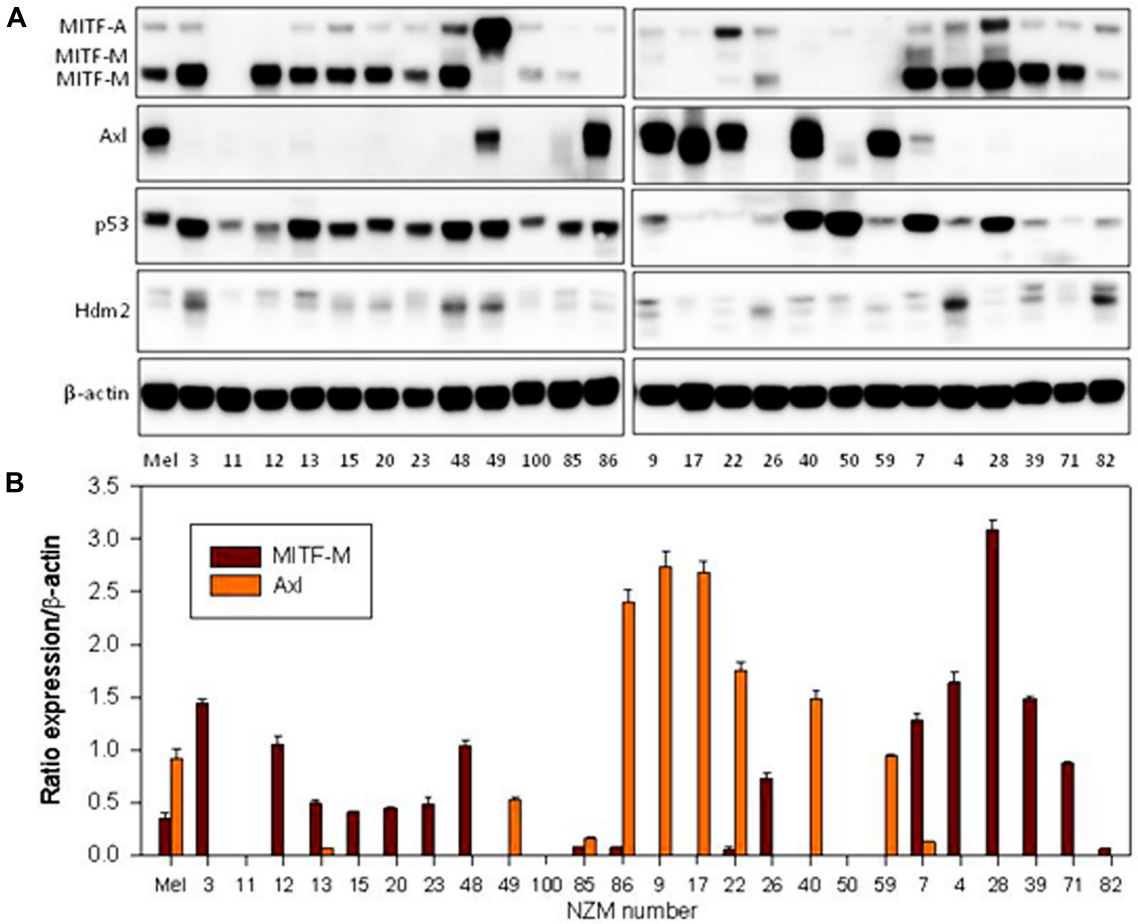

FIGURE 3 | (A) Western blots of extracts from cultures of normal melanocytes and of a number melanoma lines, indicating expression of MITF-M (bottom two bands), MITF-A (top band), Axl, p53, and Hdm2. The numbers indicate the identities of members of the New Zealand melanoma collection; Mel indicates data for normal melanocytes. The western blot shown is representative of three independent repeats. **(B)** Western blot quantification of MITF-M and Axl as ratios to β-actin loading controls. Bars show SEM.

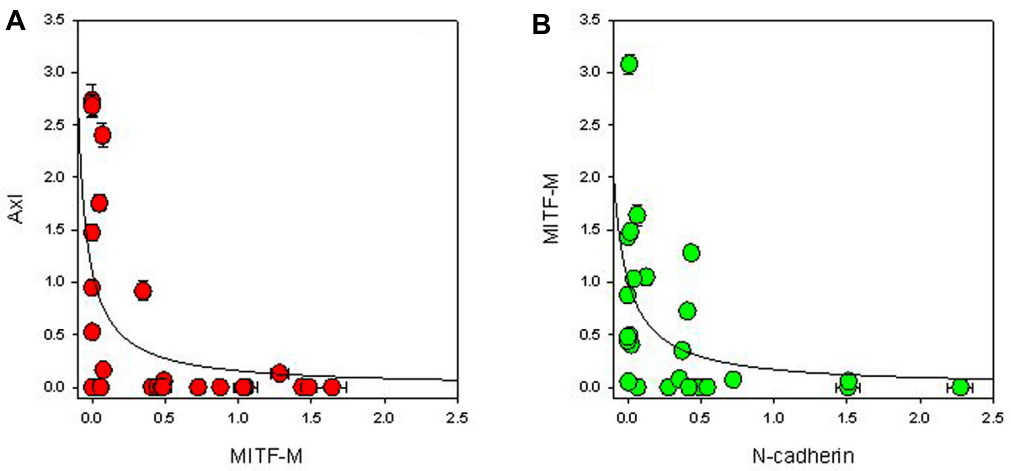

FIGURE 4 | Relationship between expression of (A) MITF-M and Axl, and (B) MITF-M and N-cadherin normalized to β-actin expression. The lines indicate best-fit hyperbolae.

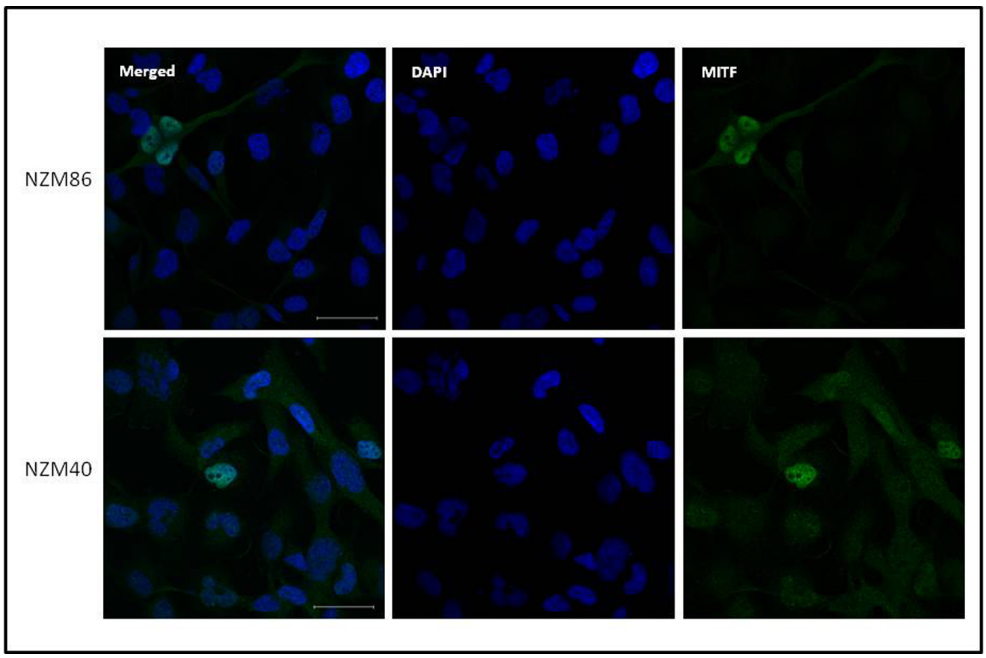

FIGURE 5 | Immunofluorescent images of NZM40 and NZM86 stained for MITF (green) and for DNA (diaminophenylindole: blue). Scale bars on the merged image indicate 50 μm.

issue (Eccles et al., unpublished). The mechanistic basis of such switching has not yet been elucidated but the concept is consistent with evidence that melanomas cells do not have a defined hierarchical organization with stem cells at one end and differentiated cells at the other (Quintana et al., 2008). Rather, each cell in a population may have a certain probability of switching to or from a phenotype with stem cell characteristics. There are speculations as to what could induce or decrease MITF activity (Strub et al., 2011) and determine the invasiveness or the stemness of the melanoma cells in response to hypoxia (Cheli et al., 2012) or to other factors in the tumor microenvironment (Li et al., 2003). One interesting observation is that even though NZM40 and NZM86 show low MITF expression by western blotting, we clearly see by microscopy that some cells highly express MITF (**Figure 5**), which is evidence of a heterogeneous population of cells (Quintana et al., 2010).

Histological studies on *in vivo* human melanoma tissue have shown considerable heterogeneity by individual cells in expression of markers associated with EMT (Shirley et al., 2012) and this is consistent with the *in vitro* histological data shown in **Figure 5**. It is possible that melanoma tissue *in vivo* shows even greater phenotypic diversity than the derived cell lines. Thus, as shown diagrammatically in **Figure 6**, the *in vivo*, population develops, by phenotypic switching, a diverse population with individual cells exhibiting a high E-cadherin/high MITF-M expression on one hand or a high N-cadherin/high Axl expression on the other. Melanomas *in vivo* generally have cell cycle times of approximately 1 week, while derived cell lines have cell cycle times of 1–2 days (Baguley, 2011). Development of cell lines thus exerts a strong selective pressure for outgrowth of more rapidly cycling cells and

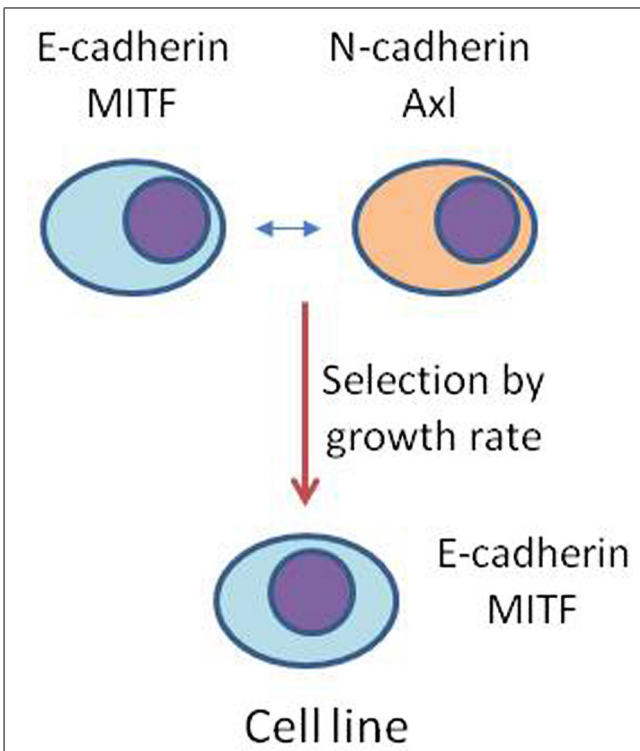

FIGURE 6 | Possible model for the generation of melanoma cell lines. Phenotypic switching *in vivo* generates a highly heterogeneous population of cells that vary in expression of proteins such as E-cadherin, N-cadherin, Axl, and M-MITF. Derivation of a cell line, by selecting for rapid proliferation, may select for an individual phenotype.

may tend to select one of these phenotypes. Thus, melanoma tissue may be characterized as a mixture of phenotypes, some expressing high MITF-M and E-cadherin with more differentiated non-invasive behavior, and others expressing high N-cadherin, Slug, and Axl and with a more invasive behavior.

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MITF and PAX3 play distinct roles in melanoma cell migration; outline of a "genetic switch" theory involving MITF and PAX3 in proliferative and invasive phenotypes of melanoma

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Melanoma is a very aggressive neoplasm with a propensity to undergo progression and invasion early in its evolution. The molecular pathways underpinning invasion in melanoma are now just beginning to be elucidated, but a clear understanding of the transition from non-invasive to invasive melanoma cells remains elusive. Microphthalmia-associated transcription factor (MITF), is thought to be a central player in melanoma biology, and it controls many aspects of the phenotypic expression of the melanocytic lineage. However, recently the paired box transcription factor PAX3 was shown to transcriptionally activate POU3F2/BRN2, leading to direct repression of MITF expression. Here we present a theory to explain melanoma phenotype switching and discuss the predictions that this theory makes. One prediction is that independent and opposing roles for MITF and PAX3 in melanoma would be expected, and we present empirical evidence supporting this: in melanoma tissues PAX3 expression occurs independently of MITF, and PAX3 does not play a key role in melanoma cell proliferation. Furthermore, we show that knockdown of PAX3 inhibits cell migration in a group of "lower MITF" melanoma cell lines, while knockdown of MITF promotes cell migration in a complementary "higher MITF" group of melanoma cell lines. Moreover, the morphological effects of knocking down PAX3 versus MITF in melanoma cells were found to differ. While these data support the notion of independent roles for MITF and PAX3, additional experiments are required to provide robust examination of the proposed genetic switch theory. Only upon clear delineation of the mechanisms associated with progression and invasion of melanoma cells will successful treatments for invasive melanoma be developed.

Keywords: melanoma, phenotype switching, paired box transcription factors, microphthalmia-associated transcription factor, migration and invasion, pax3

INTRODUCTION

Melanoma is a malignant neoplasm of the neural crest-derived melanocytes, the pigment-producing cells. Approximately 65% of cutaneous melanomas are thought to arise from individual cutaneous melanocytes, while ∼25% arise from a pre-existing nevus. The remaining melanomas (4–12%) appear to arise *de novo* with no identifiable primary tumor. Melanoma is a very aggressive neoplasm with a high risk of metastasis early in tumorigenesis. Despite numerous studies, the mechanisms underlying metastasis are complex, and a clear understanding remains elusive.

Acquisition of the ability of tumor cells to migrate represents a defining characteristic of cancer metastasis. However, cell migration is also necessary during embryogenesis and homeostasis of multicellular organisms. Indeed, recent studies suggest that melanoma cells revert to an embryonic program of gene expression involved in neural crest cell migration to support developmental plasticity and metastasis (1). Numerous factors are involved in the differentiation of melanocytes, and also in the control of cell migration.

PAX3, a member of the paired box family of genes, is a key developmental regulator of the neural crest and its derivatives, including melanocyte progenitors (2). PAX3 is expressed in melanoma tissues and cell lines, melanocyte cell lines (3, 4), and circulating melanoma cells. Several groups (5, 6) have shown that PAX3 protein is expressed in normal skin melanocytes and melanocytic lesions. *PAX3* expression is thought to contribute to cell survival and growth (3, 4) in the melanocytic lineage. Several studies have suggested that PAX3 expression is important in regulating the transition from an early melanoblast derived from the neural crest into mature melanocytes. Knockdown of PAX3 expression in melanoma cells leads to reduced or arrested cell growth, and the induction of apoptosis and/or senescence (3, 4).

Microphthalmia-associated transcription factor (MITF) is another important developmental regulator of neural crest and its derivatives (7). MITF has been suggested to be an important melanoma growth and survival factor (8). For instance, FOXD3, a neural crest-associated transcription factor, is able to repress MITF through non-canonical mechanisms, and regulate the lineage commitment of bi-functional neural crest-derived glial/melanocyte precursor cells into either the melanocyte or glial lineages (9). Analysis of MITF expression in melanoma cell lines, as well as melanoma tissues reveals marked variability in expression level, with some melanoma cell lines expressing up to 10-fold higher levels of MITFm, a melanocyte-specific isoform of MITF, than in other melanoma cell lines (10).

The variable levels of MITF expression in melanoma may have important consequences. Low levels of MITF expression have been shown to identify a small group of melanoma patients with high mortality. Agnarsdottir and colleagues showed that patients with melanomas where 25–75% of tumor cells stained with weak intensity for MITF expression using an anti-MITF antibody were at higher risk of death than patients with an overall strong MITF staining intensity (11). This effect of low MITF expression level on patient survival may be through various roles that MITF is thought to play in cell invasion- and proliferation-associated pathways. High MITF levels are thought to promote cell proliferation through the direct activation of the *DIAPH1* gene, one of many MITF target genes (12). High MITF expression has also been shown to transcriptionally activate microRNA *miR-211* expression, expressed from within the MITF target gene, *TRPM1*, which results in reduction in the levels of *POU3F2* (*BRN2*) mRNA (13). In contrast, under conditions of low MITF expression there is increased ROCK activity downstream of Rho, an important mediator of cell migration (12).

High expression of POU3F2 in melanoma represseses *MITF* expression (14). Moreover, POU3F2 both transcriptionally activates (including transactivation of *PAX3*) and represses genes leading to enhanced cell migration/invasion and stem cell-like characteristics (15–20). Indeed, POU3F2 is part of the phosphatidylinositol 3-kinase (PI3K)-PAX3-POU3F2 (BRN2) axis that has been proposed to promote melanoma cell invasion (21). Bonvin and colleagues showed that inhibiting the PI3K pathway causes down-regulation of POU3F2 and PAX3 expression, and that PAX3 directly bound to and transactivated the *POU3F2* promoter, up-regulating *POU3F2* expression. These findings implicate PI3K signaling in PAX3-dependent enhancement of *POU3F2* expression and melanoma cell invasion, while simultaneously inhibiting *MITF* expression (21).

A second signaling pathway that leads to cell migration also involves the downstream activation of PAX3 expression; fibroblast growth factor 2 stimulates STAT3-mediated regulation of *PAX3* expression in melanocytes (22). Moreover, STAT3 activation promotes cell migration through phosphorylation of STAT3, requiring Rho Kinase (ROCK) and JAK activity (23). Phosphorylated STAT3 transcriptionally activates *PAX3* expression in melanocytes, and the silencing of *STAT3* or *PAX3* using RNAi was recently shown to inhibit the growth of melanoma cells, particularly in melanoma cells that have acquired resistance to the BRAF inhibitor, vemurafenib (24). These studies suggest that PAX3 expression can promote melanoma progression, and that PAX3 plays an important role in acquired resistance and recurrence of melanoma following treatment with tyrosine kinase inhibitors.

Over the last 20 years a unique series of cell lines (NZM cell lines) from metastatic melanomas (MMs) occurring in New Zealand patients has been developed (25). To date the NZM cell lines have been characterized for cell cycle time, drug sensitivity, and driver gene mutation status. We recently profiled global gene expression in a panel of 25 of these cell lines, and showed that NZM and other melanoma cell lines could be classified into two major groups represented by relatively lower (6/25) or higher (19/25) *MITF* transcript levels. In the gene expression signature that distinguished the two groups there were 96 differentially expressed genes, many of which are known targets of MITF, which differed in expression in a similar fashion to MITF (26). The lower MITF cell lines were characterized as having higher rates of migration than higher MITF cell lines in Boyden chamber transwell assays and scratch assays (26).

Here we extend a hypothesis that we previously suggested; that PAX3 and MITF play independent roles in melanoma progression (10). Moreover, we showed previously that PAX3 does not regulate *MITF* in melanoma cells (10) and we now propose a "genetic switch" theory to explain phenotype switching (27), whereby a PAX3-POU3F2 axis and a MITF-*miR-211* axis function to negatively regulate each other. This predicts that PAX3 and MITF play distinct roles in signaling pathways that promote melanoma progression, and also predicts additional features expected in melanoma cells undergoing phenotype switching.

We present here several lines of experimental evidence supporting the notion that PAX3 and MITF expression indeed play independent roles in melanoma progression and cell migration. Firstly, we show that in melanoma tissues expression of MITF and PAX3 occur independently, and are variable from region to region, and furthermore that the expression of PAX3 is not correlated with Ki67 expression, a marker of cell proliferation. In addition, we show that in melanoma cell lines with lower levels of MITF expression, knockdown of *PAX3* expression inhibits melanoma cell migration, whereas in melanoma cell lines with higher levels of MITF, knockdown of *MITF* enhances cell migration. In addition we show that PAX3 promotes increased *POU3F2* transcript levels, which then leads to repression of *MITF* transcript levels. Lastly, we show that the morphological effects of knocking down *PAX3* versus *MITF* in melanoma cells differ. Taken together with earlier published data (10), the evidence suggests that there are distinct roles for PAX3 and MITF in melanoma progression and melanoma cell migration, thus providing supporting evidence for one of the key predictions of the genetic switch theory.

OUTLINE OF A GENETIC SWITCH THEORY FOR THE INVOLVEMENT OF PAX3 AND MITF AXES DURING "PHENOTYPE SWITCHING" IN MELANOMA

The genetic switch theory is outlined in **Figure 1** and embodies the concept of two interacting signaling pathways represented by

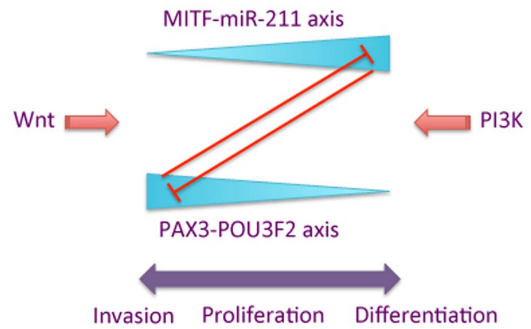

FIGURE 1 | A schematic of the "genetic switch" model. The MITF-miR-211 axis is counterbalanced by the PAX3-POU3F2 axis, with high levels of each pathway inhibiting the corresponding other pathway. "Wnt" and "PI3K" represent one of several possible signaling pathways promoting the activity of MITF-miR-211 axis versus the PAX3-POU3F2 axis, respectively. High levels of the PAX3-POU3F2 axis (represented by the thick end of the wedge) are associated with cell migration and invasion of melanoma cells, while high levels of the MITF-miR-211 axis are associated with proliferation and/or differentiation of melanoma cells.

PAX3-POU3F2 and MITF-*miR-211*, two key pathways contributing to the strength of lineage commitment and phenotypic choice of individual melanoma cells.

In the genetic switch model we firstly note that PAX3 does not transcriptionally activate *MITF* in melanoma cells. Our earlier studies (10) support this notion. While in normal cells of the melanocytic lineage during melanocyte development PAX3 transcriptionally activates *MITF*, it is clear there is a difference in melanoma cells (10), and we propose this difference might be an important feature underlying the malignant potential of melanoma cells.

Our theory is consistent with data presented by Carreira and colleagues (12), who proposed that MITF functions like a "rheostat" with respect to cell migration. In their paper they suggested that, depending on the expression level, MITF plays a role in stem cell-like properties, and proliferation of melanocytes and melanoma cells, with an effect on terminal differentiation or senescence of cells at very high levels. Our genetic switch theory extends this model, and suggests that the rheostat model may only be half the story. In the genetic switch model we propose that the MITF-*miR-211* axis inhibits cell migration and promotes cell differentiation in cells where the relative expression of MITF is high. Conversely, we propose that when MITF levels are low, the expression of the PAX3-POU3F2 axis is high, and that this then promotes cell migration and stem cell-like properties (**Figure 1**).

The patterns of gene expression that we, and others, have previously characterized in melanoma cell lines (26–28) might reflect the bi-modal nature that would be predicted by the genetic switch model. In the NZM cell lines high expression of MITF and many of its target genes, and the low expression of another set of genes, were found to be typical of one gene expression signature, while the low expression of MITF and its target genes, and the high expression of the other gene set were typical of an alternative gene expression signature. Evidence of *in vivo* switching between two such alternative gene expression signatures has been suggested (27). Moreover, expression of the "lower MITF" gene signature corresponds to melanoma cells with a higher rate of migration, and migration rates in the "higher MITF" melanoma cell type were able to be enhanced by knocking down expression of *MITF* (26).

Within melanoma tissues, depending on localized exposure to external signals or cues, signals such as PI3K or STAT3 in the external "milieu" could activate the PAX3-POU3F2 axis, and therefore initiate migratory stem cell-like properties in melanoma cells (21, 22). Alternatively, external Wnt signals (for instance) might activate the MITF-*miR-211* axis, and so promote the expression of adhesion molecules to anchor migrating melanoma cells in order to colonize and proliferate in distal sites (8). Given predictions that relatively high numbers of stem-like cells may exist in melanoma, it may be that, *in vivo*, there is a relatively high frequency of the conversion rate from the proliferative phenotype to the migratory "stem cell-like" phenotype in melanoma cells compared to the reverse conversion rate.

Furthermore, accumulating evidence supports two models of how melanoma cells move, a cytoskeletal model of dynamic actin microfilaments, and a membrane flow model of plasma membrane deposition and recycling (23). In the former of these models it has been shown that STAT3 signaling plays an important role, which again provides supporting evidence for the role of a PAX3-POU3F2 axis in promoting cell migration.

Aside from what we have discussed above, several predictions arise from the proposed genetic switch theory. The first of these is that MITF and PAX3 should both have independent roles and expression patterns in melanoma cells. The second prediction is that the MITF-*miR-211* axis will prevail precisely when the PAX3-POU3F2 axis wanes, and vice versa. This prediction will need to be investigated in *in vitro* and *in vivo* models. *In vitro*, it is predicted that *MITF* and/or *miR-211* expression would be enhanced in melanoma cells with knockdown of the *PAX3-POU3F2* axis, and that *PAX3* and/or *POU3F2* expression will be enhanced in melanoma with knockdown of *MITF-miR-211* axis. We have already obtained preliminary evidence that the knockdown of *MITF* leads to increased *POU3F2* mRNA levels in NZM12 melanoma cells (He, Jeffs et al., unpublished data). The third prediction is that during periods of enhanced cell migration, melanoma cells would be under the influence of the PAX3-POU3F2 axis, and that melanoma cells not enhanced in migration would be under the influence of the MITF-*miR-211* axis. The fourth prediction is that high activity of the PAX3-POU3F2 axis would lead to stem cell-like features, with reduced pigmentation, reduced mitotic activity of melanoma cells, and enhanced resistance to drugs that inhibit proliferation, while high activity of the MITF-*miR-211* axis would lead to more strongly differentiated melanocytic features, enhanced pigmentation, and enhanced mitotic activity, with reduced resistance to drugs that inhibit proliferation. A fifth prediction that perhaps arises from all the above predictions, but is important nevertheless in translating to patients, is that the use of treatments in patients targeting MITF might result in enhancement of melanoma metastasis.

RESULTS

PAX3 AND MITF EXPRESSION VARY IN THEIR RELATIVE INTENSITY IN DIFFERENT REGIONS OF MELANOMA

Several experimental approaches were used to investigate whether PAX3 and MITF expression and function were independent in melanoma cells and tissues. In the first approach we used dual label immunofluorescence to compare the relative expression of PAX3 and MITF in adjacent regions within the same melanoma tissue section. To do this PAX3 and MITF were immunolabeled with different fluorophores, and the captured immunofluorescent images merged. The relative saturation (intensity) of the signals in the merged image were then visualized (**Figure 2**). Relative levels of MITF expression in normal skin melanocytes (observed as a single layer of cells in the basal layer of the epidermis – see **Figure 2**) seemed to vary in relation to each other more than the variation in PAX3 expression (**Figures 2A,B**). In melanoma the expression of MITF appeared to be generally more intense relative to PAX3, and tended to involve cells immediately underlying the epidermal surface ("Top dermis," **Figure 2B**). Cells that expressed lower levels of MITF relative to PAX3 were often located deeper below the epidermal surface of the tissue ("Deep dermis," **Figure 2B**). This was also observed in dysplastic nevi (not shown). We show two representative melanoma tissues (MM and lentigo maligna melanoma) where cells more distal to the epidermal surface showed a lower intensity of MITF expression relative to PAX3 expression, and a change in color saturation was observed in melanoma cells more distal to the epidermal surface compared to cells immediately below the epidermal surface (**Figures 2A,B**). These data suggest that variations in the relative levels of *PAX3* and *MITF* protein occur from region to region in melanoma tissues. Such variations in the expression of these factors could impact on the invasive behavior of melanoma cells.

PAX3 IS RELATIVELY INFREQUENTLY CO-EXPRESSED WITH Ki67 IN MELANOMA TISSUE

We next investigated whether expression of PAX3 is associated with loss of growth control in melanoma, which is a role that MITF has been implicated (8), as might be expected if PAX3 and MITF were to function in the same or similar pathways. We investigated whether PAX3 expression is co-localized with the cell proliferation marker, Ki67, scoring cells that were positive in immunofluorescence for both PAX3 and Ki67 as a percentage of the total number of PAX3-positive cells. Expression of PAX3 practically never co-localized with Ki67 expression in nevi (**Figure 3**), suggesting that the expression of PAX3 was in general not associated with proliferation in nevi. Co-localization of Ki67 with PAX3 was also relatively infrequent in melanomas including superficial spreading, lentigo maligna melanoma and nodular melanoma, and MMs, with an average of only ∼20% of cells co-expressing Ki67 and PAX3 in the latter (**Figure 3**). The observation that the expression of PAX3 does not markedly overlap with Ki67-positive melanoma cells (as the majority of PAX3-positive cells were Ki67-negative) suggests that PAX3 expression is not associated with cell proliferation in melanoma. The observed low frequency (∼20%) of incidental co-expression of PAX3 and Ki67 could simply reflect progressive deregulation of growth control in melanoma cells, as marked by Ki67 expression.

RNAi-MEDIATED KNOCKDOWN OF PAX3 GENE EXPRESSION LEADS TO REDUCED POU3F2 mRNA LEVELS AND MIGRATORY BEHAVIOR AND INCREASED MITF mRNA LEVELS IN MELANOMA CELL LINES

We previously characterized a panel of melanoma cell lines for expression levels of MITF and PAX3, and cell migratory behaviors (10, 26). Four melanoma cell lines chosen from this panel were transfected with siRNAs against *PAX3* to determine whether migration of the melanoma cells depended on *PAX3* expression. Knockdown of *PAX3* in NZM9 and NZM40, characterized to have lower levels of MITF expression and a high cell migration potential (10, 26), led to a significant decrease in migration rate ($p < 0.001$, **Figure 4**), whereas the knockdown of *PAX3* in NZM6 and NZM15 (characterized to have higher levels of MITF expression and a low migration potential) did not cause a significant change in the migration rates in transwell assays. In contrast, knockdown of *MITF* in NZM6 and NZM15 cell lines caused an average of ∼4-fold increase in migration rate in the transwell assays ($p < 0.001$, **Figure 4**), whereas in the highly motile NZM9 and NZM40 cell lines there was no significant change in migration potential observed with *MITF* knockdown (**Figure 4**).

In two different NZM melanoma cell lines (NZM11 and NZM12), one of which has previously been characterized to be a "lower MITF" cell line (NZM11), and the other cell line previously characterized as a "higher MITF" cell line (NZM12) (10, 26), the knockdown of *PAX3* expression resulted in decreased levels of *POU3F2* transcripts in both cell lines (**Figure 5**). In the NZM12 cell line there was a concomitant increase in *MITF* transcript levels, consistent with the proposed genetic switch hypothesis (**Figure 5**). An increase in the levels of both *MITF* mRNA and protein in NZM12, NZM11, NZM9, and NZM15 cell lines in response to PAX3 knockdown has also previously been reported (10). Interestingly, in two other "lower MITF" expressing melanoma cell lines (i.e., NZM9 and NZM40) there were undetectable levels of *POU3F2* expression. It is possible that these "lower MITF" melanoma cell lines have an alternative pathway to suppress *MITF* that does not involve *POU3F2* expression.

These data suggest that relatively higher expression of *PAX3* compared to *MITF* in "lower MITF" cell lines may facilitate cell migration in those melanoma cell lines, while relatively higher expression of *MITF* to *PAX3* in "higher MITF" cell lines may inhibit cell migration.

RNAi-MEDIATED KNOCKDOWN OF PAX3 OR MITF GENE EXPRESSION LEADS TO DIFFERENT PHENOTYPIC MORPHOLOGIES IN MELANOMA CELLS

The knockdown of either PAX3 or MITF expression had different effects on the morphology of NZM15 cells in culture (**Figure 6**). Knockdown of MITF in NZM15 cells led to cells with a fibroblast-like spindle-cell phenotype with dendrites protruding from the cells. In contrast, knockdown of PAX3 led to the cells acquiring an epithelial-like rounded phenotype with few dendrites.

DISCUSSION

The data presented here are consistent with and extend our previous work, in which we showed that melanoma cell lines with

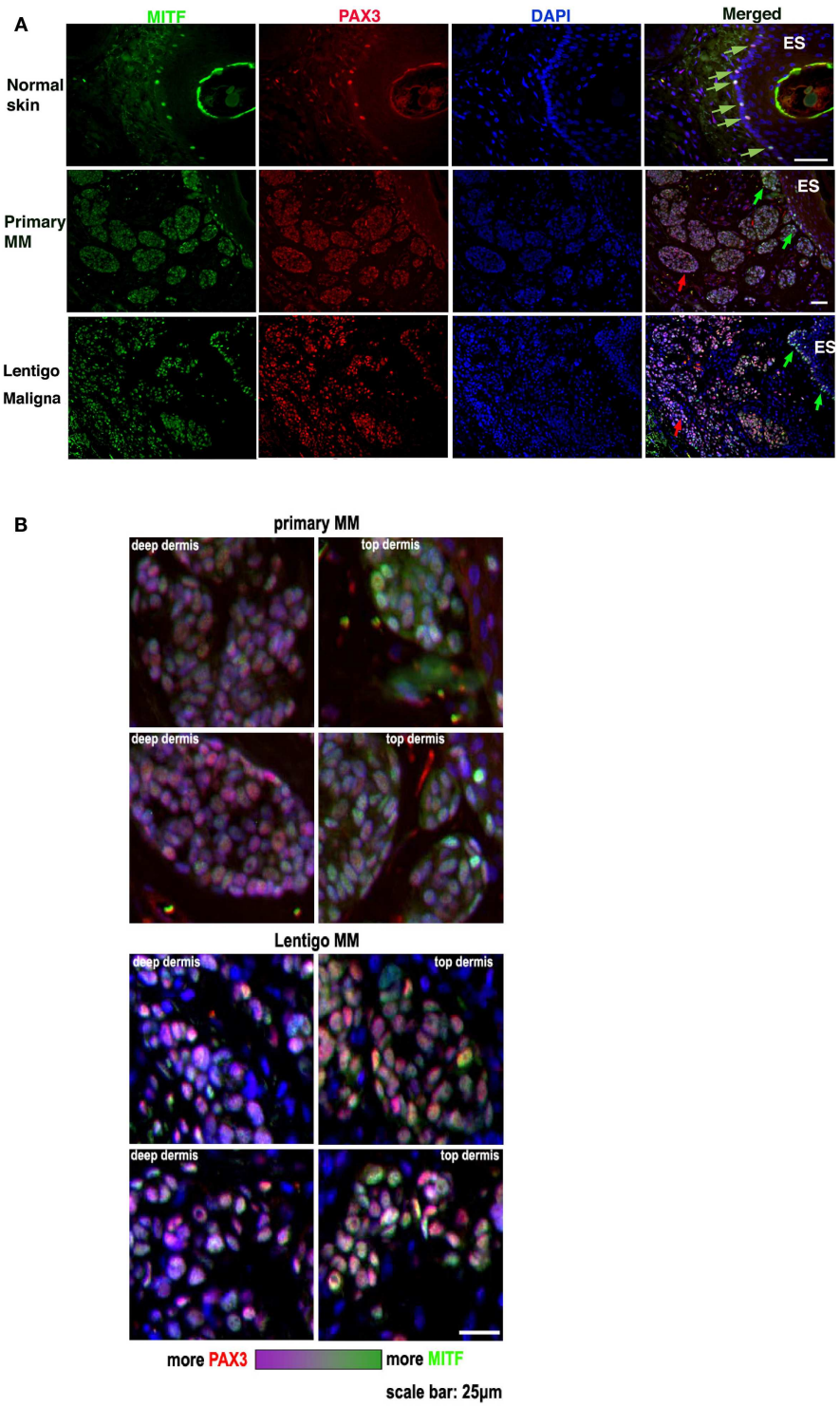

FIGURE 2 | PAX3 and MITF expression vary in their relative intensity in different regions of melanoma. **(A)** The figure shows photomicrographs of dual immunofluorescent staining of MITF expression (green label), PAX3 expression (red label), DAPI nuclear staining (blue label), and a merged image in normal skin, metastatic melanoma (Primary MM), and Lentigo maligna melanoma. The scale bar, which varies in length on the images, represents 50 μm. **(B)** Higher magnification photomicrographs taken from the merged image in **(A)** of the Primary and Lentigo maligna melanomas show in greater detail the difference in the relative immunofluorescence color intensity of MITF labeling versus PAX3 labeling in the tumor cells immediately below the skin surface ("Top dermis") versus cells located deeper in the tumor ("Deep dermis"). Below the panels is shown a color intensity scale, with one end representing relatively strong MITF intensity, and the opposite end representing relatively strong PAX3 intensity. The scale bar in the bottom right image is for all the panels and represents 25 μm.

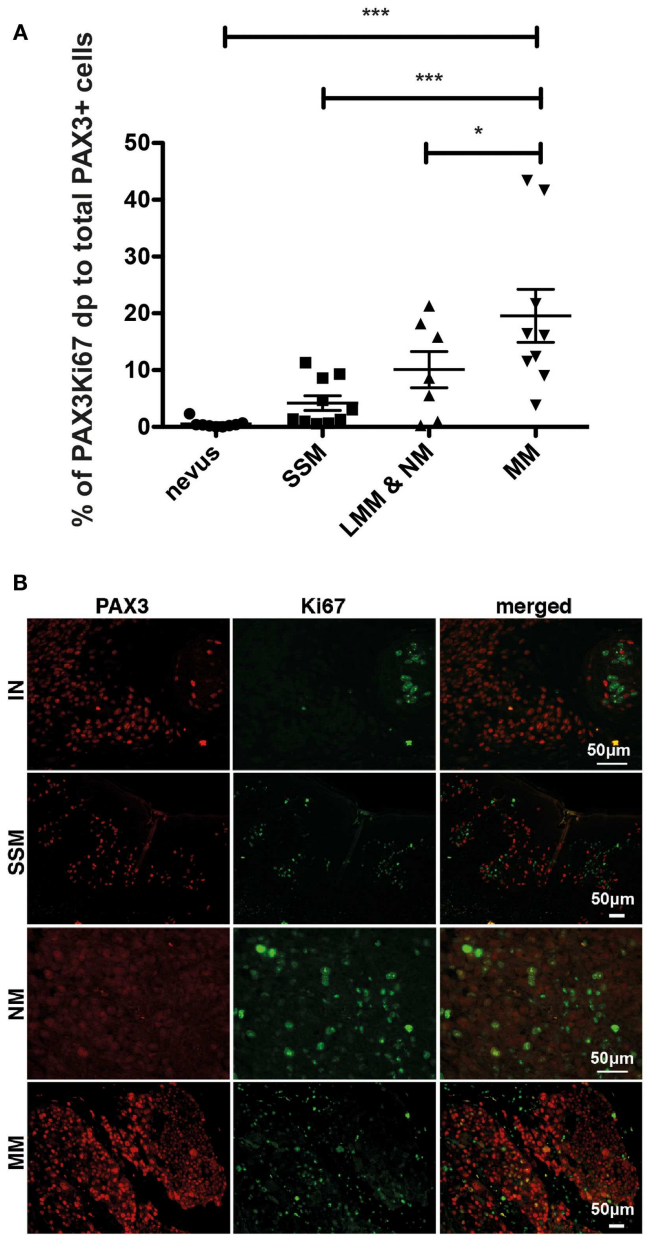

FIGURE 3 | Dual immunofluorescent staining of Ki67 and PAX3 in benign and malignant melanocytic lesions. **(A)** Graph showing the percentage of PAX3 and Ki67 double positive (dp) cells as a percentage of the total number of PAX3-positive cells for superficial spreading melanoma (SSM), lentigo maligna melanoma and nodular melanoma (LMM & NM), and metastatic melanoma (MM), $***p < 0.001$. **(B)** Photomicrographs showing the results of immunofluorescent staining for PAX3, Ki67, and merged images for an intradermal nevus (IN), superficial spreading melanoma (SSM), nodular melanoma (NM), and metastatic melanoma (MM). The scale bar, which varies in length on the images, represents 50 μm.

low levels of MITF expression (NZM9, NZM11, NZM22, NZM40, and NZM52) have higher (∼23-fold) migratory potential than melanoma cell lines with high MITF expression levels (NZM6, NZM12, NZM15, NZM42, NZM45). The latter cell lines have a low migratory potential using scratch and transwell (Boyden chamber) migration assays (26). Furthermore, we previously showed that knockdown of MITF expression in NZM6 and NZM15 melanoma cell lines led to an almost 4-fold increase in the migration rates of the cells (26).

Our results suggest that PAX3 might play a role in melanoma cell invasion (rather than in proliferation), and our data predict that the effect of increased signaling through the PAX3/POU3F2 pathway on cell migration would be most pronounced in melanoma cells *in vivo* where the MITF expression levels are relatively low. In addition, reduced MITF expression levels would occur when POU3F2 expression is elevated, and this would also correspond to instances *in vivo* in melanoma tissues when pigment production is reduced (19). In contrast, in melanoma cells *in vivo* where the MITF expression is high there is likely to be a minimal role of the PAX3-POU3F2 axis in promoting melanoma cell invasion.

We reported previously that PAX3 is extensively expressed in melanocytes, nevi and melanoma tissues (6), and that expression levels of PAX3 and MITF are highly variable in melanoma cell lines, and are not concordant with each other, especially comparing individual melanoma cells in culture (10). We have also previously reported that PAX3 does not transcriptionally activate *MITF* in melanoma cells (10), an observation contrary to that outlined in a number of contemporary melanoma research papers. Current belief has it that in melanoma, PAX3 transcriptionally activates MITF and therefore functions in an epistatic relationship with *MITF*. This is a notion held by many in the melanoma field, primarily because in neural crest development and during melanocyte differentiation PAX3 transcriptionally activates *MITF* [reviewed in Ref. (7, 8)]. Our earlier investigations (10) are, to our knowledge, the only comprehensive investigations systematically addressing this notion, demonstrating that PAX3 does not transcriptionally activate *MITF* in melanoma cells. Further, as suggested in **Figure 2**, the relative expression levels of PAX3 and MITF are variable in different regions of melanoma tissue, which is not inconsistent with observations of transient changes in pigment production and of POU3F2 expression associated with melanoma dissemination (19). Indeed, amongst several melanoma cell lines that we have examined previously, we observed relatively large fluctuations in MITFm expression, and the variations in PAX3 expression level were not as great as MITFm (10).

Our RNAi data in **Figure 4** suggest that PAX3 and MITF expression contribute in distinct ways to cell migration, leading to the suggestion that it is the relative strengths of signals in the PAX3-POU3F2 axis versus the MITF-*miR-211* axis that define the strength of lineage commitment in melanoma cells, and the migratory behavior of the melanoma cells. This latter interpretation could be related to the mechanisms involved in phenotype switching of melanoma cells, where melanoma cells are induced to de-differentiate from a relatively strongly differentiated and proliferative melanoma cell lineage to a more stem cell-like phenotype with reduced proliferation and enhanced cell migration (10, 17, 29).

In conclusion, here we have presented new evidence that PAX3 and MITF expression have independent and opposing effects in

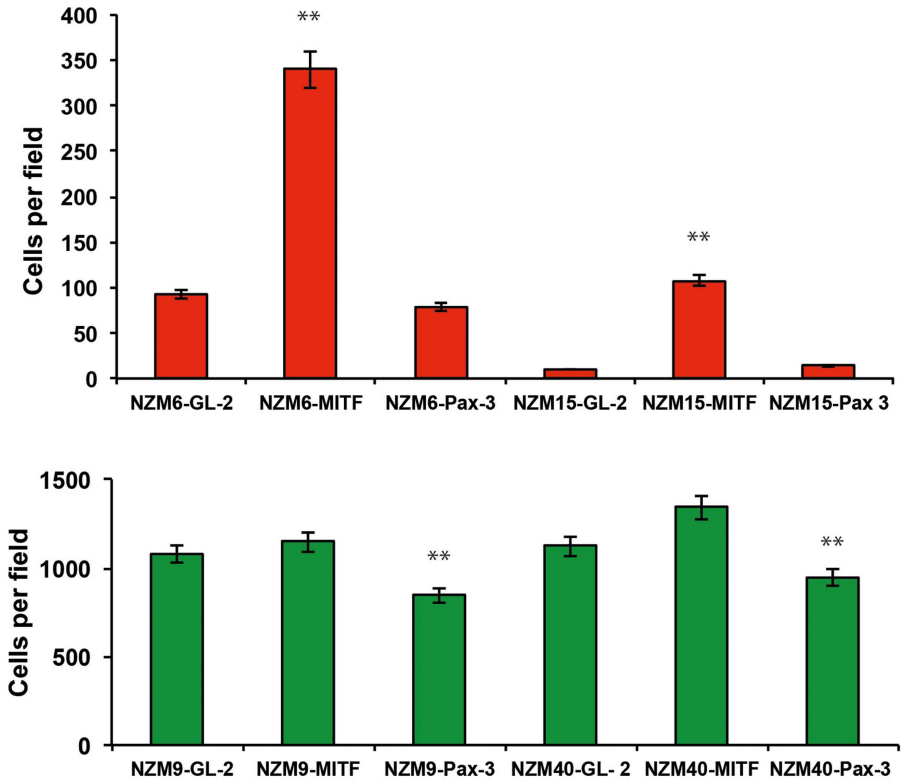

FIGURE 4 | Knockdown of PAX3 or MITF results in differential effects on the migration of melanoma cell lines *in vitro*. siRNA-mediated knockdown of *PAX3* inhibits the migration of melanoma cells expressing lower levels of MITF (green bars), but not melanoma cells expressing higher levels of MITF (red bars). Conversely, siRNA-mediated knockdown of *MITF* does not enhance the migration of melanoma cells expressing lower levels of MITF (green bars), but does enhance the migration of melanoma cells expressing high levels of MITF (red bars). $^{**}p < 0.001$.

melanoma. In line with these data we are proposing a genetic switch theory as a working model to guide future experimental approaches investigating the mechanisms underlying melanoma progression, and the acquisition of resistance and invasiveness. As more work is carried out to test the predictions made from the genetic switch theory, this in turn should lead to a better understanding of mechanisms associated with melanoma progression. Developing a clear description of the mechanisms in melanoma associated with key molecular pathways and phenotype switching is highly likely to be important for the successful treatment of invasive melanoma.

MATERIALS AND METHODS

HUMAN TISSUES, CELL LINES, AND CELL CULTURE

Normal human skin, human nevus, and melanoma tissues, which were formalin-fixed and embedded in paraffin blocks were obtained from Dunedin hospital. Approval for the use of the archival formalin-fixed paraffin-embedded tissues in research was from the New Zealand Multi-Region Ethics Committee. A panel of metastatic human melanoma cell lines, NZM1-NZM48, established in culture from human MM tissue explants (25) were grown at 37°C in a low oxygen (5% O_2, 5% CO_2) humidified atmosphere in ITS (Roche, Penzberg, Germany) medium comprising α-modified minimal essential medium (Invitrogen, Carlsbad, CA, USA) insulin (10 μg/mL), transferrin (10 μg/mL), selenite (10 ng/mL), and 10% fetal bovine serum (FBS) as previously described (25). These cell lines were then subsequently cultured in 5% CO_2 and 95% air humidified atmosphere in ITS DMEM medium and 10% FBS.

IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENCE STAINING

Tissue sections were cut at 4 μm thickness. Antigen retrieval was performed by heating in 10 mM Tris, 1 mM EDTA buffer, at pH 9, for 30 min. Non-specific antigen reactivity in the sections was blocked by incubation in 1× BSA (ImmSolv LLC, Seattle, DC, USA) for 30 min at room temperature, followed by incubation with PAX3 antibody [Developmental Studies Hybridoma Bank (DSHB, Iowa, IA, USA)] diluted 1:50 or MITF antibody (Invitrogen, Clone C5 + D5) diluted 1:100 in 0.3 × BSA/PBS buffer and incubated at 4°C overnight. The slides were then washed and incubated with horse anti-mouse biotin conjugated antibody and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). DAB was used as the substrate chromagen and hematoxylin as the counterstain. For dual immunofluorescence staining, mouse anti-PAX3 antibody (DSHB) and rabbit anti-MITF antibody (Atlas Antibodies, 1:50) were co-incubated for 2 h at room temperature, then followed by washing, and secondary antibodies (goat anti-mouse – Alexa fluor-568 and goat anti-rabbit-Alexa fluor-488,

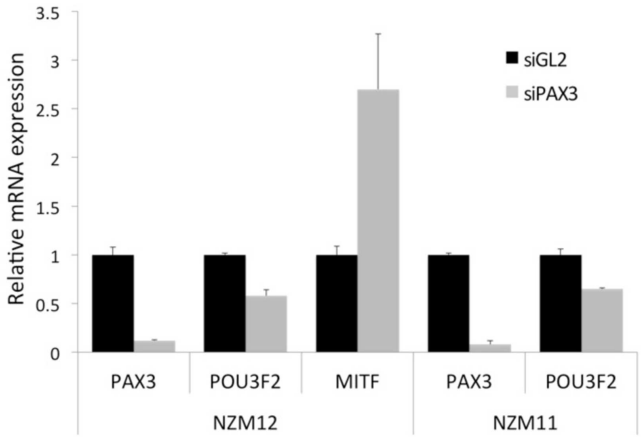

FIGURE 5 | Knockdown of PAX3 results in a decrease of *POU3F2* and an increase of *MITF* gene transcript levels in melanoma cell lines *in vitro*. Transcript levels of *PAX3*, *POU3F2*, and *MITF* mRNA were examined by RT-qPCR in NZM12, and in NZM11 (*PAX3* and *POU3F2* only) melanoma cell lines following RNAi treatment of the cell lines with either siRNAs against luciferase (siGL2) as a negative control, or against PAX3 (siPAX3). The results were calculated as the fold difference in transcript level relative to the level of the housekeeping gene *GNB2L1*, normalized to the siGL2 data.

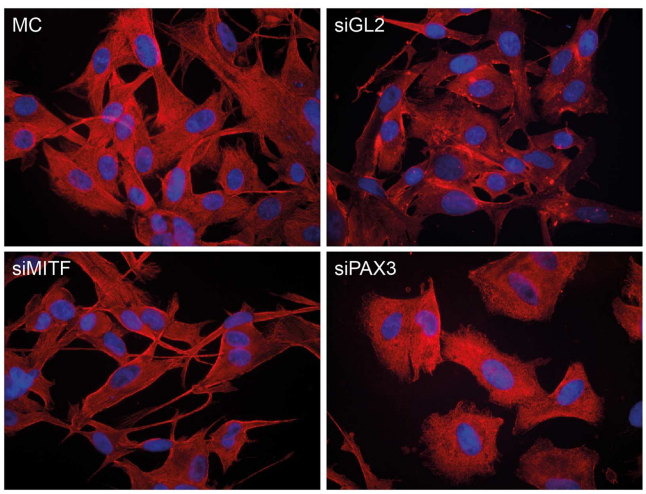

FIGURE 6 | Knockdown of PAX3 or MITF results in differential morphological effects in NZM15 melanoma cells. NZM15 melanoma cells were grown in media without any transfection (MC), or transfected with siRNA to *luciferase* (siGL2) as a non-targeting control, siRNA to MITF (siMITF), or siRNA to *PAX3* (siPAX3) and then stained after 48 h with β-tubulin antibody.

both from Invitrogen Molecular Probes, 1:1000 dilutions) incubation for 1 h. The β-tubulin antibody (clone E7) was purchased from DSHB, and the secondary antibody was goat anti-mouse – Alexa fluor-568. The cell fixation and staining processes were the same as described previously (10). Negative control incubations using the same secondary antibody, but omitting the primary antibodies were also carried out and showed negative staining as expected. Images were captured with using a Zeiss Axioplan (Germany) microscope, Diagnostic digital camera (Model# 9.4 Slider-6) and Spot software (USA). Fluorescent light source was from EXFO X-Cite 120.

Table 1 | PCR primers and amplicon sizes.

Gene		Primer sequence (5′→3″)	Amplicon (bp)
PAX3	F	ACGCGGTCTGTGATCGAAACA	126
	R	TCTCGCTTTCCTCTGCCTCCTT	
MITF	F	GAGCACTGGCCAAAGAGAGG	82
	R	ATGCGGTCATTTATGTTAAATCTTCTTC	
POU3F2	F	TTTCCTCAAATGCCCCAAG	108
	R	TTTCTGTCTCCTGTTACAAAACCA	
GNB2L1	F	CACAACGGGCACCACCAC	138
	R	CACACACCCAGGGTATTCCAT	

siRNA TRANSFECTIONS

Cells were cultured in 5% CO_2 and 95% air humidified atmosphere in ITS DMEM medium and 10% FBS prior to and during *PAX3*-siRNA, and *MITF*-siRNA treatments. A reverse transfection technique was used to deliver siRNAs to melanoma cell lines according to the manufacturer's instructions (Lipofectamine RNAiMAX; Invitrogen, cat. no. 13778-075). Briefly, 1 μl of Lipofectamine RNAiMAX and 6 pmol of siRNA were used for each well of 24-well-plate, in 100 μl of OPTI MEM I media and 500 μl of cells (6×10^4/mL). *PAX3*-siRNA from Ambion (ID#: 215907): sense, GCCGCAUCCUGAGAAGUAAtt; antisense, UUACUUCUCAGGAUGCGGCtg. MITF-siRNA from Ambion (ID#: 3816): sense, GGACAAUCACAACCUGAUUtt; antisense, AAUCAGGUUGUGAUUGUCCtt. An siRNA against Luciferase (from Dharmacon Research Inc.) was used as negative control scramble siRNA, mRNA target sequence AACGUACGCGGAAUACUUCGA.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-PCR ANALYSIS

Total RNA was extracted from cell lines and subject to RT-qPCR analysis as previously described (10), with the exception that a Roche Lightcycler was used for amplification and analysis. The primer sequences and the amplicon sizes of the PCR products are shown in **Table 1**.

TRANSWELL MIGRATION ASSAYS

Transwell migration assays were carried out using 1×10^5 cells seeded into transwell inserts with 8 μm micropore filters (Becton Dickinson) as previously described (26).

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Melanoma biomolecules: independently identified but functionally intertwined

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The majority of patients diagnosed with melanoma present with thin lesions and generally these patients have a good prognosis. However, 5% of patients with early melanoma (<1 mm thick) will have recurrence and die within 10 years, despite no evidence of local or metastatic spread at the time of diagnosis. Thus, there is a need for additional prognostic markers to help identify those patients that may be at risk of recurrent disease. Many studies and several meta-analyses have compared gene and protein expression in melanocytes, naevi, primary, and metastatic melanoma in an attempt to find informative prognostic markers for these patients. However, although a large number of putative biomarkers have been described, few of these molecules are informative when used in isolation. The best approach is likely to involve a combination of molecules. We believe one approach could be to analyze the expression of a group of interacting proteins that regulate different aspects of the metastatic pathway. This is because a primary lesion expressing proteins involved in multiple stages of metastasis may be more likely to lead to secondary disease than one that does not. This review focuses on five putative biomarkers – melanoma cell adhesion molecule (MCAM), galectin-3 (gal-3), matrix metalloproteinase 2 (MMP-2), chondroitin sulfate proteoglycan 4 (CSPG4), and paired box 3 (PAX3). The goal is to provide context around what is known about the contribution of these biomarkers to melanoma biology and metastasis. Although each of these molecules have been independently identified as likely biomarkers, it is clear from our analyses that each are closely linked with each other, with intertwined roles in melanoma biology.

Keywords: melanoma, CD146, CSPG4, galectin-3, MMP2, Pax3, biomarker

INTRODUCTION

The incidence of cutaneous melanoma has risen faster than any other malignancy in Causasian populations in the last 30 years, making it a global health problem (1). Although some of this increase may be due to improved surveillance, early detection and changes in diagnostic criteria, most is considered to be linked to increased sun exposure. Fortunately, the majority of patients present with thin, localized melanoma which in most cases is curable by surgical resection (2, 3). However, because melanoma metastasizes very early in the disease process, approximately 3% of patients who present with lesions <0.75 mm thick, 15% with lesions between 0.75 and 1.00 mm, and 30% with lesions >2.00 mm develop metastatic disease and die within 10 years (4, 5). The prognosis is significantly worse for those patients who present with regional and distant metastases at diagnosis, with 10 year survival rates of 64 and 16% respectively (6).

These poor survival rates are a reflection of the two main challenges in the management of metastatic melanoma – (1) the inadequacy of current prognostic markers and (2) the lack of effective treatment options. Currently, prognosis is based on a small set of clinical and histological features, e.g., tumor thickness, level of invasion, and ulceration (7), which have limited predictive power for individual patients and no direct implications for personalizing treatment (8). Therefore, there is an urgent need for a prognostic tool that can triage patients into high and low risk of metastatic melanoma, particularly for patients with thin melanoma, who show significant heterogeneity in survival (9). This would enable high-risk patients to receive necessary follow-up and adjuvant treatment while minimizing the interventions received by low risk patients. Moreover, melanoma is refractory to standard treatments such as chemo- and radiotherapy (10, 11), and new therapies are either effective for a relatively short time, e.g., BRAF inhibitors (12), or have serious side effects, e.g., ipilimumab, an immune-modulating antibody that targets CTLA-4 on activated T lymphocytes and suppressor T regulatory cells (13, 14).

Clearly, there is a significant need for both new biomarkers and new therapeutic options in melanoma. Intuitively, a biomarker with high predictive value may also be a potential therapeutic target. However, the discovery of new biomarkers and development of new treatments is challenging, as one molecule on its own is unlikely to have sufficient predictive value to be an effective biomarker. Similarly, therapies targeting a single molecule will also lack efficacy. The complexity of the metastatic process suggests an accurate prognostic tool-kit will include additional biomarkers to the current histological features used, while an effective

treatment will require simultaneous targeting of multiple steps in the metastatic pathway (15).

Recent systematic reviews by Gould Rothberg et al. (16), Schramm and Mann (8), and Tremante et al. (17) used REMARK criteria (REporting recommendations for tumor MARKer prognostic studies) (18) to select high quality studies investigating melanoma biomarkers. From these reviews and others we have identified five melanoma biomarkers consistently associated with melanoma progression – melanoma cell adhesion molecule (MCAM), galectin-3 (Gal-3), matrix metalloproteinase-2 (MMP-2), chondroitin sulfate proteoglycan 4 (CSPG4), and paired box 3 (PAX3). They comprise a transcription factor (PAX3), cell surface glycoproteins (MCAM and CSPG4), a secreted protein (Gal-3), and a matrix-degrading enzyme (MMP-2). These molecules were chosen because of their apparent involvement in different aspects of the disease process. Yet intriguingly, these five melanoma biomarkers are all linked by a network of overlapping functions in melanoma progression.

PAIRED BOX 3

PAX3/Pax3 (PAX3 and Pax3 represent the human and mouse factors respectively) is a member of the Pax family of transcription factors that are highly conserved throughout phylogeny. All play a crucial role in embryogenesis but all are also implicated in tumorigenesis – [for reviews see (19–21)]. Pax3 protein contains two DNA binding domains, a paired domain and a homeodomain which can be used alone or in combination to bind downstream target genes (22–25). In addition Pax3 contains a C-terminal transcription activation domain and an octapeptide (24, 26, 27). The ability of Pax3 to employ one or both DNA binding domains accounts for its ability to regulate numerous downstream targets. A single Pax3 gene encodes multiple transcripts produced by alternate splicing (28–31). The resultant protein isoforms provide functional diversity for Pax3, as they differ in structure and in the activity of their paired, homeodomain and alternate transactivation domains (31–33). Pax3 functions by activating or repressing expression of its downstream target genes, thereby affecting the target gene-mediated regulatory pathways. Moreover, certain protein modifications, e.g., acetylation, can switch Pax3 from an activator to a repressor on the same target gene promoter (34). In addition, different PAX3 isoforms seem to have a different (and even opposing) effect on the same cellular process (35).

PAX3 expression and function has been extensively studied in embryogenesis and its role here is well described [reviewed in Ref. (36)]. Its expression during early embryogenesis is critical for development of cells of neural crest origin, the cells that give rise to skin melanocytes. PAX3 is considered a key player in melanocyte development, from lineage specification and maintenance of melanoblast stemness, to regulation of cell proliferation and migration to their final location where they terminally differentiate into melanocytes (28, 37, 38). Pax3 is crucial for melanoblast specification and differentiation, being at the pinnacle of the hierarchy of melanocyte-specific gene regulators. In addition, Pax3, along with other factors, activates the key melanocytic regulator MITF (microphthalmia transcription factor) which initiates the activation of the cascade of melanogenic genes (39, 40). It is interesting to note that even though activation of Mitf by Pax3 during embryogenesis is well described, this regulatory axis does not seem to be operational in melanoma cells (41), where MITF and PAX3 regulate diverging pathways.

The involvement of PAX proteins in cancer is well known (20). Many studies show PAX3 expression in melanoma, but also in tumors arising from other neural crest-derived tissues, such as medulloblastoma, benign peripheral glial tumor neurofibroma (precursor of malignant nerve sheath tumor), Erwin's sarcoma, supratentorial primitive neuroectodermal tumor, and pediatric alveolar rhabdomyosarcoma (ARMS) (42–51). In melanoma, PAX3 expression is evident at all stages of disease progression, including the primary lesion, circulating melanoma cells, and metastatic lesions (29, 42–46, 52–55). PAX3 is also expressed in benign naevi and in normal melanocytes (53, 56), although its precise role here is not clear. This suggests PAX3 is best described as a lineage marker rather than a marker of disease progression.

However, the recently proposed theory that melanoma progression is driven by those melanoma cells showing a highly motile, less differentiated (stem-like) phenotype (57–60), and the crucial roles PAX3 plays in melanocyte development, implies that it is more than just a lineage marker. It might actively drive melanoma progression. It has been suggested that the ability of a melanoma cell to respond to micro-environmental changes by switching between a highly proliferative (low metastatic potential, leading to tumor growth), and highly invasive phenotype (motile and stem cell-like, resulting in tumor dissemination) contributes to the aggressive nature of melanoma (60, 61). PAX3 is a nodal point in melanocyte differentiation, as it simultaneously functions to initiate the melanogenic cascade while preventing terminal differentiation, thus keeping the cell in a lineage restricted stem cell-like state (19). The evidence that PAX3 protein modifications, such as phosphorylation and acetylation, can alter cell functions, from stem-like to differentiated (34, 62–64), strengthens this hypothesis.

PAX3 has been shown to prevent apoptosis in melanoma cells (56, 65) via a range of mechanisms. Several known anti-apoptotic factors, such as tumor suppressors p53, PTEN, and Bcl-Xl, are mediators of Pax3-induced cell survival, in both embryogenesis and tumorigenesis. Pax3 has a dual effect on p53; it represses transcription of p53-dependant genes, *BAX* and *HDM2-P2*, and promotes p53 protein degradation (66). Knock down of PAX3 induced increased cell detachment, growth reduction, and increased apoptosis in melanoma cell lines (65). Inactivation of the tumor suppressor gene PTEN is often found in PAX3-positive tumors (67). PAX3 binds directly bind to the *PTEN* promoter (68), down regulating its expression and decreasing apoptosis (69). PTEN regulates progression through the G1 cell cycle checkpoint, by negatively regulating PI3K/AKT signaling. Transcription of *BCL-XL*, a member of the *BCL-2* family of anti-apoptotic genes, is also directly regulated by PAX3 (68, 70). Treatment with *PAX3* or *BCL-XL* antisense oligonucleotides, individually or in combination, decreased cell viability to a similar extent, suggesting that *PAX3* and *BCL-XL* lie in the same anti-apoptotic pathway (70). Additionally, MITF regulates another member of the same gene family, *BCL-2* (71), providing an alternative indirect mechanism to regulate melanoma cell survival.

During embryogenesis Pax3 plays a crucial role in controlling the correct migration of cells, by directly regulating the

transcription of TGFα and TGFβ (72, 73), growth factors that are involved in remodeling the extracellular matrix (ECM) and cell cytoskeleton as required for cell migration (73–75). A similar role is suspected in melanoma cells, where PAX3 has been found to directly target the *TGF*β promoter in metastatic melanoma cell lines (68). Involvement of PAX3 in melanoma migration is further supported by evidence showing that other genes associated with cell migration, including *MCAM*, *CSPG4*, and *CXCR4*, are targeted by PAX3, as shown by ChIP assay in A2058 melanoma cells (68). Up-regulation of MCAM expression following *Pax3*-transfection in melanoma cells confirmed that MCAM is a downstream target of Pax3 (76, 77), and the number of cells co-expressing MCAM and PAX3 is increased in highly metastatic melanoma (53). CXCR4 is also associated with metastatic spread of melanoma (78). CXCR4, and its ligand CXCL12, regulates chemotactic migration and "homing" of tumor cells to a secondary organ/site, and facilitates tumor cell extravasation (79, 80).

Medic et al. (68) suggested the traditional developmental roles of PAX3 in regulating differentiation, proliferation, cell survival, and migration, are retained in melanoma cells. They showed that PAX3 promoted a less differentiated, stem-like (via HES1, SOX9, NES, DCT), motile (via MCAM, CSPG4, and CXCR4) phenotype, characteristic of melanomas with high metastatic potential (81). PAX3-mediated regulation of melanoma cell survival and proliferation is through BCL2L1 and PTEN, and TPD52 (tumor protein D52) respectively (82). By controlling crucial cell processes (proliferation, cell survival, and migration), as well as promoting a less differentiated stem-like phenotype, PAX3 "ticks all the boxes" as an intrinsic factor driving melanoma development and progression.

From these studies it is evident that PAX3 is involved in melanoma progression on multiple levels, and it is likely that at different stages of disease progression, PAX3 plays different roles. Most recently, PAX3 has been identified as the mediator of anti-senescence and induced drug resistance in melanoma cells (83–85). Consistent with its crucial roles in normal melanocytes and melanoma cells, PAX3 appears to be expressed on similar percentages of circulating tumor cells (CTCs) in patients with different stages of metastatic disease (AJCC stages 0–V). However, this percentage decreased in patients following surgical removal of metastatic lesions, suggesting PAX3 expression could be used to monitor the tumor load in patients undergoing surgery and other treatments (55).

MELANOMA CELL ADHESION MOLECULE

Melanoma cell adhesion molecule (CD146, Muc18, S-Endo-1) is a cell surface glycoprotein belonging to the immunoglobulin (Ig) superfamily. It has five extracellular Ig-like domains, a short transmembrane region, and a cytoplasmic tail, which includes two putative endocytic motifs (86, 87). MCAM was initially identified as a marker of melanoma progression in 1989 (88), and recently was recognized as a more accurate prognostic marker than all other clinico-pathological characteristics (89). MCAM is expressed on approximately 70% of primary melanoma and 90% of lymph node metastases, and MCAM expression in a primary lesion is predictive of lymph node metastases and metastases at other sites (90). MCAM expression is also associated with significantly lower 5 year survival rates: approximately 95% of patients with MCAM negative primary lesions survive 5 years post-diagnosis, compared to 40% of patients with MCAM positive primary lesions. Stratification of patients by MCAM expression in the primary tumor may therefore enable more accurate identification of patients who are likely to have a positive lymph node, and those patients that have high-risk of recurrence despite a negative lymph node (90).

In addition to melanoma, MCAM expression has been linked to progression of breast, prostate, and ovarian cancer (91–95). Interestingly, MCAM also plays a role in trophoblast invasion during pregnancy (96, 97) and is used as a marker of mesenchymal stem cells (98, 99). In normal adult tissue, MCAM is primarily expressed by the vascular endothelium and smooth muscle (100, 101). Most studies on MCAM have focused either on its contribution to melanoma metastasis or its role in endothelial cell function and angiogenesis.

On melanoma cells, MCAM mediates cation independent cell–cell adhesion (102), moderates cell-matrix interactions (103) and is associated with increased cell migration and invasion, as seen in *in vitro* scratch wound and invasion assays (104, 105). A blocking antibody to MCAM decreased cell–cell adhesion and cell invasion *in vitro*, and decreased primary tumor growth and lung metastases *in vivo* (106). Other murine studies suggest MCAM influences the later stages of metastasis, such as the establishment of a secondary lesion (107). In endothelia, MCAM has been implicated in maintenance of endothelial cell–cell junctions (101, 108), endothelial cell proliferation, migration, and angiogenesis (109).

Data from human studies also suggest that MCAM expression may be linked to the development of metastatic melanoma lesions. MCAM expression on CTCs in melanoma patients has been associated with increased tumor burden and poorer outcome in Stage IV disease (55, 110). In addition, MCAM expression on CTCs was found to be a useful marker for monitoring response to therapy, as patients with poor outcomes had an increased incidence of MCAM positive CTCs compared to patients with more positive outcomes (55, 110). Reid et al. (55) also suggest that MCAM expression on CTCs may help identify patients that respond poorly to conventional treatments and may benefit from alternative regimes. Despite the overwhelming evidence that MCAM expression on a melanoma lesion is associated with a poor prognosis, details of the key molecular interactions in melanoma progression that involve MCAM remain unclear. We, and a small number of other groups, have been exploring how the structural features of MCAM contribute to its role in melanoma progression as a way of redressing this issue.

Melanoma cell adhesion molecule has eight potential N-glycosylation sites (88) and is heavily glycosylated during post-translational processing, with approximately 35% of its weight due to carbohydrate modifications (111). Sialic acid, the HNK-1 antigen (CD57), and β1–6 branched *N*-acetylglucosamine side-chains (β1–6 branches) (111) are among the carbohydrates moieties carried by MCAM, although the carbohydrate structures decorating MCAM vary according to the cell-type which is expressing this protein. MCAM exists as monomers and dimers on the surface of both endothelial and melanoma cells (112); with dimerization mediated through a disulfide bond occurring between cysteine residues in the most membrane proximal Ig domain (113). There are two isoforms of MCAM: MCAM-long contains a 63 amino acid

intracellular domain including two putative endocytic domains and five potential protein kinase recognition sites (100), while MCAM-S contains a truncated cytoplasmic tail that lacks both of the endocytosis motifs and one of the protein kinase sites (87). Melanoma cells express primarily the long isoform whereas endothelial cells express both (87, 103). A soluble form of MCAM has also been detected in cell culture supernatants and serum from normal healthy subjects (114).

The intracellular tail of MCAM-long binds to hShroom1 (87) and ezrin-radixin-moesin (ERM) proteins (115), both of which bind to the actin cytoskeleton. Luo et al. (115) found that the ERM proteins link MCAM to the actin cytoskeleton and promoted the formation of microvilli. In addition, the MCAM-ERM protein complex recruited Rho guanine nucleotide dissociation inhibitory factors 1 (RhoGDI1) and sequestered it from RhoA. The release of RhoA from RhoGDI1 inhibition led to RhoA activation, downstream signaling and widespread microfilament reorganization (115). Activation of the PI4P5K-PIP_2 pathway during this process formed a positive feedback loop, further promoting the phosphorylation and activation of the ERM proteins and the MCAM-ERM interaction (115). The regulation of cytoskeletal reorganization and migration by RhoA in melanoma cells in response to the chemokine CXCL12 (SDF-1), has previously been described (116, 117). Thus, Luo et al. (115) proposed the overexpression of MCAM in melanoma cells drives RhoA activation, cytoskeletal reorganization, and cell migration.

Witze et al. (118) describe a different model for the contribution of MCAM to cell polarity and migration of melanoma cells. They described Wnt5-mediated recruitment of MCAM, actin, and myosin IIB into intracellular bodies known as Wnt5a-mediated receptor-actin-myosin polarity (W-RAMP) structures. In the presence of CXCL12, these structures distributed asymmetrically and directed membrane retraction at the trailing edge of the cell. Membrane retraction then promoted nuclear movement and influenced the direction of cell migration (118). This process required membrane internalization, endosomal trafficking, and the intracellular translocation of MCAM, and in contrast to other Wnt-cytoskeletal interactions and the model proposed by Luo et al. (115) it is regulated by RhoB rather than RhoA.

Endothelia and melanoma express high levels of MCAM, and as melanoma cell interactions with vascular endothelia are a key part of the metastatic process, it is likely MCAM on both of these cells contributes to melanoma metastasis. Although a homophilic interaction between MCAM cannot be demonstrated (102, 119), it is possible that melanoma and endothelial cells both express MCAM and its ligand, and these interact bi-directionally. It is known that MCAM contributes to cell–cell adhesion in the vascular endothelium (108) and that engagement of the extracellular domain of MCAM initiates outside-in signaling resulting in calcium flux and the phosphorylation of a panel of intracellular proteins, including $p125^{FAK}$ and paxillin, which leads to focal adhesion formation (120). Collectively, these data suggest the localization and function of MCAM at endothelial cell junctions involves dynamic interactions with, and reorganization of, the actin cytoskeleton (121). There is also evidence that MCAM expression in melanoma cells modulates the expression (103) and/or activity of integrin chains. The most compelling evidence involved the β1 chain. MCAM overexpression also appears to stimulate the expression of MMP-2. The association of MCAM with MMP-2 expression was first reported in the late 1990s (106, 122, 123). A recent study further revealed that MCAM is involved in signaling cascades that affect the expression of the transcriptional regulator, inhibitor of DNA binding-1 (Id-1) and activating transcription factor (ATF)-3 (124). This study showed that MCAM silencing increased the expression of ATF-3 and decreased the expression of Id-1. Interestingly, Id-1 expression was shown to positively regulate MMP-2 transcription. As AFT-3 binds to the Id-1 promoter and represses its transcription, the suggestion was that MCAM indirectly led to an increase in MMP-2 levels via decreasing AFT-3 and increasing Id-1 levels (124). These examples illustrate that MCAM expression may shift the balance between cell–cell and cell-matrix adhesion, in addition to increasing migration and invasion via the up-regulation of pro-invasive enzymes.

Jiang et al. (125) showed that MCAM interacts with vascular endothelial growth factor receptor 2 (VEGFR-2) on endothelia and acts as a co-receptor for the binding of vascular endothelial growth factor A (VEGF-A). The interaction of the extracellular domain of MCAM with VEGFR-2 occurred independently of VEGF-A, and was a crucial step in VEGFR-2 activation. When associated with VEGFR-2, the cytoplasmic tail of MCAM recruited ERM proteins and the actin cytoskeleton, to assemble a "signalosome," which was required for signal transduction from VEGFR-2 to AKT and P38 MAPKs. The result was increased endothelial cell migration (125). MCAM can also function independently of VEGFR-2, and VEGF-A (109, 113). The interaction of MCAM with VEGFR-2 on melanoma cells remains to be confirmed, although it is known melanoma express VEGF and VEGFR-2, and overexpression of VEGF-A in a melanoma cell line with VEGFR-2 favored cell growth and survival through MAPK and PI3K signaling pathways (126).

Laminin 411 (laminin 8) and galectin-1 (Gal-1) have also been described as ligands for MCAM (127, 128). Flanagan et al. (128) reported that MCAM expressed by a subset of CD4+ T-cells (Th17 cells) binds laminin 411 from the vascular endothelia and this interaction was blocked by an anti-MCAM antibody and soluble recombinant MCAM (MCAM-Fc). Animal studies showed that an anti-MCAM antibody administered *in vivo* reduced Th17 lymphocyte infiltration into the central nervous system. The interaction of MCAM with laminin 411 is consistent with the interaction of gicerin (the avian homolog of MCAM) with neurite outgrowth factor, a member of the laminin family (129, 130), and basal cell adhesion molecule (an immunoglobulin superfamily member) with laminin 511 (131). The interaction of MCAM on melanoma with laminin 411 has not been investigated, but it is known that MCAM does not interact with laminin 111 (105), 511, or 332. The interaction of MCAM with Gal-1 is carbohydrate mediated. Gal-1 is produced by vascular cells and binds to carbohydrates on cell surfaces and ECM proteins (132). It has been implicated in angiogenesis (133) and melanoma progression and Jouve et al. (127) hypothesized that the interaction of MCAM with Gal-1 protects cells from Gal-1 induced apoptosis.

In conclusion, MCAM expression in a primary melanoma appears to increase the likelihood of metastatic spread and may assist to stratify patients into low and high-risk of recurrence at diagnosis (90). In addition, it is also useful as a marker on CTCs,

as MCAM-expressing CTCs appear to correlate with tumor burden and disease progression (55). In melanoma, MCAM appears to facilitate cell migration by the rearrangement of the cellular cytoskeleton via activation of Rho proteins (115, 118), and potentially via activation of the AKT and P38 MAPK pathway in association with VEGRF (125). MCAM expression is also correlated with up-regulation of MMP-2 (124), and a modulation of integrin-mediated cell spreading and migration.

GALECTIN-3

Galectin-3 belongs to a family of lectins that bind β-galactosides. It is found in the nucleus, cytoplasm, and on the cell surface of many cell types, and is also secreted into the extracellular space. It has a C-terminal carbohydrate recognition domain (CRD) and an N-terminal tail that mediates the oligomerization of Gal-3 molecules, which is vital for its extracellular functions (134). Gal-3 also contains an amino acid motif, NWGR, which is involved in its anti-apoptotic function. This motif is also found in Bcl-2 and has been called an "anti-death" motif. Like Bcl-2 family members Gal-3 exerts its anti-apoptotic activity at the peri-nuclear mitochondrial membranes (135). Extracellular Gal-3 binds with high affinity to *N*-acetyllactosamine containing glycans and binds to both cell membrane and ECM proteins that carry these glycosylation structures. Gal-3 binds a host of membrane proteins including integrins (e.g., β1, αv, αM), cell adhesion molecules (e.g., N-cadherin, NCAM, VCAM), lysosomal membrane associated glycoproteins (Lamps)-1 and -2, growth factor receptors (e.g., epidermal growth factor receptor, transforming growth factor β receptor), and molecules associated with the immune response including the T lymphocyte receptor (136, 137). Its ECM protein ligands include laminins-111, -332, -511, fibronectin, collagen IV, vitronectin, and elastin (137). The N-terminal domain of Gal-3 can be post-translationally modified via phosphorylation at Ser 6. Phosphorylation of this site influences the intracellular distribution of Gal-3 and therefore its ability to regulate transcription of downstream genes, anti-apoptotic functions, and carbohydrate binding properties. Specifically, phosphorylation is required for Gal-3's anti-apoptotic function, and dephosphorylation for realization of its full ability to bind carbohydrate ligands (138).

Galectin-3 is expressed in the nucleus, cytoplasm, and plasma membrane of melanoma cells (139). The intra- and extracellular distribution of Gal-3 and its variety of extracellular binding partners, both on the cell surface and in the tumor microenvironment, suggests Gal-3 could affect metastatic progression via a range of mechanisms (139).

There is a growing literature indicating Gal-3 expression is associated with tumor progression in melanoma. Consistently the data indicate primary melanomas express significantly more Gal-3 than naevi (140–142). Gal-3 expression has also been positively correlated with tumor thickness, Clarke and Breslow tumor stage, lymphatic invasion, lymph node positivity, and distant metastases (143), although Brown et al. (144) recently reported that Gal-3 expression showed a bi-modal distribution, with increased levels in thin primary melanoma compared to naevi, and a progressive decrease in expression in thicker and metastatic melanoma. The decrease in Gal-3 expression in metastatic melanoma was particularly evident in the nucleus (144). This bi-modal distribution of Gal-3 was also reported by Vereecken et al. (142). Brown et al. (144) suggest that high Gal-3 in thin melanoma may contribute to resistance to apoptosis (145), but as a lesion progresses, intracellular Gal-3 may be released by the cell into the extracellular environment. Once in the extracellular environment, Gal-3 can interact with cell surface and ECM proteins. Melanoma progression may be associated with a decrease in intracellular stores of Gal-3, such that a decrease in Gal-3 expression may be associated with metastatic spread and a worse prognosis in melanoma (144). Curiously Gal-3 expression was reported to vary depending on the extent to which the melanoma lesion was exposed to the sun, chronically sun-exposed melanoma displayed nuclear Gal-3, whereas melanomas on intermittently sun-exposed sites had cytoplasmic staining for Gal-3. The authors of this study concluded that UV light may be involved in Gal-3 activation and that the translocation of Gal-3 to the nucleus is associated with a more aggressive lesion (140). The prognostic significance for melanoma of serum Gal-3 has also been investigated. This work suggested Gal-3 could be of prognostic value, as American Joint Committee on Cancer (AJCC) stage 3 and 4 melanoma patients had higher serum Gal-3 levels than patients with AJCC stage 1 and 2 melanoma, and serum measurements could have a role in follow-up and management of stage 3 and 4 melanoma patients (146).

Nuclear Gal-3 contributes to melanoma metastasis by regulating multiple genes such as VE-cadherin, MMP-1, MMP-2, interleukin 8 (IL-8), and autotaxin (135, 147–150). Wang et al. (150) reported that Gal-3 directly interacts with the transcription factor activating protein 1 (AP-1) to increase expression of MMP-1, which breaks down the collagens, types I, II, and III, thus enabling the migration of melanoma cells through interstitial connective tissue. In addition, Gal-3 expression in melanoma has also been associated with increased levels of VE-cadherin and IL-8, both of which are implicated in angiogenesis though the stimulation of vascular endothelial cell proliferation and migration. Gal-3 induced up-regulation of IL-8 has also been associated with increased MMP-2 expression (151). Recently, silencing Gal-3 expression in melanoma was shown to reduce expression of the transcription factor NFAT1 and so decrease the transcriptional activation and expression of autotaxin (lysophospholipase D) (149). Autotaxin was first identified from a human melanoma cell line due to its chemotactic and motility activity for melanoma cells (152). Autotaxin catalyzes the conversion of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), which acts as ligand for a range of G-protein coupled receptors to induce downstream signaling associated with migration, invasion, and angiogenesis in a range of cancers (153, 154). In melanoma, decreased autotaxin lowers melanoma growth and metastasis as well as affecting cell motility.

Gal-3 is also believed to play a role in the organization of cell membrane micro-domains. The cell membrane is a dynamic structure, with proteins clustered in non-random, functional domains held together by cohesive forces between proteins and lipids (155, 156). Most cell-surface proteins are glycosylated and oligomeric lectins such as Gal-3 bind to specific glycan structures on cell surface glycoproteins and help organize proteins into functional groups on the cell membrane (157, 158). These

galectin-protein lattices are thermodynamically stable due to multiple low-affinity interactions, but are modulated by changes in protein glycosylation or galectin expression (159). Fluorescence recovery after photobleaching (FRAP) experiments revealed Gal-3 lattices on endothelial cells are stable and resistant to lateral movement once the Gal-3 oligomers have been formed (160). Further work has indicated Gal-3 lattices contribute to cell proliferation, migration, and apoptosis (155). By stabilizing glycoproteins in the cell membrane, Gal-3 lattices reduce receptor endocytosis (161) and influence the turnover of focal adhesions (162). Goetz et al. (162) found that Gal-3 lattices promoted integrin clustering, and with Caveolin-1 tyrosine phosphorylation, this stabilized focal adhesion kinase (FAK), paxillin, and α5 integrin in focal adhesion (FA) complexes. This decreased the exchange of FA components with the cytosol and facilitated FA maturation and turnover. The control of FA dynamics is critical for cell motility, as the assembly, maturation, translocation, and disassembly of FAs mediate cell attachment, contraction, protrusion of the leading edge, and retraction of the trailing edge during cell migration (163). Saravanan et al. concluded from their experiments with epithelia that on these cells Gal-3 cross-linked and clustered α3β1 integrins at the leading edge of migrating cells. Integrin clustering activated FAK and Rac1, which promoted lamellipodia formation and cell migration (164). We are currently performing experiments with melanoma cells to determine whether this model also holds for melanoma cell migration.

In addition to binding to cell and matrix components, Gal-3 is also cleaved by MMP-2 and MMP-9 to produce a biologically active fragment that that may be involved with cell invasion (147) and angiogenesis. These enzymes cleave extracellular Gal-3 to separate the C-terminal CRD from the N-terminal domain. Curiously, the 22-kDa cleaved fragment containing an intact CRD was found to bind its glycan ligands more strongly than the intact protein, under conditions when the concentration of the intact protein is such that oligomerization is prevented (147). Moreover, the data suggested that truncated Gal-3 effectively competes with full length Gal-3 to inhibit its homophilic cross-linking and other types of protein–protein interactions as treatment with the truncated form showed reduced tumor growth and metastasis in a breast cancer model (165).

Exogenous Gal-3 (secreted by melanoma cells) could also influence melanoma progression as a result of its role in angiogenesis. Gal-3 been shown to stimulate capillary tube formation of endothelial cells *in vitro* and angiogenesis *in vivo* (166). Interestingly the angiogenic activity of Gal-3 involves CSPG4 and the integrin α3β1. The binding of soluble CSPG4 to endothelial cell surfaces induced cell motility and the formation of a multicellular network on type I collagen gels. Antibody blocking studies indicated that both Gal-3 and α3β1 were involved in CSPG4 endothelial cell motility and that these molecules formed a complex on the endothelial cell surface (167). CSPG4 is expressed by microvascular pericytes whereas, Gal-3 and α3β1 are expressed by vascular endothelial cells, but as the regulation of the development of new vessels involves cross-talk between pericytes and endothelial cells it is likely that the signaling complex of α3β1, Gal-3, and CSPG4 is involved in pericyte endothelial cell cross-talk during early stage angiogenesis (167). Vascular endothelial expressed Gal-3 was also shown to important for the adhesion of melanoma cells to lung endothelia, which led to the suggestion that Gal-3 on lung endothelia could serve as the first anchor for circulating melanoma cells undergoing extravasation (168). Oligomerization of Gal-3 on endothelial cells to form lattices has been observed experimentally, with most Gal-3 concentrated in the cell–cell junctions. Fluorescent energy transfer (FRET) experiments with neutrophil adhesion suggested that oligomerized Gal-3 mediated neutrophil adhesion to endothelial layers primarily at the endothelial cell–cell junctions (160). It is very likely that melanoma cells similarly interact with endothelial cells via Gal-3 lattices. This conclusion is supported by Gal-3 knock-out studies that revealed Gal-$3^{-/-}$ mice were resistant to lung melanoma metastases and melanoma cells bound less well to lung tissue from Gal-$3^{-/-}$ mice (169).

The involvement of the immune system in checking melanoma progression has been an avenue for exploration for many years. It now seems that Gal-3 expression contributes to the effectiveness of leukocyte interactions with melanoma. A melanoma biopsy study reported a correlation between Gal-3 expression and the level of apoptotic tumor-associated lymphocytes (170).

The studies reviewed here indicate that Gal-3 is involved in many aspects of melanoma progression. Nuclear Gal-3 has been implicated in melanoma cell proliferation (probably in the earlier stages), while secreted Gal-3 in the tumor microenvironment has been linked to migration and invasion of melanoma cells and angiogenesis. Thus, the location of Gal-3 as well as the overall levels of Gal-3 expression could be useful as a biomarker or prognostic indicator at different stages of melanoma progression.

CHONDROITIN SULFATE PROTEOGLYCAN 4

Chondroitin sulfate proteoglycan 4 (CSPG4) was first identified over three decades ago as a surface antigen on human melanoma cells (171). This molecule has been variously named high molecular weight melanoma associated antigen (HMW-MAA), melanoma chondroitin sulfate proteoglycan (MCSP), and nerve/glial antigen 2 (NG2), the latter originally identified on rat glia. CSPG4/NG2 positive cells make up about 5–10% of glia in the developing and adult central nervous system and these cells are believed to comprise a progenitor population, which matures into oligodendrocytes and subpopulations of astrocytes. Immature Schwann cells of the peripheral nervous system also express CSPG4/NG2 (172) as do pericytes in newly formed blood vessels (173), and cells of mesenchymal lineages, such as immature chondrocytes, osteoblasts, and myoblasts. In addition, cells in the basal layer of human epidermis and in the outer root sheath of hair follicles that co-express CSPG4 and high levels of β1-integrin are interfollicular epidermal stem cells and the numbers of these cells decrease with age (174, 175). CSPG4 has thus been called a stem cell marker.

CSPG4 is a single pass type I membrane glycoprotein. The intact core protein of 250 kDa has a large extracellular domain which consists of three structural domains: (1) a globular domain of two laminin G-Type regions, (2) a central region of 15 CSPG4/NG2 repeats containing 7 Ser-Gly motifs, one of which is the consensus motif SGXG for glycosaminoglycan attachment, and (3) a membrane proximal globular domain (D3) that contains 6 of the 15

potential sites for N-linked glycosylation. This domain also contains a number of possible proteolytic cleavage sites; cleavage here would give rise to soluble CSPG4 that can be detected in sera. The first globular domain has a compact configuration containing 8 of the 10 extracellular cysteines and 3 potential N-linked glycosylation sites. The 76 amino acid cytoplasmic domain contains threonines that can be phosphorylated by PKCα and ERK 1,2 (residues 2256 and 2314, respectively); a proline rich region that may contain a non-canonical Src Homology type 3 (SH3) domain binding motif, and a C-terminal 4 residue PDZ binding motif (176, 177) that binds to the PDZ domain of scaffold proteins like syntenin and MUPP1 (178, 179). Despite its name, CSPG4 can be expressed without a covalently attached chondroitin sulfate chain making it a "part-time" proteoglycan. As the presence of the chondroitin sulfate chain affects the cell surface distribution of CSPG4 and various functions of the glycoprotein, it has been suggested that regulation of chondroitin sulfate chain attachment may be a way tumor cells control CSPG4 activities (176).

Like MCAM, CSPG4 is widely expressed on melanoma cells, appearing on >85% of cutaneous melanoma lesions and melanoma cell lines (180, 181). This antigen can distinguish metastatic melanoma cells in sentinel lymph nodes by immunohistochemistry and qRT-PCR assays, and CSPG4 is more sensitive and more specific than MART-1, a commonly used melanoma marker (182). The level of CSPG4 expression is similar between lentigo maligna, nodular, and superficial spreading melanoma lesions but it is lower in primary acral lentiginous melanoma lesions. Recent data indicate that approximately 54% of primary acral lentiginous melanoma lesions express the antigen and staining levels are generally weak (183). CSPG4 is, however, a sensitive marker for desmoplastic melanoma; 95% of desmoplastic primary lesions stained for CSPG4, and 86% of nodal metastases were CSPG4 positive (184). When qRT-PCR was used for diagnosis, CSPG4 mRNA was detected in metastatic desmoplastic lesions that did not express MART-1 (184). The use of CSPG4 in diagnosis of desmoplastic melanoma could potentially be very useful, as these lesions display unusual spindle cell morphology and lack the common clinical and histological characteristics of cutaneous melanoma, which complicates diagnosis. CSPG4 immunoreactivity is also an important diagnostic indicator in the two forms of ocular melanoma (conjuctival and uveal). CSPG4 expression levels clearly separate conjuctival melanoma from conjuctival nevi and in one study lower CSPG4 expression appeared to be correlated with increased risk of recurrence (185). Most uveal melanoma also stain for CSPG4, with normal retinas and choroid displaying low immunoreactivity. CSPG4 may also be detected in the serum of some melanoma patients, but is not a reliable predictor of melanoma as only 29% of 117 melanoma patients had elevated serum CSPG4 (186). Immunomagnetic selection of CTCs from peripheral blood using antibodies to CSPG4 has been performed by a number of groups, using either one antibody or an antibody cocktail that recognizes different epitopes of CSPG4 (187–191). This method appears effective in enriching for circulating melanoma cells from peripheral blood samples. Collectively these studies provide convincing evidence that CSPG4 is a useful biomarker for melanoma.

Useful biomarkers generally have functions that aid either the initial development of the primary lesion or progression to metastases. The functions of CSPG4 could contribute to both of these processes. A number of reports have indicated that CSPG4 expression enhances the proliferation of melanoma cells *in vitro* and *in vivo*. This is true for murine melanoma cells (B16F1 and B16F10) transfected with NG2 and human melanoma cells (M14 and WM1552C) transfected with CSPG4 (192, 193). CSPG4 expressing WM1552C cells were also capable of anchorage-independent growth *in vitro* and had activated extracellular signal-regulated kinase (Erk)1,2, activities that required the cytoplasmic domain of CSPG4. Inhibition of CSPG4 expression by siRNA in melanoma cells expressing endogenous CSPG4 reduced Erk1,2 activation and anchorage dependent growth (193). Constitutive activation of the Erk1,2 pathway is associated with more advanced melanomas and the results of activation include entry into the cell cycle and increased expression of key melanoma transcription factors. CSPG4 can bind to and present growth factors, like FGF-2 and PDGF-AA, that impact on the Erk1,2 pathway. Although many advanced melanoma present with a mutation in BRAF, this BRAF-V600E mutation, although contributing to Erk1,2 phosphorylation, is not sufficient for sustained activation. Instead, full length CSPG4 and BRAF-V600E both appear to be required for sustained Erk 1,2 activation (193) and a CSPG4-specific mAb enhanced and increased the duration of the effects of a BRAF inhibitor in melanoma cells (194).

Transfection of CSPG4 stimulated melanoma cell motility in a scratch wound assay (193), an effect believed to be indicative of metastatic potential. Interestingly, CSPG4 stimulates α4β1-integrin-mediated adhesion and spreading, as well as FAK phosphorylation. Signaling through CSPG4 induces the recruitment and phosphorylation of p130cas indicating that CSPG4 signaling may intersect integrin-mediated signaling pathways even though it can signal independently of integrins (195). Interestingly, β1-integrin activation occurs as a result of CSPG4/NG2 phosphorylation and phosphorylation of different threonines trigger different β1-integrin-mediated events; either proliferation (Thr2314 phosphorylation) or motility (Thr2256 phosphorylation) (196).

Other evidence implicates CSPG4 in integrin-controlled cell activities. Chondroitin sulfate binds to the SG-1 site on α4 integrin subunits, and activation of this site is important for α4β1 binding to its ligand, the CS1 site on fibronectin (197). On melanoma, it is predominately chondroitin sulfate carried by CSPG4 that binds and activates the SG-1 site.

The chondroitin sulfate chain addition to CSPG4 also allows CSPG4 to interact directly with fibronectin through its heparin-binding domain. Ligand induced clustering of α4β1 causes the co-localization of CSPG4 and α4β1 (197). NG2/CSPG4 also associates with α3β1 via an interaction with galectin-3. Galectin-3 binds to N-linked oligosaccharides within the D3 domain of the CSPG4 core protein (198) and to oligosaccharides on β1 to form a complex that can be immunoprecipitated from human melanoma cell surfaces (167). It has been suggested that galectin-3 mediated clustering of NG2/CSPG4 and α3β1 leads to enhanced α3β1 signaling (167) and the promotion of melanoma invasion and migration through laminin containing extracellular matrices, because α3β1

selectively binds laminin and galectin-3 binds oligosaccharides on laminin.

Another mechanism by which CSPG4 facilitates melanoma metastasis is by its interaction with MMP-2. This complex comprises the inactive zymogen of the matrix metalloproteinase MMP-2, pro-MMP-2, which binds to the chondroitin sulfate chains of CSPG4. This interaction facilitates the generation of active MMP-2 (discussed later in this review) (199).

Collectively, the data suggest that CSPG4 acts as a scaffold at the cell membrane to facilitate the formation of molecular complexes that stabilize integrins and receptor tyrosine kinases, and localize active MMP-2 to the melanoma cell surface. The result of this is enhanced integrin signaling and ECM degradation, plus more effective growth factor activation of the RAS-RAF-MEK-Erk 1,2 pathway to increase cell proliferation and motility.

MATRIX METALLOPROTEINASE-2

Matrix metalloproteinases are a family of zinc-dependent enzymes that degrade different ECM proteins (200). There are at least 26 different MMPs, which are classified into five groups according to their structure and substrate specificity – collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMPs), and others (200, 201). The constitutive gene expression of MMPs is low, but when the ECM is remodeled, whether for normal physiological or pathological processes, expression of these enzymes increases. The MMPs play a crucial role in physiological and pathological remodeling of the ECM during angiogenesis, wound healing, embryogenesis, and tumor metastasis (202). Degradation and remodeling of the ECM during melanoma metastasis allows tumor cells to invade surrounding ECM, spread via the lymphatic or vascular circulation, and extravasate into distant organs (200). The role of MMPs in tumor cell invasion is not limited to degradation of matrix components – additional substrates for MMPs include proteinases, proteinase inhibitors, other MMPs, growth factors, chemokines, cytokines, and cell surface proteins (203, 204). Thus, MMPs contribute to cell migration, proliferation, and apoptosis; and regulate tumor growth, vascularization, and spread (205).

The gelatinases, MMP-2, and MMP-9, are often over-expressed in malignant cancer. These enzymes degrade basement membrane proteins, such as collagen types IV, V, VII, X, and fibronectin. In melanoma, MMP-2 has frequently been associated with malignant progression and poor prognosis (200, 201, 206). A recent study using tissue microarray and immunohistochemistry of melanoma biopsies of primary and metastatic lesions as well as nevi concluded that MMP-2 expression is a prognostic indicator in primary but not metastatic lesions (201). This suggests that strong MMP-2 expression in the primary lesion contributes to the invasiveness of primary tumor cells, leading to metastases and poor survival outcomes. These findings are in accord with an earlier immunohistochemistry study of primary melanoma biopsy tissue. This study revealed that patients with a low number of MMP-2 positive cells (5–20%) in the tumor sample survived as well as those with an MMP-2 negative melanoma (10 year disease-specific survival rate of 79%), whereas patients with a primary tumor with high MMP-2 expression (>20% of tumor cells) had a 10-year disease-specific survival rate of 51% (207). The survival rate of this patient cohort declined further when proliferative activity of the tumor cells (indicated by Ki67 protein expression levels) and activation of apoptosis (revealed by p53 immunogenicity) were considered. Patients with primary melanoma having all three of these adverse factors had a 10-year survival rate of 28% (207). Interestingly, although MMP-2 and MMP-9 act on similar substrates, and are both expressed in melanoma, MMP-2 appears to be the better prognostic indicator (16, 207, 208).

Matrix Metalloproteinase-2 is synthesized and secreted as a 72 kDa pro-enzyme. It is activated primarily at the cell surface by proteolytic cleavage by membrane type 1 MMP (MT1-MMP/MMP-14); a process that is regulated by the concentration of tissue inhibitor of metalloproteinases-2 (TIMP-2). Activation of MMP-2 requires the formation of a ternary complex consisting of MT1-MMP, TIMP-2, and MMP-2. To form this complex, TIMP-2 first binds to MT1-MMP, and pro-MMP-2 then binds to TIMP-2. This facilitates cleavage of pro-MMP-2 by a neighboring active (TIMP-2 free) MT1-MMP, generating an intermediate 64 kDa MMP-2 fragment (205). This fragment then undergoes autocatalysis (209) or is further cleaved via the plasmin-plasminogen system to produce a fully active molecule (208). At high concentrations of TIMP-2, pro-MMP-2 activation is inhibited because TIMP-2 binds to both the pro-MMP-2 already complexed with MT1-MMP and to neighboring MT1-MMP molecules, so that pro-MMP-2 is unable to undergo cleavage and activation (205). However, the balance between free MT1-MMP and the MT1-MMP-TIMP-2 complex only partially determines the degree of MMP-2 activation (210).The relative amount of active and inactive MMP-2 also depends on the ratio of MT1-MMP and TIMP-2 expression and the quantity of TIMP-2 retained by low-affinity interactions with other plasma membrane molecules (211). Other members of the MT-MMP family (MT2-MMP and MT3-MMP) can also activate pro-MMP-2, but this does not involve TIMP-2. In addition, TIMP-1, -3, and -4 can regulate MT1-MMP activation of MMP-2 (212, 213).

Membrane proteins such as the claudins, αvβ3 integrin, and CSPG4 (discussed earlier) also participate in the activation of MMP-2. The association of these membrane glycoproteins with MMP-2 activation is of particular interest because αvβ3 integrin is often highly expressed on melanoma, claudin-1 expression levels increase with increasing thickness of the primary lesion (16) and CSPG4 is potentially a useful biomarker for melanoma. The chondroitin sulfate chains of CSPG4 have been shown to bind both pro-MMP-2 and MT3-MMP, an MT-MMP that is expressed on vertical growth phase melanoma and is important for melanoma invasion into collagen gels (199). CSPG4 appears to localize pro-MMP-2 in the vicinity of MT3-MMP, thereby assisting the generation of active MMP-2 (199), and this is likely to be important on melanoma cells where the surface density of MT3-MMP is relatively low. The tri-molecular complex comprising MT3-MMP, CSPG4, and pro-MMP-2 leads to activation of MMP-2 in the absence of TIMP-2 because structural features of MT3-MMP allow direct binding to the C-terminal domain of MMP-2 (199). Interestingly, claudin-1 binds to both MT1-MMP and pro-MMP-2 in regions that involve the catalytic domain of both enzymes, and this allows MT1-MMP to activate pro-MMP-2 in the absence of TIMP-2. In a similar mechanism to that described for CSPG4, it appears that claudin-1 localizes MT1-MMPs and pro-MMP-2 on

the cell surface to produce local elevated concentrations of these enzymes, which enhances the activation of pro-MMP-2 (214). In melanoma cells, overexpression of claudin-1 is associated with increased activation of MMP-2; there is more MMP-2 associated with the cell surface than in non-transfected cells, and knockdown of claudin-1 in melanoma cells using siRNA decreases both the amount of active MMP-2 secreted and cell motility (215).

The role of αvβ3 in MMP-2 activation seems to be most important in the invasive growth phase of melanoma as expression of this integrin begins when melanoma cells switch from a horizontal to a vertical growth phase (216). A number of authors have reported data supporting the conclusion that αvβ3 binds active MMP-2 on the surface of melanoma cells (217, 218), others have found MMP-2 to be localized at the leading edge of migrating melanoma cells before αvβ3 (219), or that pro-MMP-2 did not bind αvβ3 (199). In the latter study the melanoma cells expressed MT3-MMP, not MT1-MMP. It is known that αvβ3 physically associates with MT1-MMP and the enzyme processes the integrin αv subunit into heavy and light chains connected by a disulfide bridge, which is the mature form. In cells lacking MT1-MMP, processing of αvβ3 occurs via another integrin convertase, like furin, but cleavage occurs at different sites and this mature αv chain is less able to promote adhesion and migration than the MT1-MMP processed αv chain (220).This and other data suggest the contribution of αvβ3 to MMP-2 activation depends on the co-expression of MT1-MMP. It has been reported that the MT1-MPP cleaved αvβ3 integrin can bind to the intermediate 64 kDa form of MMP-2 and enhance the autocatalytic step of the activation process to produce more of the mature MMP-2, as conversion of the intermediate to the mature form was low in the absence of αvβ3 (221).

Invadopodia, plasma membrane extensions enriched in cell-matrix adhesion molecules, actin-assembly regulators and proteases, form in the adhesive region of invasive tumor cells grown on an ECM. MT1-MMP traffics to these structures in cancer cells (222), suggesting that co-localization of αvβ3 with MT1-MMP and active MMP-2 concentrates adhesion molecules that bind matrix proteins with enzymes that degrade the matrix, thereby facilitating melanoma cell invasion. Moreover, αvβ3 dependent melanoma cell adhesion preferentially occurs on fibronectin fragments cleaved by MMP-2 rather than on intact fibronectin, and fibronectin fragments appear to promote αvβ3 recruitment into the invasive front of melanoma cells (219).

The conclusions from the *in vitro* studies are supported by *in vivo* data. In melanoma tissue sections, *in situ* zymography revealed MT1-MMP and secreted MMP-2 accumulate at the invasive front of melanoma cells, and the presence of functionally active MMP-2 is restricted to this region (223, 224). In another study of biopsies from patients with primary melanoma and patients with cutaneous or nodal metastases, MMP-2 expression was primarily in thick primary melanoma and in melanomas from patients who developed metastasis in the 3-year follow-up period (225). Thus, MMP-2 is very strongly associated with invading vertical growth melanomas. MMP-2 expression is not confined to tumor tissue as the surrounding stroma also synthesizes MMP-2, and in an experimental murine system MMP-2 expression was primarily attributed to the stroma (226), However, these data do not fit with the wealth of patient studies that suggest MMP-2 is a useful biomarker for melanoma.

CONCLUSION

Most patients diagnosed with melanoma now present with thin lesions less than 1 mm thick and 90% of these patients will be cured by surgical excision. However, approximately 5% of these patients will develop metastatic melanoma and die within 10 years, despite no evidence of metastasis at the time of diagnosis. Using diagnostic criteria, there is no way to triage these patients into high and low risk groups, which limits our ability to direct screening and early treatment to those patients at higher risk of metastasis. Moreover, the treatment of metastatic melanoma has advanced little in the last three decades, with ipilimumab (a monoclonal antibody targeting CTLA-4 on T cells) and the BRAF inhibitor, vemurafenib, the only treatments to show an increase in overall survival and an extension of survival time, respectively. Unfortunately, ipilimumab often has significant side effects and is suitable for only a small proportion of patients. In addition, virtually all patients prescribed the BRAF inhibitor will develop clinical resistance and progressive disease. The reader is referred to a recent review on immunotherapy in advanced melanoma (227). Thus, there is an urgent need for additional prognostic markers and therapeutic targets. It is clear that multiple markers will be required to provide accurate prognostic information at diagnosis, and multiple parts of the metastatic pathway will need to be targeted to improve survival in patients with metastatic melanoma.

This review has focused on five molecules involved in melanoma metastasis – MCAM, Gal-3, CSPG4, MMP-2, and PAX-3. All of these molecules are expressed by a high proportion of primary and metastatic melanoma and have been described by others as biomarkers for melanoma. The word "biomarker" can be defined as: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (228). Our goal in this review has been to examine the expression patterns and functions of each of these molecules, with a focus on whether these "biomarkers" reveal the pathogenic processes of melanoma metastases. We believe that a good biomarker could also be a therapeutic target, and that examining the expression of a combination of molecules involved in different aspects of the metastatic process will provide better prognostic information compared to that obtained from a single biomarker.

In this review we have shown that these five molecules, although they have unique roles, both interact with each other and show similarities in their function. For example, both Gal-3 and PAX3 are anti-apoptotic, Gal-3 binds CSPG4 and Gal-1 binds MCAM. MCAM downstream signaling regulates the expression of MMP-2, nuclear Gal-3 up-regulates MMP-2 expression and MMP-2 cleaves Gal-3. MCAM, CSPG4, and Gal-3 are associated with angiogenesis and CSPG4 is involved with the activation of pro-MMP-2 on melanoma (relevant references are in the review). It will be interesting to see if Gal-3 can similarly bind MCAM as although both Gal-1 and Gal-3 bind glycosylation structures presented by core proteins the binding specificities of these two galectins differ. Gal-1 can recognize a range of different complex *N*-glycans, whereas Gal-3 recognizes poly-*N*-acetyllactosamine containing glycans that may be N- or O-linked (229). **Figure 1** displays more of the cross connections that were revealed by the detailed examination of these five molecules.

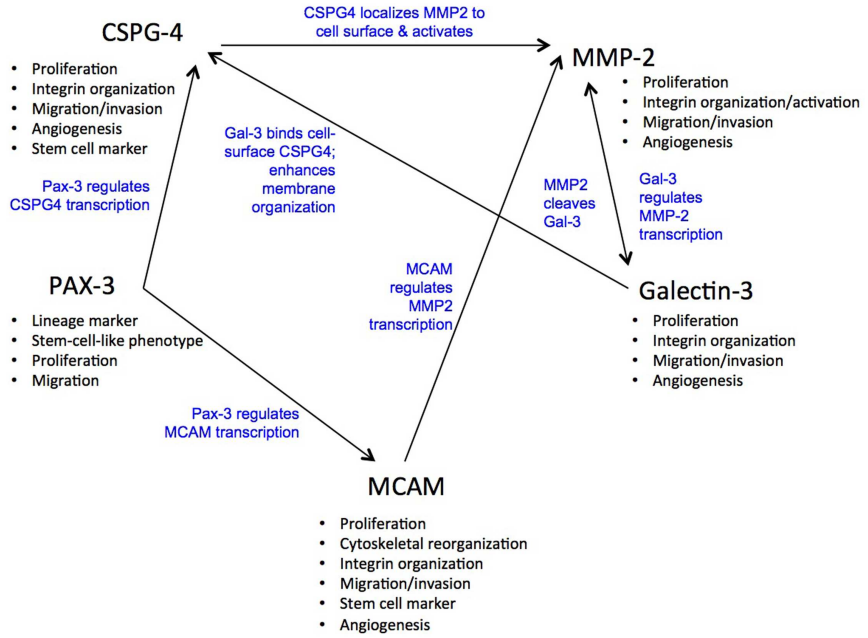

FIGURE 1 | Functional associations between MCAM, Gal-3, CSPG4, MMP-2, and PAX-3.

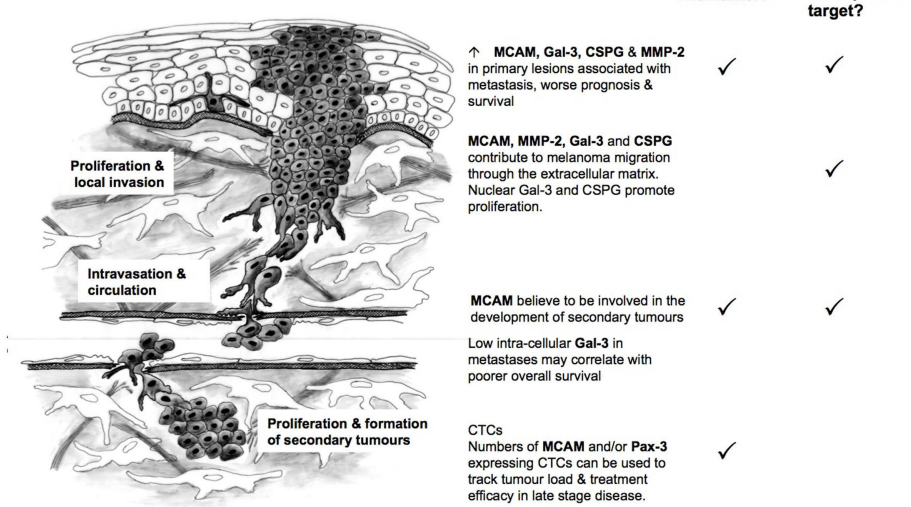

FIGURE 2 | MCAM, Gal-3, CSPG4, MMP-2, and PAX-3 as biomarkers and targets in melanoma metastasis.

It is particularly interesting that the combination of PAX3, MCAM, and CSPG4 is associated with less differentiated, motile cells of the melanocytic lineage and MCAM and CSPG4 are recognized stem cell markers. Indeed, the genes encoding these two stem cell markers are targets of PAX3 (68). The fact that the majority of metastatic melanoma express these stem cell markers, and when present, neither MCAM or CSPG4 is expressed by a minor population of cells within the melanoma leads one to think about rare cancer stem cells in melanoma. Interestingly, it has been demonstrated that approximately one in four cells from stage II, III, and IV melanomas obtained directly from patients are capable of developing tumors and moreover many markers are reversibly turned on and off *in vivo* (230). These findings directly question whether melanoma follows a cancer stem cell model and they also indicate that multiple biomarkers should be examined at each stage of melanoma progression for a reliable indication of prognosis.

MCAM, MMP-2, and Gal-3 expression in primary melanoma have been linked to poorer overall survival (89, 90, 144, 206, 207) and could be used in combination with current prognostic indicators to identify patients at high-risk of recurrence (**Figure 2**).

MCAM is believed to contribute to the later stages of metastatic spread (e.g., the formation of secondary tumors) (107), while MMP-2, and CSPG4 are likely to play a role earlier in the course of the disease. Gal-3 shows a bi-modal distribution – with increased intracellular expression early in disease progression and decreased expression in later metastatic lesions (144). This is due to Gal-3's ability to act both as a transcriptional activator within the nucleus (147–150) and as a mediator between cell surface proteins (e.g., CSPG4, MCAM, integrins) and the ECM in the extracellular environment (161, 163, 167, 231). PAX-3 is expressed by all cells of the melanocytic lineage and is a key player in melanocyte development (36). However, it has recently been suggested that melanoma may be driven by cells with a less differentiated, highly motile phenotype and that PAX-3 may actively drive melanoma progression (57, 58). Currently, PAX-3 along with MCAM appears to be a useful biomarker for assessing tumor load and the effectiveness of treatment in later stage disease (55).

Although molecular biomarkers for cutaneous melanoma have received a lot of attention in recent years the introduction of one or more molecular biomarkers into clinical melanoma staging has lagged behind other cancers. This is partly due to the nature of the disease, and is compounded by the increasing diagnosis of melanoma from thin primary lesions, which leave no tissue for study outside of the standard clinical pathology procedures. In addition, some melanoma may recur many years after the original diagnosis, whereas others may recur within 5 years (17). We have highlighted throughout our review that currently there is no way of predicting which patients with thin melanomas are likely to relapse and when. The fact that cutaneous melanoma originates in melanocytes that have arisen from the neural crest and migrated to the skin is an additional difficulty, as this suggests normal melanocytes may have a molecular signature characteristic of an invasive phenotype. Therefore, the use of multiple markers will provide the best indicator of prognosis. Specifically, we believe that further study of a panel of markers, like those examined here, which have overlapping functions and are implicated at multiple stages of the disease process, may lead to the identification of a set of genes that can reliably assist in diagnosis and prognosis. Whether or not a combination of MCAM, MMP-2, CCPG4, PAX-3, and Gal-3 can identify those thin melanomas that comprise the 5% that will develop metastases at a later stage will require further studies of clinical material.

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Variable expression of GLIPR1 correlates with invasive potential in melanoma cells

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GLI pathogenesis-related 1 (GLIPR1) was previously identified as an epigenetically regulated tumor suppressor in prostate cancer and, conversely, an oncoprotein in glioma. More recently, GLIPR1 was shown to be differentially expressed in other cancers including ovarian, acute myeloid leukemia, and Wilms' tumor. Here we investigated GLIPR1 expression in metastatic melanoma cell lines and tissue. GLIPR1 was variably expressed in metastatic melanoma cells, and transcript levels correlated with degree of GLIPR1 promoter methylation *in vitro*. Elevated GLIPR1 levels were correlated with increased invasive potential, and siRNA-mediated knockdown of GLIPR1 expression resulted in reduced cell migration and proliferation *in vitro*. Immunohistochemical studies of melanoma tissue microarrays showed moderate to high staining for GLIPR1 in 50% of specimens analyzed. GLIPR1 staining was observed in normal skin in merocrine sweat glands, sebaceous glands, and hair follicles within the dermis.

Keywords: GLIPR1, melanoma, invasion, methylation, CAP

INTRODUCTION

GLI pathogenesis-related 1 (*GLIPR1*) has been reported to act as a tumor suppressor gene that is down-regulated in prostate cancer (1–3). In contrast, GLIPR1 is up-regulated in glioma (4, 5) and Wilms' tumor (6) compared to normal tissue, and has recently been shown to be differentially expressed in ovarian cancer cell lines (7). *GLIPR1* encodes a 266 amino acid (∼30 kDa) member of the CAP superfamily (cysteine rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins) (8). The N-terminus of most of the CAP proteins includes a putative signal peptide indicating this protein is secreted or surface exposed. GLIPR1 is characterized by a postulated signal peptide and a putative C-terminal transmembrane domain (TMD) (5).

GLIPR1 has been reported to be regulated by p53 in prostate cancer (1). Increased apoptosis, accompanied by decreased tumor progression and metastasis were reported following adeno-viral delivery of *Glipr1* in an orthotopic model for metastatic prostate cancer (9). *Glipr1*-mediated proapoptotic activity was also shown to be related to the presence of the N-terminal signal peptide. Li et al. showed that GLIPR1 up-regulation resulted in elevated reactive oxygen species production, leading to apoptosis through activation of the c-Jun–NH_2 kinase signaling cascade (10). GLIPR1 has also been shown to regulate growth, survival, and invasion of glioma cells (11). Epigenetics studies have shown that hypermethylation in the promoter region of *GLIPR1* is responsible for the down-regulation of GLIPR1 in prostate cancer (2). In addition, methylation studies of *GLIPR1* showed significant hypomethylation in Wilms' tumor relative to normal tissue (6). In the development of malignant melanoma, epigenetic changes are emerging as important factors where more than 70 hypermethylated genes have been identified and hypomethylation occurs globally in tumor cells [reviewed in (12)].

Despite a growing body of literature pointing to a role for GLIPR1 in cancer, little is known of the normal function of GLIPR1 and of how disruption might contribute to cancer initiation or progression. We identified *GLIPR1* as part of a gene expression signature that predicted invasive potential in melanoma cell lines (13). Here we report on the role of GLIPR1 in melanoma in more detail, and confirm that GLIPR1 is variably expressed in melanoma cells, which is underpinned by differential promoter methylation, and that GLIPR1 levels correlated with invasive potential. We also show that GLIPR1 is variably expressed in melanoma tissue samples, and can be detected in certain adnexal structures of normal epidermis. We also show that GLIPR1 is a glycosylated transmembrane protein transported to the cell surface.

MATERIALS AND METHODS

CELL LINES

Melanoma cell lines used for this study were generated from pathologically confirmed metastatic melanoma samples obtained with ethical approval as previously described (14, 15) and cultured in MEM-α (Invitrogen) supplemented with 0.1% insulin-transferrin-sodium selenite (Roche) and 10% fetal bovine

serum (FBS; Bio International, New Zealand). Glioma cell lines U251 and SNB75 were obtained from the Developmental Therapeutics Program, National Cancer Institute and cultured in DMEM (Invitrogen) supplemented with 10% FBS. All new cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2. The NZM cell lines used for migration assays in this study were chosen based on their classification as either having a lower (NZM12, 15, 45) or higher (NZM9, 40) invasive potential based on previously published transcript and phenotype profiling (13). Given the established association in the literature between elevated GLIPR1 levels and glioma progression, glioma cell lines were included as comparative high GLIPR1 positive controls in this study.

RNA ISOLATION AND REAL-TIME REVERSE TRANSCRIPTION QUANTITATIVE PCR

Total RNA was extracted from cultured cells using RNeasy columns (Qiagen) according to the manufacturer's specification. Total RNA (100 ng) was transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen), primed with random hexamers (Invitrogen) and oligo d(T) (Invitrogen) in a 20 μL reaction volume according to the manufacturer's instructions. Transcript abundance was measured using Platinum SYBR Green qPCR SuperMix-UDG with ROX reference dye (Invitrogen) on an ABI 7300 Real-Time PCR System. Reverse transcription quantitative PCR (RT-qPCR) reactions were performed in duplicate with 2.5 ng template cDNA (RNA equivalent) per 20 μL reaction and corresponding no-template controls. Cycling conditions were 50°C for 2 min, 95°C for 2 min, then 40 cycles of 95°C for 15 s/60°C for 1 min, followed by melting curve analysis. *GLIPR1* abundance was normalized to Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), and Ubiquitin C (UBC) reference gene expression and expressed relative to *GLIPR1* levels in NZM15 (possessing the lowest level of *GLIPR1*) using qBase software and the delta–delta-C_q method.

The following primers were used in RT-qPCR experiments. GLIPR1 forward primer: AGT TCC GAT CAG AGG TGA AAC C; GLIPR1 reverse primer: GCT TCA GCC GTG TAT TAT GTG A; UBC forward primer: ATT TGG GTC GCG GTT CTT G; UBC reverse primer: TGC CTT GAC ATT CTC GAT GGT (RTPrimerDB ID 8); YWHAZ forward primer: ACT TTT GGT ACA TTG TGG CTT CAA; YWHAZ reverse primer: CCG CCA GGA CAA ACC AGT AT (RTPrimerDB ID 9) (16).

CELL LYSATE PREPARATION AND WESTERN BLOTTING

Lysates were prepared by incubating cells for 30 min in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (w/v) nonyl phenoxylpolyethoxylethanol (NP40), 0.1% (w/v) sodium dodecyl sulfate (SDS), 20× complete protease inhibitor (50 μl; Roche), 1 μM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration of the lysate was determined using a bicinchoninic acid assay (BCA kit; Pierce Chemical Co.) according to the manufacturer's instructions. Total protein was resolved by SDS-PAGE and electro transferred onto Bio Trace Polyvinylidene Fluoride (PVDF) transfer membrane (Pall Corporation, pore size 0.45 μm). The PVDF membrane was blocked with 5% (w/v) non-fat milk powder in Tris-Buffered Saline Tween-20 (TBST) followed by staining with mouse anti-GLIPR1 polyclonal antibody (Abnova, H00011010-A01) overnight at 4°C. The specific bands were detected using goat anti-mouse IgG horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich). Immuno-reactive bands were visualized using Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and exposing the blots to imaging film (Kodak MXB X-Ray film).

The quantification of non-saturated, developed western blots was carried out using a GS-700 Imaging Densitometer (BioRad) and the intensities of individual bands were quantified using BioRad Quantity One software.

SMALL INTERFERING RNA TRANSFECTION

siRNA-mediated knockdown of GLIPR1 was performed using reverse transfection with Lipofectamine RNAiMAX (Invitrogen) and pre-designed siRNAs targeting *GLIPR1* (ON-TARGET*plus* SMARTpool, L-019819-00-0020, Dharmacon) according to the manufacturer's instructions, with a final siRNA concentration of 10 nM. GLIPR1 knockdown was confirmed using RT-qPCR (24 h) and western blotting (72 h) post-transfection. Negative control experiments were performed using an ON-TARGET*plus* Non-Targeting Pool (D-001810-10-20, Dharmacon). Sense-strand *GLIPR1* siRNA target sequences were:

GAG ACC AAG UGA AAC GUU A; GCU CAA GUA CCC UAA UUU A; UAG CCU GGA UGG UUU CUU U; UGG CUG CGC AGU UCA AUU U

ON-TARGET*plus* GAPD control siRNA was used as a positive control to assess transfection efficiency (D-001830-01, Dharmacon).

CELL PROLIFERATION

Cell proliferation and viability was quantified using an MTT-based cell proliferation kit (Roche) according to the manufacturer's instructions. Briefly, 2.5×10^3 cells per well of a 96-well plate, in a final volume of 100 μl media were transfected with siRNA (see above). The cells were grown at 37°C, 5% CO_2, for 2, 4, 6, or 8 days after which they were treated with 10 μl MTT and incubated at 37°C for 4 h. The resulting formazan crystals were then solubilized with 100 μl solubilization solution and incubated overnight at 37°C. The absorbance (570 nm) was then measured with a microplate reader (Molecular Devices). Media was changed every 3 days.

TRANSWELL MIGRATION AND INVASION ASSAY

Boyden chamber migration assays were carried out in 24-well format using Transwell cell culture inserts with a polyethylene terephthalate (PET) membrane filter and an 8 μm pore size (BD Biosciences). Cell suspension in 200 μL media supplemented with 2% FBS (v/v) was added to the upper chamber (5×10^5 cells/ml). The lower chamber was filled with 600 μl medium supplemented with 10% (v/v) FBS as a chemoattractant. The cells were allowed to migrate across the membrane for 24 h at 37°C, 5% CO_2. After 24 h, cells were rinsed with PBS and fixed with pre-chilled methanol for 10 min followed by rinsing with distilled H_2O. Fixed cells were stained with hematoxylin for 5–10 min at room temperature then

rinsed with dH_2O. Cells that remained on the top side of the membrane were removed with a cotton swab, and the remaining cells imaged using an Olympus IX71 inverted microscope. Twenty-five random fields of view were captured per Transwell insert, and the number of cells that had migrated to the bottom side of the membrane was counted by using ImageJ software (17).

Invasion assays were carried out in a similar way using BioCoat Matrigel invasion chambers (BD Biosciences) consisting of a BD Falcon Cell Culture Insert with an 8 μm pore PET membrane, uniformly coated with BD Matrigel Matrix.

To investigate the effect of siRNA-mediated knockdown of GLIPR1 on the migration or invasion of cells, 3×10^4 cells/well were transfected with siRNA targeting *GLIPR1*, or non-targeting siRNA, for 24 h. Cells were trypsinized, pooled, and counted from three to four wells 24 h post-transfection, and 1×10^5 cells were seeded per insert for migration or invasion assays as described above.

METHYLATION

GLIPR1 promoter methylation status was analyzed by using bisulfite genomic sequencing. Genomic DNA was isolated from cultured cells using a Purelink Genomic DNA kit (Invitrogen) according to the manufacturer's instructions. Genomic DNA (300 ng) was bisulfite-converted and purified by using EZ DNA Methylation-Gold (Zymo Research) according to the manufacturer's instructions. One microliter of bisulfite-converted DNA was amplified by using two rounds of PCR, purified with a Purelink PCR Purification kit (Invitrogen), then visualized and quantitated by using an Agilent 2100 Bioanalyzer. Purified PCR products were then submitted to the Genetic Analysis Services, University of Otago, for Sanger sequencing. DNA methylation analysis was performed using BiQ Analyzer v2.00 (18). We amplified a 320 bp region using primers for amplification and sequencing of GLIPR1 from bisulfite-treated DNA previously published by Muller et al. (19). This region extends from −104 to −424 of the GLIPR1 translation start site, and corresponds to the CpG's referred to as "A–D" in (2). The primers were: forward, TTA TTA TGT GTT GAT ATG ATT TTA AAA AG; reverse AAC CCA CAA CTT TAC AAA CC TAA CC.

TISSUE SAMPLES AND ARRAYS

Skin specimens used in this study were archival human tissue specimens maintained by Healthlab Otago, Dunedin Public Hospital, Dunedin, New Zealand. All specimens were ethically consented for use under approval MEC/07/05/065 (with written informed consent from Multi-Region Ethics Committee, Ministry of Health, New Zealand). Melanoma tissue microarrays were purchased from US Biomax, Inc. Melanoma and nevi tissue microarrays (catalog number ME1001) consisted of 56 cases of malignant melanoma, 20 cases of metastatic melanoma, and 24 cases of benign nevus.

IMMUNOHISTOCHEMISTRY

Five micrometer paraffin-embedded tissue sections were de-waxed in xylene and rehydrated through a series of graded ethanols. Endogenous peroxidase activity of the tissue was then quenched with 3% (v/v) H_2O_2 in methanol for 10 min. Slides were washed with distilled water prior to microwave-mediated antigen retrieval in 10 mM sodium citrate with 0.05% Tween-20, pH 6.0 for 15 min. Cooled sections were rinsed in PBS, blocked with 10% normal goat serum in PBS for 30 min and incubated with a mouse polyclonal anti-GLIPR1 antibody (Abnova, A01; 1:200) overnight at 4°C. The signal was subsequently detected using Vectastain Elite ABC peroxidase-based detection system (Vector Laboratories). For melanoma and skin specimens NovaRED™ (Vector Laboratories) was used for visualization of immuno-reactivity to distinguish from melanin. To verify the specificity of the immuno-reactions, some sections were incubated in normal goat serum instead of primary antibody. Negative control incubations using the same secondary antibody, but omitting the primary antibody were also carried out and showed negative staining. Validation of the antibodies is provided in supplementary material (Figure S1 in Supplementary Material).

All tissue sections were observed and photographed using a Zeiss MC100 camera coupled to a Zeiss Axioplan universal microscope at a power of 200× or 400×. Randomly selected two to three microscopic measuring fields were analyzed for staining and identification of specific cell types. At least 100 cells were counted per microscopic field visualized.

STATISTICAL ANALYSIS

The results are presented as the average values ± standard error of mean (SEM). Data were analyzed using ANOVA and a Student's *t* test (with unequal variances). All graphs were generated by using Prism 4 (GraphPad Software, Inc.).

RESULTS

GLIPR1 EXPRESSION CORRELATED WITH PROMOTER METHYLATION AND INVASIVE POTENTIAL *IN VITRO*

GLIPR1 levels measured using RT-qPCR corresponded with the relative levels reported by previous microarray profiling of NZM cells lines (13), with NZM9 and 40 having relatively higher GLIPR1 abundance compared to NZM12, 15, and 45. Among the five melanoma cell lines, NZM15 cells had the lowest level of *GLIPR1* expression and was used as the baseline reference. The NZM40 cells had the highest *GLIPR1* expression (∼66-fold higher expression relative to NZM15, **Figure 1A**). NZM9 cells showed a greater *GLIPR1* expression level than that seen in U251 cells which were used as a positive control (∼43 and 24-fold higher than NZM15 respectively). SNB75 cells previously shown to have elevated *GLIPR1* expression (5), showed the highest expression levels within the melanoma and glioma cells tested, with 110-fold higher abundance relative to NZM15 cells. Consistent with the RT-qPCR results, NZM9, NZM40, U251, and SNB75 cells showed higher amounts of GLIPR1 protein, whereas GLIPR1 was undetectable in NZM12, NZM15, and NZM45 after exposure for 5 min and only a very small amount was evident following exposure overnight (**Figure 1B**). Variable GLIPR1 transcript levels were associated with differences in promoter methylation in a panel of melanoma cell lines of known invasive potential (**Figure 1C**; Figure S2A in Supplementary Material), with increasing promoter methylation associated with decreasing GLIPR1 abundance ($r^2 = 0.82$, $p = 0.037$). When additional cell lines from a previous study (13) were included

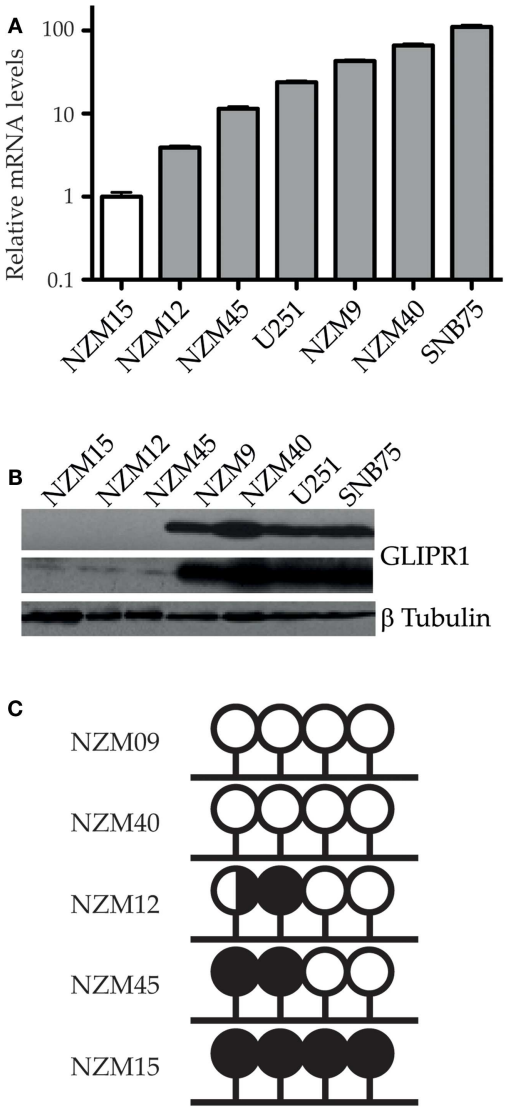

FIGURE 1 | GLIPR1 expression and promoter methylation in melanoma cells. **(A)** Relative mRNA transcript levels of GLIPR1 in different melanoma and glioma cells were quantified by RT-qPCR, normalized to reference genes YWHAZ and UBC, and reported relative to levels in NZM15. Results are mean of three independent experiments and error bars indicate SEM. **(B)** GLIPR1 protein levels in different melanoma and glioma cell lines were determined by western blotting with each lane loaded with 100 μg of total protein; the top GLIPR1 blot and β-tubulin loading control (bottom) was exposed for 5 min, and the center GLIPR1 blot exposed overnight. Two separate experiments gave similar results. **(C)** Summary of bisulfite sequencing data showing GLIPR1 CpG promoter methylation status for melanoma cell lines with known invasive potential (this study). NZM09 and NZM40 are strongly invasive compared to the remaining cell lines. Lollipops represent individual CpG dinucleotides within a CpG island in the GLIPR1 promoter. DNA from three different vials of each NZM cell line was sequenced at least twice on both strands. White, unmethylated; black, methylated; black/white, hemimethylated.

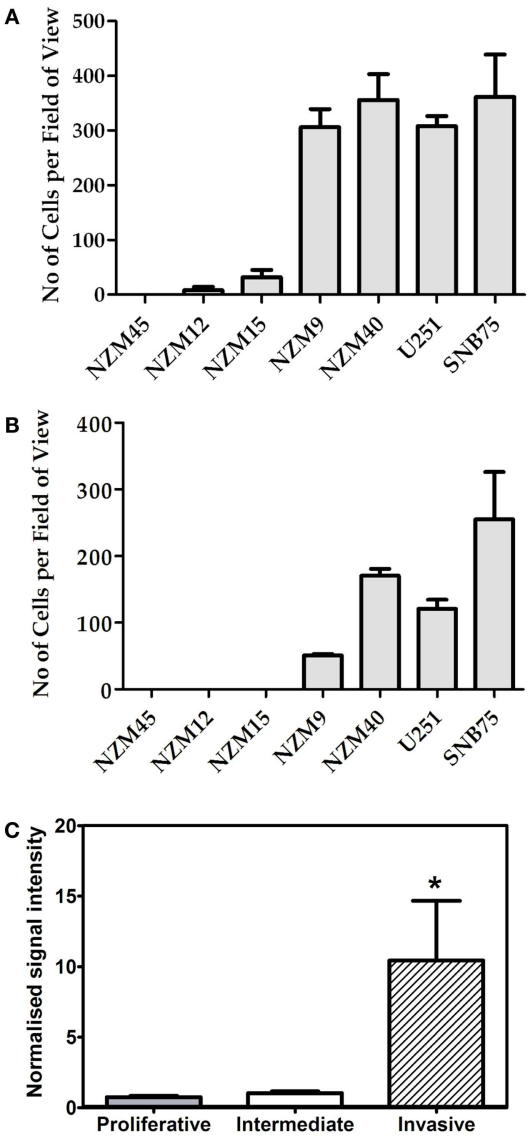

FIGURE 2 | GLIPR1 cell migration and invasion. Frequency with which melanoma and glioma cells cross a pored membrane by **(A)** migration or **(B)** invasion was assessed by microscope after 24 h. Number of migrating or invading cells was calculated by counting the number of cells per field of view in 25 microscopic fields per well. Data shown as the average number of cells per field of view ± SEM from three **(A)** or two **(B)** independent experiments respectively. **(C)** GLIPR1 transcript levels were significantly higher in an independent panel of cell lines with experimentally validated invasive potential (20).

in the analysis the correlation improved ($r^2 = 0.83$, $p = 0.002$; Figures S2B,C in Supplementary Material). Demethylation treatment with 5-azacytidine caused increased transcript abundance in NZM15, NZM12, and NZM45 (Figure S3 in Supplementary Material).

Having confirmed variable expression of GLIPR1, we then chose to investigate the relationship between GLIPR1 abundance and invasion in melanoma and glioma cells. *In vitro* cell migration and invasion was positively correlated with endogenous GLIPR1 expression levels ($r^2 = 0.94$ and $r^2 = 0.91$ respectively, **Figures 2A,B**; Figure S4 in Supplementary Material) suggesting a role for GLIPR1 in the migratory or invasive potential of

melanoma cells. Cells with the highest GLIPR1 expression (NZM9, NZM40, U251, SNB75) showed the highest number of migrating and invading cells (**Figures 2A,B**). Conversely, cells with relatively low levels of GLIPR1 (NZM12, NZM15, NZM45) showed little to no migrating cells and no detectable invasion. The total number of cells invading through the Matrigel matrix was less than in the absence of matrix, but the overall trend of invasion was similar to that of migration: cell lines with highest GLIPR1 levels (NZM40 and SNB7) showed the highest invasion. Increased GLIPR1 transcript levels were also associated with increased invasion in an independent set of publically available microarray data generated from melanoma cell lines with experimentally validated invasive potential (**Figure 2C**) (20).

GLIPR1 KNOCKDOWN CAUSED REDUCED CELL INVASION AND PROLIFERATION

To further investigate the relationship between GLIPR1 expression and cell migration and invasion, GLIPR1 expression was decreased using siRNA. siRNA-mediated knockdown of GLIPR1 resulted in a significant decrease in the number of melanoma and glioma cells migrating across the membrane relative to non-targeting controls (**Figure 3**; Figure S5 in Supplementary Material). We used glioma cell lines in which GLIPR1 had previously been shown to modulate invasive behavior (11) as positive controls to compare with melanoma cell lines in our *in vitro* invasion assays. SNB75 glioma cells, with the highest pre-knockdown migration rate, showed about a 50% decrease in cell migration 24 h after GLIPR1 knockdown (**Figure 3B**). Similarly, the high GLIPR1-expressing cell lines NZM40, NZM9 and U251 showed a 20–30% decrease in migration compared to non-targeting controls. Cells with lower GLIPR1 levels (NZM12, NZM15) showed no measurable change in the already small number of cells migrating across the membrane after knockdown which reflects the intrinsically weak invasive potential of these cells.

GLIPR1 knockdown led to a significant reduction in the number of cells invading through Matrigel matrix (**Figure 3C**). The largest decrease in invasion was observed in NZM9 cells (∼38%) followed by NZM40 (30%) and SNB75 (24%) cells, with U251 cells (8%) showing a small but significant reduction in cell invasion (**Figure 3C**). Overall, the results of our Transwell assays and knockdown experiments support the notion that GLIPR1 is involved in mediating the invasive potential of melanoma cell lines.

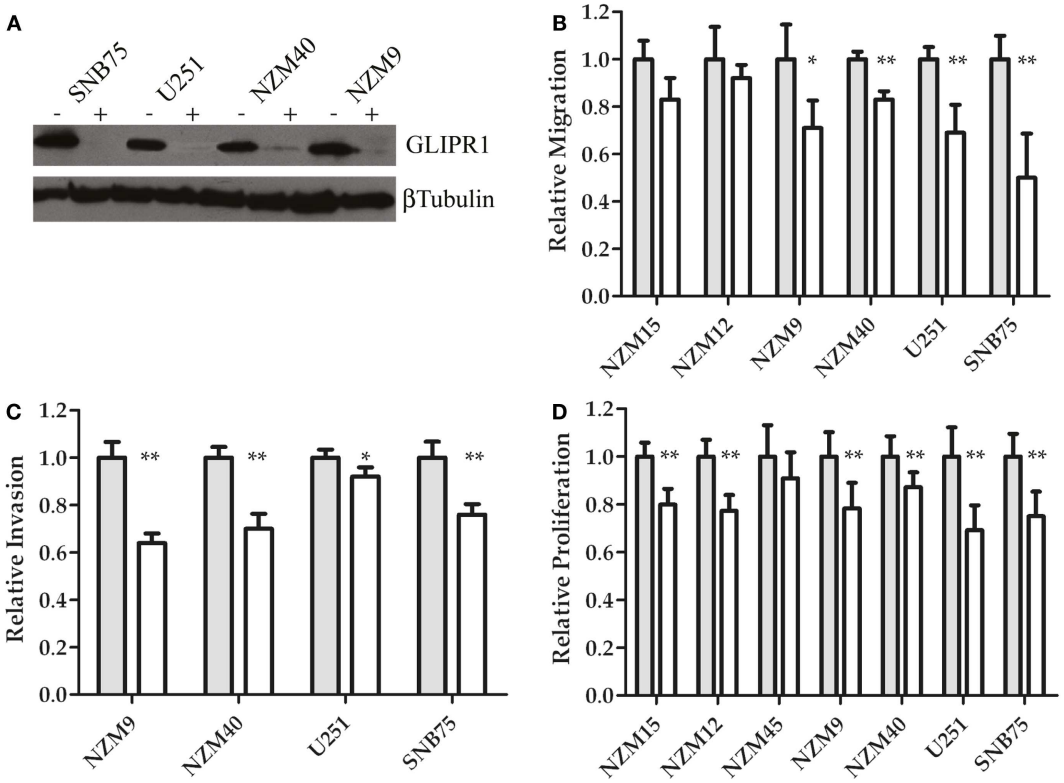

FIGURE 3 | GLIPR1 siRNA knockdown decreases cellular invasion and proliferation. (A) GLIPR1 protein levels following knockdown were determined by western blotting (40 μg of total protein per lane) from cells 72 h following transfection with siRNA. GLIPR1 knockdown with siGLI (+) and control treatment with non-targeting siRNA siNT (−) are indicated above gel. Low levels of endogenous GLIPR1 prevented assessment of the extent of knockdown in NZM15, NZM12, and NZM45 by western blotting. Relative migration **(B)** and invasion **(C)** of cells across the membrane of transwell inserts was measured 24 h after siGLI. Data shown as the average number of cells per field of view ± SEM from three **(B)** or two **(C)** independent experiments. Error bars indicate SEM; $*p < 0.005$, $**p < 0.001$. No migration was observed for NZM45 (**Figure 2A**) and no invasion was seen for NZM45, NZM12, or NZM15 (**Figure 2B**). **(D)** Cell proliferation was quantified using MTT-based colorimetric assay. Results are mean of two independent experiments, $n = 4$. Results in **(D)** are shown as data for cells 4 days after transfection with siGLI relative to data for cells transfected with siNT. Results for **(B,C)** are shown for cells 24 h following transfection.

siRNA-mediated knockdown of GLIPR1 resulted in a 10–22% decrease in proliferation in melanoma cells compared to 31 and 25% decrease for U251 and SNB75 glioma cells respectively, 4 days after transfection (**Figure 3D**). Reduced proliferation in glioma cell lines was observed by Rosenzweig et al. (11) who also reported that silencing of GLIPR1 induced apoptosis in some glioma cells. However, we saw no evidence of increased apoptosis or cell cycle-related growth arrest in any of the glioma and melanoma cells tested in this study (data not shown).

CELLULAR LOCALIZATION OF GLIPR1

To help understand how GLIPR1 could mediate invasion we examined cellular localization. GLIPR1 contains a predicted signal peptide and C-terminal TMD suggesting it may be translocated to the endoplasmic reticulum (ER) and trafficked to the cell surface as an integral membrane protein. *In vitro* translation assays showed GLIPR1 is processed to a higher molecular weight form in the presence of ER membranes (Figure S6A in Supplementary Material). This higher molecular weight form is sensitive to Endoglycosidase H digestion, indicating that it is glycosylated (Figure S6B in Supplementary Material). GLIPR1 segregates in the pellet fraction upon sodium carbonate extraction (Figure S6C in Supplementary Material), which confirms that it is integrated in the membrane. The higher molecular weight glycosylated GLIPR1 is protected from protease digestion in the absence of detergent (Figure S6D in Supplementary Material). Taken together these results indicate that GLIPR1 has a functional signal peptide and TMD, and is translocated into the ER where it is glycosylated at a site in its ER-lumen exposed soluble domain. Cell surface biotinylation assays in GLIPR1-expressing NZM9 cells demonstrated that GLIPR1 is present at the cell surface (Figure S7 in Supplementary Material).

GLIPR1 EXPRESSION IN MELANOMA AND SKIN TISSUE

Having demonstrated a relationship between GLIPR1 expression and migration/invasion in melanoma cell lines, we investigated GLIPR1 expression in melanoma and skin tissue samples. Immunohistochemical staining of malignant melanoma tissue samples showed variable expression of GLIPR1 (**Figures 4A–C**) in a similar fashion to the NZM cell lines. Of the 76 melanoma specimens analyzed, 50% showed moderate to high immuno-reactivity (++ and +++) for GLIPR1. The other 50% of the specimens (38/76) showed either no (26/76) or low (12/76) staining for GLIPR1. However, unlike glioma, there was no obvious relationship between GLIPR1 positivity and melanoma progression. In normal skin, all layers of the epidermis except for the stratum corneum were immuno-positive for GLIPR1 (**Figure 5A**). Cells of the basal layer showed positive staining for GLIPR1 (**Figure 5A**, red). Most of the fibro-elastic tissue in the dermal layer of skin was found to be immuno-negative for GLIPR1. However, merocrine sweat glands, sebaceous glands, and hair follicles within the dermis were found to be immuno-positive for GLIPR1 (**Figures 5B–D**).

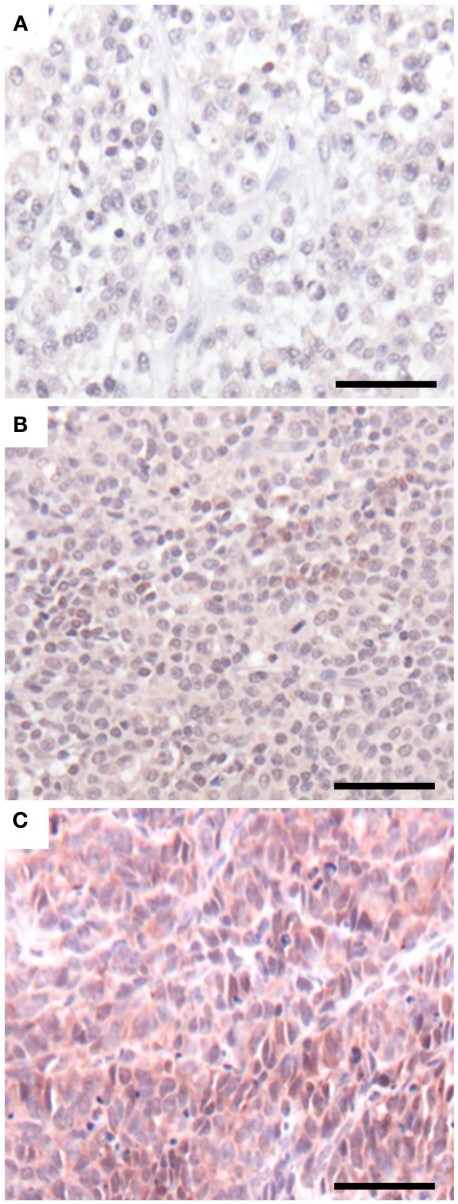

FIGURE 4 | GLIPR1 staining in malignant melanoma. GLIPR1 immuno-staining in **(A)** malignant melanoma of the heel (+, low staining intensity), **(B)** malignant melanoma of the pate/crown (++, moderate staining intensity), and **(C)** malignant melanoma of the thumb (+++, high staining intensity). GLIPR1 immuno-positive regions were stained with NovaRED. All images were photographed at a power of 200×. Scale bar = 50 μm. Samples shown are from Melanoma tissue microarrays (US Biomax, Inc.).

DISCUSSION

GLIPR1 transcript and protein have been reported in various tissues including heart, lung, liver, spleen, skin, colon, pancreas, lymphocytes, muscle, bone marrow, placenta, adrenal gland, prostate, glioma, and prostate cancer (5, 11, 21). More recently, *GLIPR1* has been found to be differentially expressed in ovarian cancer and acute myeloid leukemia (7, 22). However, GLIPR1 expression analysis in melanoma has not been previously reported. Using RT-qPCR and western blots we found variable expression of *GLIPR1* mRNA and protein in different metastatic melanoma cell lines with two melanoma cell lines having similar levels to glioma cell lines previously reported as having high GLIPR1 expression

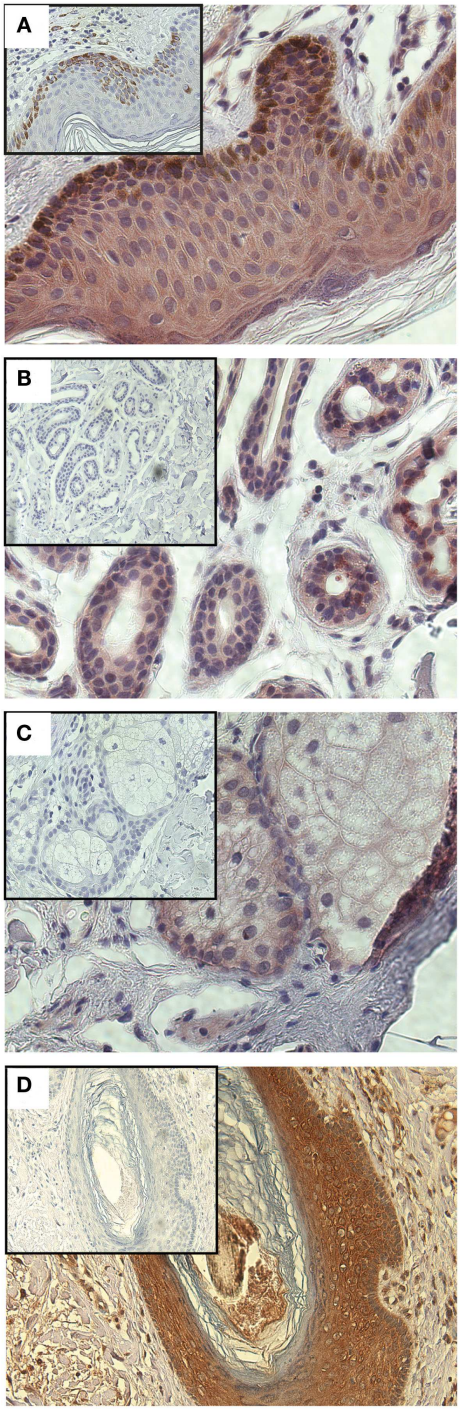

FIGURE 5 | Immunohistochemical staining of GLIPR1 in skin specimens. Immuno-reactive cells of normal skin **(A)**, sweat glands **(B)**, sebaceous glands, **(C)**, hair follicles **(D)**. Skin specimens **(A–C)** were stained with NovaRED to give a red color for GLIPR1 immuno-positive regions, while hair follicles **(D)** were stained with DAB to give brown color for GLIPR1. Cell nuclei are stained blue with hematoxylin. Inset images show negative control reacted with the non-specific goat serum instead of anti-GLIPR1 antibody. Lack of brown staining in the negative control indicates this is due to GLIPR1 and not melanin. All images were photographed at a power of 400×.

(**Figures 1A,B**). These data add to the growing number of cancer cell types in which GLIPR1 has been reported to be variably expressed.

Epigenetic modulation of GLIPR1 expression by promoter methylation has been reported previously in prostate cancer, glioma, and Wilms' tumors, and we show that it is also the case in melanoma cells in the samples tested, with decreasing promoter methylation associated with increasing levels of GLIPR1. Our study focused on metastatic melanoma cells. Whether GLIPR1 promoter methylation is dynamic during disease progression or shows higher or lower levels of methylation in metastatic melanoma cells compared to normal melanocytes, benign nevi and primary melanoma remains unknown and requires further investigation.

We investigated the relationship between endogenous GLIPR1 levels and migration and invasion potential *in vitro*, which revealed that melanoma cells with higher endogenous GLIPR1 levels displayed significantly greater migration and invasion capability than cells with relatively lower levels of GLIPR1. siRNA-mediated knockdown of GLIPR1 inhibited migration and invasion in melanoma cell lines (**Figures 3B,C**). The low level of migration and complete lack of invasion in melanoma cells with relatively lower GLIPR1 levels (NZM15, NZM12, and NZM45) suggests that a threshold level of GLIPR1 expression may be present in melanoma cells with a strongly invasive phenotype as GLIPR1 levels in NZM9, NZM40, U251, and SNB75 cells after siRNA-mediated knockdown were still higher than that in untreated and weakly invasive NZM15, NZM12, and NZM45 cells. Overexpression of GLIPR1 has previously been shown to increase invasion of several glioma cell lines (11), and is consistent with our findings in melanoma cells suggesting GLIPR1 acts as an oncoprotein rather than a tumor suppressor in melanoma. GLIPR1 knockdown caused a modest but reproducible decrease in proliferation in both melanoma and glioma cells, suggesting that GLIPR1 may play a role in cell growth at some level. It is possible that the reduced invasion we observed after GLIPR1 knockdown was a consequence of the observed reduction in proliferation.

We demonstrated for the first time that GLIPR1 contains a functional signal peptide and TMD (Figure S6 in Supplementary Material). We confirm that it is a glycoprotein, most likely at the predicted glycosylation site at asparagine 92 (5), which is shown by homology modeling of GLIPR1 to be on the surface of the protein (data not shown). Glycosylation was also seen in the recent expression and structural studies by Asojo and co-workers (23, 24). GLIPR1 is translocated to the cell surface where its soluble N-terminal domain will be exposed to the extracellular space. The exposure of GLIPR1's soluble domain to the extracellular matrix is consistent with a role of GLIPR1 in invasion and migration. Future investigations as to whether GLIPR1 possesses proteolytic activity as displayed by another member of the CAP family (25) would prove invaluable and provide insight as to whether such an activity is directly associated with the cell invasion and migration properties similar to that of matrix metalloproteases.

The present study characterized GLIPR1 expression by immunohistochemistry in normal and cancerous skin. Previously, mRNA levels of *GLIPR1* transcripts in skin have been reported as very low or undetectable (11, 21). Immunohistochemistry showed

GLIPR1 was detectable in normal skin (**Figure 5**) within certain tissues such as epithelial cells of epidermis, sebaceous glands, merocrine sweat glands, and hair follicles. Immunohistochemical staining of melanoma tissues confirmed variable expression of GLIPR1, similar to that observed in NZM cell lines, with almost an equal proportion of melanoma specimens expressing relatively higher or relatively lower levels of GLIPR1. It does not appear that GLIPR1 levels are uniformly elevated with increasing melanoma stage, which is again consistent with GLIPR1 being sensitive to dynamic gene expression changes that are associated with the postulated phenotype switching capacity of melanoma cells during disease progression. Although we demonstrated variable GLIPR1 staining in melanoma tissue microarray samples, these samples lacked clinical information, so the clinical significance of variable GLIPR1 expression remains unknown. A survey of publically available array data shows that GLIPR1 levels are variable in melanoma (13), which is in agreement with our observations in this study. However, there is little useful published melanoma array data that includes patient information to determine if GLIPR1 transcript abundance is correlated with clinical outcome. We analyzed GLIPR1 levels in the study of metastatic melanoma samples by Bogunovic and colleagues (26), which includes clinical data, and found that elevated GLIPR1 levels were significantly positively correlated with survival (Figure S8 in Supplementary Material). Given elevated GLIPR1 levels are part of a multi-gene expression signature that is associated with a phenotypic balance between invasive and proliferative states in melanoma, we speculate that metastatic melanomas with an invasive phenotype may proliferate more slowly (according to the phenotype switching model), and thus offer a survival advantage to the patient, compared to less invasive but more rapidly proliferating tumors. Further data are required to confirm this observation. Yang and co-workers (27) identified *GLIPR1* as part of a group of extracellular matrix-related genes involved in a melanocyte growth arrest program, which further implicates GLIPR1 as having a potential role in the dynamics of melanocyte biology via interaction with the extracellular microenvironment. Further investigation is required to clarify this.

The MITF and POU3F2 (BRN2) transcription factors have been reported to be inversely correlated in melanoma cells (28), are both key markers of the phenotype switching model of invasive potential [reviewed in (29)], and are both possible regulators of GLIPR1. GLIPR1 is inversely correlated with MITF in NZM cell lines (13), suggesting that perhaps MITF might negatively regulate GLIPR1. However, the evidence to support this is lacking: there are no MITF binding sites in the GLIPR1 promoter (not shown), and recent studies by others – although not taking epigenetic silencing into account – did not identify GLIPR1 as a direct target of either MITF or POU3F2 in melanoma cells (30–32). Based on our GLIPR1 promoter methylation data, we speculate that perhaps genome-wide epigenetic re-programing can occur in melanoma cells, to which GLIPR1 is sensitive, and that is associated with phenotype switching mechanisms in melanoma cells.

Malignant melanoma is an aggressive and unpredictable cancer. Currently there is no cure for metastatic melanoma, and no way of determining which patients will respond to current treatment options. The mechanisms underlying melanoma progression and resistance to therapeutic agents are not well understood. There are few treatment options once metastasized, and new biomarkers that aid diagnosis, predict clinical outcome, and suggest new therapies are required. Based on the data presented here, future studies will focus on identifying whether GLIPR1 levels and/or promoter methylation status may be a clinically beneficial marker of metastatic melanoma phenotype.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cancer_Genetics/10.3389/fonc.2013.00225/abstract

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Circulating melanoma cells: scoping the target

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CIRCULATING MELANOMA CELLS

Molecular markers have been increasingly applied in cancer to assess metastatic risk and to guide treatment, including in melanoma, where assessment of BRAF mutations in tumor tissues to determine suitability for treatment with vemurafenib is now routine (1). Static assessment of tumor tissues, however, does not indicate whether tumor cells are being shed or whether treatment is reducing metastasis. Because melanoma metastasizes hematogenously, examination of circulating melanoma cells (CMC) is a logical as well as a convenient alternative to the examination of tumor tissues. A reliable assessment of CMC numbers and molecular signatures could have major clinical impact. The failure to demonstrate a survival advantage for adjuvant treatment might be linked to inadequate disease staging and, consequently, inadequate assessment of relapse risk. Because CMC may indicate systemic subclinical disease, their detection and analysis may be useful not only for staging/prognosis but also for assessing response to adjuvant therapy. The discovery of CMC theoretically would also allow for earlier detection of metastasis. This could potentially increase the effectiveness of existing therapies. Serial CMC assessments during treatment may allow for the earlier assessment of response, sparing non-responding patients toxicities. Serial CMC assessments could help determine the mechanisms of resistance and suggest interventions to address them. Furthermore, not all patients with melanoma are candidates for surgery to obtain tissue for analysis of molecular markers, and CMC would provide a liquid biopsy of sum total of tumors at all the sites in the patient.

POLYMERASE CHAIN REACTION APPROACHES

Polymerase chain reaction (PCR)-based techniques that detect the expression of the mRNA/transcripts of melanocyte-associated factors, such as tyrosinase and Melan-A, in nucleated blood cells and present in cell free fraction have been best studied clinically. They have demonstrated promise in melanoma surveillance and in monitoring adjuvant and metastatic therapy (2–4). They can also be combined with assessment of circulating DNA for melanoma-associated mutations, which in itself can be used to infer the presence of CMC (5). No PCR-based approach, however, has been validated for clinical use, due to limitations in consistency and high false negative rates. These approaches cannot quantify the number of CMC, and morphologic evaluation of the cells cannot be obtained. The presence of normal cellular transcripts by leukocytes, which contribute most of the total nucleotides extracted, may dilute those that are tumor-related, even following substantial enrichment for CMC (6). Furthermore, tumor heterogeneity may lead to clones of cells that do not express the melanocyte marker. There are technical issues. RNA is inherently labile (7). Differences in the PCR methodologies applied as well as differences in data interpretation may also be responsible for the disparate findings of various studies. Because cells are not captured, the ability to evaluate changes in targets or biological characteristics is limited, particularly in the context of the tumor heterogeneity that characterizes melanoma (8).

CYTOMETRIC APPROACHES

Techniques that isolate and enumerate morphologically identified CMC have also been studied. Although several steps can be involved, molecular characterization of the CMC isolated has been accomplished. Typically, cytometric approaches have two components: a preparative and an analytical one. Preparative enrichment is required because CMC are rare in the blood, at counts lower than 10/ml of whole blood (as low as 1 CMC per 1,000,000 leukocytes). The goal is to increase sensitivity. The analytical step eliminates the non-relevant blood cells in the enriched fraction. The goal is to increase specificity. Here the greatest concern is again false negative results. Despite an abundance of potential markers, a consensus on how melanoma cells circulate, their phenotype, and the optimal capture reagent have not been established. Tumor cells in circulation may not always exhibit the criteria used to identify them in the context of tissue biopsy. The CellSearch® system (Veridex LLC, Raritan, NJ, USA), a cytometric approach based on the immunomagnetic capture of circulating EpCAM-positive tumor cells, has been approved by the U.S. Food and Drug Administration to monitor the effectiveness of therapy in patients with metastatic breast, colorectal, and prostate carcinomas (9). No cytometric method has been validated for clinical use in melanoma. Representative approaches applied include the following:

PHYSICAL

Density gradient separations with for example, Ficoll, combined with elimination of erythrocytes using isotonic ammonium chloride lysis method have been applied (10). The advantages of these approaches are simplicity and lower costs. However, these techniques typically have unacceptably high cell losses and thus lack sensitivity. Several platforms, including filter-based microdevices, or microfluidic devices, using size as the capture method have been described (11, 12). Given their heterogeneity, it is not clear that large size is a sufficient criterion to capture all CMC. Dielectrophoretic forces have been applied, and cells of different types have been separated, without interfering with their viability, according to their dielectric and hydrodynamic flow properties (13). These approaches may be applicable to CMC but have not yet been effectively applied clinically.

IMMUNOMAGNETIC – POSITIVE SELECTION

Circulating melanoma cells have been positively selected using immunomagnetic cell enrichment technique with antibody to, for example, the high molecular weight-melanoma-associated antigen (HMW-MAA). Cells isolated have been assessed for BRAF status (14). CellSearch® technology has also been applied to CMC (15). Magnetic particles are tagged with antibodies against the melanoma-associated cell-surface antigen CD146 and captured in a magnet. These cells are detected using microscopy by fluorescently tagged antibody to HMWMAA (15). The caveat of this technology is the non-specificity of the capture antigen. CD146 is also expressed on circulating endothelial cells. It is also not known whether all CMC express CD146. Hence, sensitivity of this approach is unclear. Multiple antibody-bound beads were suggested to increase the sensitivity of positive selection of CMCs, however, the approach may add to the complexity of the microscopic image analysis and interpretation (16).

IMMUNOMAGNETIC – NEGATIVE SELECTION

Negative immunomagnetic selection is an attractive approach for isolating CMC in the absence of reliable CMC surface markers. Antibodies tagged with magnetic particles against CD45 antigen present ubiquitously on leukocytes are used to magnetically deplete a blood sample of white cells. These remaining non-magnetic cells are analyzed for CMC with melanocyte-associated markers, such as Melan-A/MART-1, HMB-45, and S100B. The advantage of this technique is unbiased capture of non-leukocytic cells, and disadvantage is lower purity of the CMC due to less than 100% capture of leukocytes. Negative separation has been successfully used to isolate CMC, and also offers the possibility of molecular characterization (17, 18). Although CMC prepared by this method are not pure, due to less than complete depletion of leukocytes, this disadvantage may be outweighed by the presumed complete capture of all types of CMC.

AUTOMATED CYTOMETRIC METHODS

Flow cytometry methods using antibodies against melanocyte determinant have been used to identify and capture CMC. However, the throughput is low, and the rarity of CMC make this technique by itself less practical (19). High speed scanning microscopy techniques such as fiber-optic based automated scanning technology and laser microdissection may be used in identifying and enumerating CMC that are identified with fluorescently tagged melanocyte-associated markers (20, 21). Combining these technologies with unique chip-based substrates has made possible the molecular characterization of single melanoma cells (21).

SUMMARY

Polymerase chain reaction-based approaches to enumerate rare and heterogeneous CMC have demonstrated promise but do not allow for morphologic or molecular analysis of specific cell populations. Although cytometric approaches are in clinical use in the management of patients with carcinomas, the development of similarly approved technology for CMC has proven challenging because of the lack of specific, cell-surface, CMC capture antigen(s). Analysis of the captured cells, such as the identification of molecular targets or special biological characteristics, with current methods can also be cumbersome. Thus, there remains a need for the development of a reliable, efficient platform to isolate, enrich, and characterize CMC in blood. Molecular assessments are now impacting melanoma management. Given the multitude of therapeutic targets emerging, whole genome sequencing adapted to enriched CMC obtained from peripheral blood samples will be necessary for meaningful evaluation of therapeutic directions, and given tumor heterogeneity, it will most likely need to be aimed at the single cell level.

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Why does melanoma metastasize into the brain? Genes with pleiotropic effects might be the key

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INTRODUCTION

Melanoma is the most aggressive type of skin cancer. It is the seventh most common type of cancer among men and the eighth most common among women with a lifetime risk about 2% (Feng et al., 2011). The incidence of melanoma is rising faster than that of any other cancer type in the US (Tsao et al., 2004). Melanoma is a multifactorial disease whose risk depends on genetic susceptibility (around 10% of melanoma cases have a family history of the disease) as well as on external factors, among which an exposure to ultraviolet (UV) radiation and sunburn play an important role.

Brain metastases is a major challenge in melanoma and one of the least understood aspects of this disorder (Skibber et al., 1996; Fidler et al., 1999). Average survival in advanced metastatic melanoma is only 6–10 months with <5% of patients living 5 years after diagnosis (Jemal et al., 2002). More than half of all melanoma deaths occur from brain metastasis. A key event in brain metastasis is the migration of cancer cells through the blood brain barrier (BBB) (Arshad et al., 2010). The BBB is formed by specialized endothelial cells lining capillaries in the central nervous system. Brain capillary walls are more difficult to penetrate due to a tight layer of endothelial cells, tight junctions (TJs), and other structures that restrict the diffusion of microscopic objects (e.g., bacteria) and large or hydrophilic molecules into the cerebrospinal fluid.

In order to allow the melanoma cell to metastasize into the brain, the integrity of the BBB has to be compromised. This suggests that some germ line mutations contributing to metastatic melanoma may also increase the permeability of the BBB. Finding such mutations and understanding the mechanisms of their action could make substantial contributions to reducing mortality from melanoma. Currently, very little is known about the molecular mechanisms by which melanoma cells can penetrate the BBB.

The literature on germ line mutations contributing to melanoma and its metastases provides some clues about relevant genes and their functions. Udart et al. (2001) gave evidence that a number of genes which are likely to play a role in melanoma and metastases are located on chromosome 7. The list includes the EGFR gene encoding the epidermal growth factor receptor; the BRAF gene, which is a member of the Raf kinase family of serine/threonine-specific protein kinases involved in the MAP kinase/ERKs signaling implicated in many cancers (Wangari-Talbot and Chen, 2012); the PDGF-A gene encoding for platelet-derived growth factor alpha; the PAI-1 encoding for plasminogen activator inhibitor type 1; the MET proto-oncogene, encoding for a membrane receptor protein with tyrosine–protein kinase activity, and others. The PDGF-A is expressed in primary and malignant melanoma and might function as an autocrine growth factor as well as an angiogenesis factor in tumor development. The PAI-1 is expressed in highly invasive metastatic human melanoma cell lines. The EGFR gene and the MET gene were independently amplified in human glioma. In malignant melanoma, the MET gene was shown to be expressed in metastatic lesions.

The permeability of this BBB is essentially regulated by TJ, the intercellular junction, in which the outer cell membranes are joined tightly together by rows of membrane proteins. TJ regulates the flow of ions, nutrients, and cells into the brain (Dejana, 2004; Abbott et al., 2006). The germ line mutation in genes involved in TJ regulation could disrupt BBB functioning. A number of recent studies strongly support the connection between melanoma metastasizing and TJ destabilization (Leotlela et al., 2007; Fazakas et al., 2011; Jayagopal et al., 2011). The important components of TJ are a family of proteins called "claudins." Twenty-four such proteins are currently known. Genes CLDN2, CLDN3, CLDN4, CLDN11CL, CLDN12, CLDN14, and CLDN15 encoding for different claudins are also located on the chromosome 7 (Paperna et al., 1998; Hillier et al., 2003; Lal-Nag and Morin, 2009). One more TJ gene on chromosome 7 is OCLN, encoding for "occludin" protein. The localization of all these genes on one chromosome indicates that these genes together with other (not yet detected) genes on the same chromosome might represent an important part of the genetic mechanism linking the development of melanoma and brain metastases. If so, then performing association study of melanoma using SNP data from chromosome 7, and investigating functions of corresponding genes, may provide important insights about biological mechanisms connecting melanoma and brain metastases.

DATA FROM LONG LIFE FAMILY STUDY SUPPORT THE IDEA ABOUT ROLE OF PLEIOTROPIC GENES IN MELANOMA BRAIN METASTASES

The Long Life Family Study (LLFS) involves four field centers (Boston, New

York, and Pittsburg in the USA and Denmark in Europe). The recruitment and enrollment were conducted between 2006 and 2009. Potential probands were identified via Medicare enrollee, Danish Social Register lists, and articles appearing on the internet. Altogether, the LLFS enrolled 583 families with 1493 probands and their siblings and 192 spouses in the older generation, 2437 offspring and 809 of their spouses. The analyses did not include individuals with missing data on prevalence of melanoma or observed covariates. The remaining sample included 4638 individuals; 110 (2.4%) of them have prevalent melanoma. Among those, there were 2551 females [51 (2%) with melanoma] and 2087 males [59 (2.8%) with melanoma]. The tag SNPs for LLFS were produced by running HaploView with windows of size of 2000 markers at a time and $r^2 = 0.8$. These markers were used in the LLFS principal component analyses (PCA). Tag SNPs were calculated for SNPs with MAF $\geq$ 5%; HWE p-value $\geq$ 1E-6.

The association study of this disease used LLFS data and tag SNPs located on chromosome 7. The prevalence of melanoma among the LLFS participants was considered as phenotype of interest. Note that according to SEER data the lifetime risk of melanoma is about 2%. Assuming that the genetic variant we are looking for is responsible for not more than 75% of melanoma cases and the lower boundary for a penetrance function corresponding to genetic variant associated with melanoma is not <0.1 the genetic frequency of the corresponding gene should not exceed 15%. The p-value threshold correcting for multiple testing was 1.7E-5. We used the EMMAX software package which allowed us to evaluate relatedness among family members using SNP data and take it into account in the analyses of family data (Kang et al., 2010). Observed covariates included gender, field center, generation (probands/offspring), and smoking habit (ever or never smoked). Twenty principal components were used to control for possible population stratification.

The analyses resulted in one genetic variant reaching chromosome-wide level of significance. The minor allele (T) of the rs208353 SNP was found to be associated with melanoma ($p = 7.07$E-6). Note that the estimate of MAF of this allele is about 7% which is in agreement with the assumption of MAF < 15% used in calculation of p-value threshold. The detected rs208353 SNP is located in the intron region of the GNA12 gene (synonyms: GNA12 | *MGC*104623 | MGC99644 | NNX3 | RMP | gep), which encodes guanine nucleotide binding protein (G protein) alpha 12. This finding supports recent result of Cardenas-Navia et al. (2010) who found that GNA12 and six other G-protein genes are frequently mutated in melanoma (somatic mutations). The literature review showed that the GNA12 gene plays a critical role in regulating TJ, which in turn is an essential component of the BBB permeability. The loss of endothelial TJ function was suggested to be an important event in the disruption of the BBB and promoting tumor metastases (Förster, 2008; Feng et al., 2011). The role of GNA12 is not limited to its involvement in melanoma and corresponding brain metastases. Several studies demonstrated the involvement of GNA12 in other cancers, potentially through compromised regulation of TJ and BBB permeability in carriers of some variants of this gene. Meyer et al. (2003) have shown that GNA12 directly affects Zona-Occludens proteins (ZO-1) and (ZO-2) which are usually localized at sites of intercellular junctions. It also interacts with the Src gene. ZO-1, ZO-2, and Src genes are involved in cancer growth and metastasis (Kaihara et al., 2003; Satomi et al., 2011; Creedon and Brunton, 2012). Sabath et al. (2008) have shown that TJ can be disrupted by GNA12-stimulated Src phosphorylation of ZO-1 and ZO-2 (TJP2). Kumar et al. (2006) and Kelly et al. (2006, 2007) demonstrated the ability of GNA12 to promote neoplastic transformations. Gan et al. (2011) showed that GNA12 is over-expressed in oral squamous cell carcinoma, and the over-expression drives migration and invasion of oral cancer cells. Juneja and Casey (2009) provided evidence that the G12 subfamily has been implicated in cancer cell invasion and metastasis. G12 signaling promotes prostate, breast, and ovarian cancer cell invasion *in vitro*, and these proteins are highly expressed in metastatic cancer tissues. Other genes that interact with GNA12 include tumor suppression gene TP53 and TJ gene TJ1. GNA12 also influences non-cancerous health disorders, such as ulcerative colitis and depression (Anderson et al., 2011; Lees et al., 2011; Zhang et al., 2012).

Thus, the GNA12 has pleiotropic health effects. Its germline variants have been significantly associated with melanoma in the LLFS data; it was also detected in an independent study of somatic mutations in melanoma (Cardenas-Navia et al., 2010); it is involved in TJ regulation important for permeability of BBB; and it plays role in many cancers as well as some other health disorders.

The variant from chromosome 7 next most significantly associated with melanoma in the LLFS data is the minor allele (T) of the rs55750236 SNP located in the KIAA1549 gene. Despite the fact that the p-value ($p <$ 8.7E-5) of this analysis slightly exceeded the chromosome-wide significance level, this association is likely to be true positive. The KIAA1549 gene is known for its fusion with BRAF gene involved in the MAPK/ERKs signaling pathway which is thought to play a pivotal role in melanoma as well as other cancer development (Dahiya et al., 2012; Lin et al., 2012; Wangari-Talbot and Chen, 2012; Lewis et al., 2013). The KIAA1549-BRAF fusion itself was implicated in brain tumors (Badiali et al., 2012; Lin et al., 2012).

The analysis described above does not preclude association studies of melanoma using genetic variants located on other chromosomes. Several melanoma-related genes were found on other chromosomes, including G-protein-coupled receptors (e.g., GRM1) that are also involved in brain function (see, e.g., Wangari-Talbot and Chen, 2012). Thus, additional studies are needed to develop a more complete picture of genetic mechanisms connecting melanoma and brain metastases, as well as connecting the pathological effects of genes located on different chromosomes. The benefit of focusing on chromosome 7 is related to the specific research question addressed in this paper exploiting the fact that quite a number of genes involved in melanoma development and BBB regulation are located in this part of the genome. Another benefit deals with a smaller number of hypotheses

testing in a genetic association study that substantially reduces the number of false positives compared to the genome wide association study dealing with SNPs from the entire genome. The important finding of this study is that mutation in the GNA12 gene can influence both the development of melanoma and the permeability of the BBB, and thereby contribute to the progression of melanoma to its metastatic state. The results of this paper also indicate the important role of genetic variants with pleiotropic effects in the developing of multiple health disorders. Recently, Jornsten et al. (2011) used data on glioblastoma available at The Cancer Genome Atlas (TCGA) to construct network models of mRNA expression. They found that the GNA12 gene is involved in network of disease-relevant hub genes that influence patient survival. The data on Skin Cutaneous Melanoma were just recently included into TCGA, so this resource can be used in the near future to validate roles of pleiotropic effects of genes in melanoma metastases. Targeting the pleiotropic genes could be an efficient strategy for simultaneous prevention and treatment of many health conditions.

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In vivo modeling and molecular characterization: a path toward targeted therapy of melanoma brain metastasis

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Brain metastasis (B-Met) from melanoma remains mostly incurable and the main cause of death from the disease. Early stage clinical trials and case studies show some promise for targeted therapies in the treatment of melanoma B-Met. However, the progression-free survival for currently available therapies, although significantly improved, is still very short. The development of new potent agents to eradicate melanoma B-Met relies on the elucidation of the molecular mechanisms that allow melanoma cells to reach and colonize the brain. The discovery of such mechanisms depends heavily on pre-clinical models that enable the testing of candidate factors and therapeutic agents *in vivo*. In this review we summarize the effects of available targeted therapies on melanoma B-Met in the clinic. We provide an overview of existing pre-clinical models to study the disease and discuss specific molecules and mechanisms reported to modulate different aspects of melanoma B-Met and finally, by integrating both clinical and basic data, we summarize both opportunities and challenges currently presented to researchers in the field.

Keywords: melanoma brain metastasis, melanoma, brain metastasis, brain tropism, therapy-related, animal models, metastasis

BRAIN METASTASIS FROM MELANOMA – A CLINICAL CHALLENGE

Brain metastasis (B-Met) occurs in 5–15% of all melanoma patients and is the cause of death in half of metastatic melanoma patients (Johnson and Young, 1996; Sampson et al., 1998; Davies et al., 2011). Disseminated melanoma cells are able to extravasate through the highly restrictive blood brain barrier (BBB) and mostly inhabit the parenchyma, with less frequent leptomeningeal or cerebral spinal fluid (CSF) metastasis.

Currently, treatments for B-Met are determined by their number, anatomic location, surgical risk, systemic disease burden, and leptomeningeal involvement. Patients with a limited number of resectable B-Met may undergo surgical resection or stereotactic radiosurgery. These procedures appear to prolong survival in a subset of patients as reported by retrospective analyses (Lonser et al., 2011; Salvati et al., 2012). Patients with inoperable disease are usually treated with whole-brain radiation therapy (WBRT) or chemotherapy such as temozolomide (Eichler and Loeffler, 2007). Response rates to single-agent chemotherapy are <10%, and treatment simply attempts to slow disease progression (Ewend et al., 2001; Agarwala et al., 2004; Eichler and Loeffler, 2007). It is becoming clearer that the genetic background of a certain patient (i.e., germline mutations) or a tumor should dictate its treatment regimen, and that targeted therapy against these tumor-specific alterations (if available) may be more efficacious. In the case of familial melanoma, germline inactivating mutations in the *CDKN2A/B* locus (mainly p16 and p14) are common (Straume et al., 2002; Gast et al., 2010), leading to aberrant CDK4/cyclin D activity that drives melanoma cell cycle progression. It is plausible that germline mutations contribute to tumor progression by affecting non-melanocytic tissues as well and by that, affecting metastatic potential. For example, certain mutations may affect blood vessels permeability, predisposing patients to increased metastatic spread. The systemic effects of prevalent germline mutations in cancer patients may prove relevant for the development of future tailored personalized medicine. On the other hand, prevalent somatic mutations in melanoma are the subject of intense studies. More than 50% of metastatic melanoma tumors harbor an activating mutation in codon 600 of the *BRAF* gene (V600E or, to a lesser extent, V600K) (Davies et al., 2002). Recently, selective *BRAF* inhibitors such as PLX4032 (vemurafenib) and GSK2118436 (dabrafenib) have shown clinical efficacy in *BRAF* mutant metastatic melanoma patients (Flaherty et al., 2010) with significant tumor regression in approximately 60% of patients (Flaherty et al., 2010). Clinical trials using *BRAF* inhibitors to treat patients with melanoma B-Met were initiated recently with promising results despite the small sample size. A phase I study tested the effects of dabrafenib in 10 patients with untreated and asymptomatic B-Met. Nine of ten patients displayed reductions in size of brain lesions (Falchook et al., 2012). In addition, an ongoing phase II trial is designed to assess the efficacy, pharmacokinetics, safety, and tolerability of dabrafenib administered to a large cohort of subjects with *BRAF V600E/K* mutation-positive B-Met (ClinicalTrials.gov Identifier: NCT01266967). However, resistance to the *BRAF* inhibitor is already evident. In part, this phenomenon is attributed to addiction or functional redundancy within the MAPK pathway, which likely buffers the impact of a single gene/target modification on the

malignant process (Johannessen et al., 2010; Nazarian et al., 2010). Moreover, Poulikakos et al. (2011) have identified an additional resistance mechanism in which a splicing variant of mutated *BRAF* that lacks the region encompassing the RAS-binding domain, showed enhanced dimerization in vemurafenib-treated cells.

Another promising, potent agent used lately in late stage melanoma patients is ipilimumab, a monoclonal antibody against the CTLA-4 molecule expressed mainly on regulatory T cells. This antibody blocks CTLA-4 signaling that acts as an immune checkpoint to inhibit T-cell activation [reviewed in (Melero et al., 2007)]. The use of ipilimumab improved overall survival with 10.9% of patients exhibiting complete response, with mostly reversible adverse effects (Hodi et al., 2010) in around 15–20% of patients. Recent reports have suggested that ipilimumab can promote the regression of melanoma B-Met. Case studies reported that ipilimumab significantly benefited individuals with central nervous system (CNS) metastasis (Hodi et al., 2008; Schartz et al., 2010). In a phase II trial of 72 patients with B-Met treated with ipilimumab, 18% of participants that had asymptomatic B-Met and were not treated previously with steroids achieved disease control (partial response or stable disease). The study revealed long-term survival rates comparable to those seen in patients without B-Met, with approximately one-third of patients alive at 12 months. Patients treated with steroids did not show similar responses (Margolin et al., 2012).

The progression-free survival, for both ipilimumab and dabrafenib/vemurafenib-treated patients, although significantly improved, is still very short. Nevertheless, these studies showing unprecedented efficacy against melanoma B-Met exemplify that targeted therapy could be key to the eradication of these highly aggressive metastases.

WHY DO MELANOMAS METASTASIZE TO THE BRAIN?

The concept that metastases arising in different locations in the body carry site-specific characteristics that facilitate tissue colonization is a subject of intense research in various types of cancers. Several studies over the past few years were dedicated to elucidate the molecular and cellular basis of melanoma B-Met, using both experimental and pre-clinical models for this condition.

Interestingly, when melanoma becomes metastatic, it has the highest risk among all tumors for B-Met development with 44–64% of patients (Davies et al., 2011). Moreover, in melanoma patients, a higher proportion of B-Met represent the only site of metastatic disease compared to other solid tumors that frequently metastasize to the brain (Thompson et al., 2004). Strikingly, in a retrospective analysis of more than 2000 melanoma patients our group showed that 36% of melanoma B-Met represent the first and isolated site of metastasis (Ma et al., 2012). Primary tumors of patients from this subgroup displayed distinct clinicopathological features with thinner (mostly stage 1), non-mitotic lesions. Another study by our group of 900 primary melanoma patients showed that location of the primary tumor on the head and neck was an independent predictor of B-Met (Zakrzewski et al., 2011). However, the correlation between anatomical site and B-Met does not hold when analyzing only tumors with B-Met as first isolated site (Ma et al., 2012), suggesting that the predilection to metastasize to the brain is already molecularly "encoded" in some primary melanomas that represent a distinct clinicopathological and possibly molecular entity. It was hypothesized that the high CNS involvement of melanoma may be due to a "homing" effect, since melanocytes and neuronal subpopulations such as glial cells and sensory neurons share a common neural crest progenitor (Herlyn et al., 2000). However, this hypothesis has not yet been yet thoroughly investigated experimentally.

From a molecular point of view it is imperative to ask whether a specific set of conditions need to occur in order for melanoma cells to seed and proliferate in a certain tissue. Multiple studies, mainly in the context of breast cancer, demonstrated how metastasis to different sites involves unique programs that facilitate tumor cell seeding and proliferation within the myriad of specialized cell types and extracellular matrices of the foreign tissue (Padua et al., 2008; Bos et al., 2009; Zhang et al., 2009a). Organ specificity can also be achieved by differential expression of molecules on resident cells of the invaded tissue. For example, the adhesion molecule Lu-ECAM-1 was reported to be specifically expressed on distinct branches of lung blood vessels, facilitating the arrest and binding of melanoma cells with higher affinity to it (Zhu et al., 1991). As for tropism of cancer cells to the brain, a study by Weiss (1992) estimated that the arrival of 66% of hematogenous B-Met may be explained by blood circulation while the remaining metastases may reflect site specificity.

IN VIVO MODELS OF MELANOMA BRAIN METASTASIS

Several groups have reported the development and use of *in vivo* models of melanoma B-Met (Fujimaki et al., 1996; Yano et al., 2000; Küsters et al., 2003; Xie et al., 2006; Huang et al., 2008; Zhang et al., 2009b) (summarized in **Table 1**). Nonetheless, there are considerable shortcomings in most of them. The 'spontaneous' B-Met model induced through subcutaneous transplantation of tumor cells in the flank allows sufficient time for primary tumor cells to disseminate and establish distant metastases (Cruz-Munoz et al., 2008). In such model, a melanoma cell line was used to generate a systemic metastatic disease in NOD/SCID mice. Mice were then subjected to a metronomic chemotherapy and surviving mice developed spontaneous B-Met. Cell lines established from B-Met were then proven to metastasize to the brain parenchyma efficiently and with shorter latency. This model recreates the multiple sequential steps that are associated with the metastatic cascade, making it closely resembled to the clinical disease. However, the long latency period needed for metastatic disease in the brain to become evident, the relatively low incidence, and the limited number of syngeneic and xenograft spontaneous B-Met models available makes this approach less appealing when compared to other models.

Mouse models in which melanoma B-Met is induced through direct injection of cancer cells into the circulation, known as 'experimental' models, do not reflect the complete series of events involved in the metastatic process. Nevertheless, they allow for both controlled delivery of cancer cells and a short time for metastatic disease to manifest. These models are particularly suitable to study later stages of B-Met such as seeding and tissue colonization. These characteristics, along with the availability of many well-characterized cell lines, make these models attractive to study B-Met in pre-clinical settings. Notably, nearly all of the

Table 1 | *In vivo* models of melanoma brain metastasis.

Model	Technique	Advantages	Limitations
Spontaneous brain metastasis (Cruz-Munoz et al., 2008; Cruz-Muñoz et al., 2012)	Subcutaneous implementation of pre-selected clones followed by tumor resection. Metastatic disease in the brain is allowed to occur spontaneously from metastasizing cells leaving the subcutaneous implementation site	Recreates the multiple sequential steps that are associated with the metastatic cascade, making it closely resembled to the clinical disease Suitable for pre-clinical testing of adjuvant therapies	Relatively low throughput Very long latency period needed for metastatic disease in the brain to become evident Relatively low incidence Limited number of available pre-selected cell lines to be used
Intra-carotid injection (Fujimaki et al., 1996; Yano et al., 2000; Xie et al., 2006; Huang et al., 2008; Zhang et al., 2009b)	Cells are injected into the internal carotid artery	Allows for controlled delivery of cancer cells Offers a short time for metastatic disease to manifest Availability of many well-characterized cell lines	Technically challenging Does not reflect the complete series of events involved in the metastatic process Extremely short latency between tumor induction and mortality Mostly leptomeningeal metastases are formed
Intra-cardiac injection (Izraely et al., 2012; Tekle et al., 2012; Morsi et al., 2013; Sundstrøm et al., 2013)	Cells are injected into the left ventricle of the heart	Relatively high-throughput Recapitulates most relevant stages of the metastatic spread to the brain Technically feasible May produce parenchymal lesions Reasonable latency between inoculation to appearance of brain metastasis – may be used for pre-clinical testing of adjuvant therapies	Does not reflect the complete series of events involved in the metastatic process Limited number of available pre-selected cell lines to be used
Injection into chick embryo (Busch et al., 2012)	Cells are injected into the rhombencephalic brain vesicle of a 2-day-chick embryo. Two to three days post-injection tumor formation is studied	Fast Controlled delivery of cells May be used with multiple cell lines	Physiological relevance is not yet established Limited to study certain processes such as extravasation and local invasion

experimental melanoma B-Met studies use internal carotid artery injections (Fujimaki et al., 1996; Yano et al., 2000; Küsters et al., 2003; Xie et al., 2006; Huang et al., 2008; Zhang et al., 2009b). This method of tumor induction, although still a commonly used methodology, is time consuming and requires certain level of surgical expertise. In addition, this route of injection proved to be "artificially" invasive, with extremely short latency between tumor induction and mortality, putting its physiological relevance in question. Moreover, the B16 syngeneic cell line used vastly in this model develops exclusively leptomeningeal metastasis, as opposed to the more prevalent parenchymal dissemination. This considerable shortcoming renders the B16 a less clinically relevant model with low translational potential.

Recently, intra-cardiac injection has been established as a less invasive and less technically demanding route of B-Met induction. In these studies, human cells are injected directly into the left ventricle of the heart of immuno-deficient mice to develop a more clinically relevant *in vivo* model. Following this methodology, human melanoma cell lines directly injected in athymic nude (Izraely et al., 2012) or Balb/c mice (Tekle et al., 2012) successfully developed parenchymal lesions. A new model developed recently by Sundstrøm et al. (2013) utilized intra-cardiac injection of melanoma cells labeled with superparamagnetic iron oxide nanoparticles (SPIONs). These cells were effectively visualized by magnetic resonance imaging (MRI) followed by automated analysis. Our group combined ultrasound-guided intra-cardiac injection of melanoma cells as a minimally invasive, high-throughput method of induction, with MRI-assisted tumor segmentation, 3D reconstruction and quantitative volumetric analysis, to precisely map and measure parenchymal B-Mets (Morsi et al., 2013). This approach takes advantage of the paramagnetic nature of melanin, which renders a signal brightening endogenous effect in tracer-free T1-weighted MRI (Isiklar et al., 1995). Importantly, the metastatic pattern observed in both studies resembled the one seen in patients and was highly reproducible. This type of in-depth characterization of the growth pattern of B-Met lesions developing within *in vivo* models, using various imaging techniques, will allow to faithfully assess melanoma brain tropism, seeding and adaptation, study the molecular mechanisms that control these processes, and may be used to test potential therapeutic agents.

Lately, a study by Busch et al., made use of the chick embryo model to study melanoma B-Met. Melanoma cell lines were injected into the rhombencephalic brain vesicle of the 2-day-chick

embryo. Two to three days post-injection, tumor formation was studied in serial paraffin sections (Busch et al., 2012). The chick embryo model is inherently limiting in studying crucial stages of melanoma dissemination to the brain but can be exploited to study early phases such as extravasation and local invasion in the brain.

HOW DO MELANOMAS REACH AND ADAPT TO THE BRAIN MICROENVIRONMENT?

The exact sequence of events required by tumor cells for successful colonization of the brain remains obscure. Kienast and colleagues used multiphoton laser scanning real-time microscopy to follow single steps of B-Met formation. Their innovative experimental system enabled them to follow melanoma cells injected into the internal carotid artery arrest at vascular branch points, extravasate early, remain in close and persistent contacts to microvessels, and co-opt the vessel for nutrients. This final step was unique for melanomas that, as opposed to lung cancer-derived cells, did not induce early angiogenesis (Kienast et al., 2010). This particular finding is intriguing and suggests that B-Met originating from different tumor types possess distinct molecular properties and may respond differently to certain therapies and thus, should not necessarily be treated uniformly as one entity.

The multistage process of metastatic spread to the brain requires the involvement and integration of multiple biological events. *In vitro* and *in vivo* models studying the nature of the alterations required for melanoma cells' tropism to the brain revealed a number of effectors to mediate different aspects of that process. Interestingly, most reports do not claim the alterations found to be exclusive of B-Met. While those are relevant for the elucidation of the mechanisms that govern melanoma B-Met, the discovery of site-specific molecular alterations may be key for the development of potent, site-specific therapy. This approach may be highly beneficial for patients, especially since current data clearly point to a model in which melanoma B-Met are not always a late stage metastatic disease but may also be a unique entity with possibly distinct molecular profile. Below we summarize some of the molecular factors implicated so far in melanoma B-Met models (depicted in **Figure 1**).

JAK-STAT

The JAK-STAT pathway, that promotes survival, growth, and angiogenesis, was reported to increase melanoma B-Met mainly via STAT3 activation by phosphorylation, or downregulation of its inhibitor SOCS-1 (Xie et al., 2006; Huang et al., 2008). The main effects observed in both studies were increased expression of MMP-2, bFGF, and VEGF, possibly supporting melanoma cell invasion and angiogenesis. Importantly, STAT3 activation and consequential effects were a more general pro-metastatic phenomenon, not restricted to B-Met. In fact, a recent study showed that melanoma lung metastases exhibited the highest level of p-STAT3 expression and that p-STAT3 expression was not associated with an increased risk of developing B-Met or time to B-Met (Lee et al., 2012). It still remains to be determined whether the effects of SOCS-1 are more specific to B-Met, but since its main downstream target reported in the study was STAT3, a brain metastatic-specific mechanism seems unlikely.

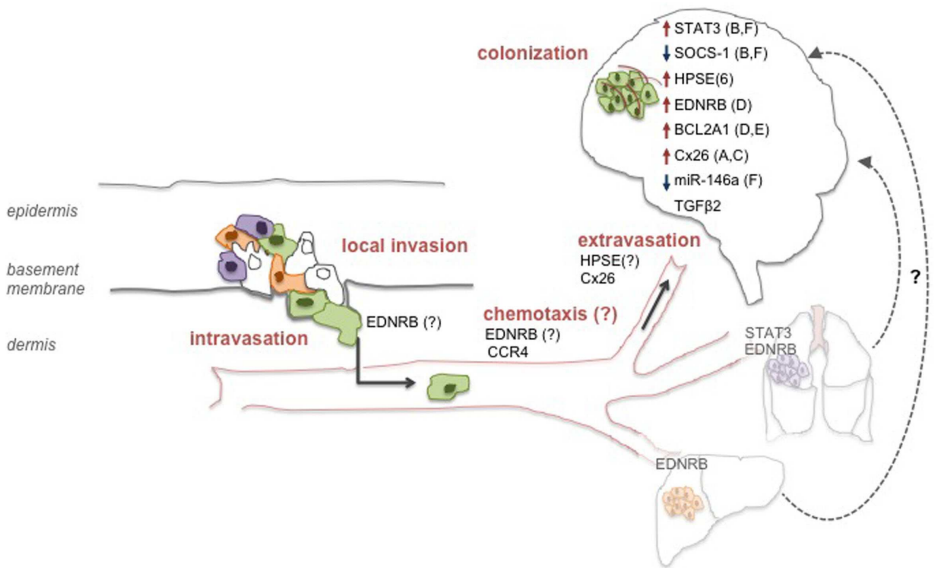

FIGURE 1 | Molecular determinants of melanoma brain metastasis. Molecular alterations may occur on all consecutive steps that support the metastatic process of melanoma cells to the brain. Alterations in specific factors may support processes at the primary tumor site and endow a subset of cells with the ability to reach the brain (represented by green cells). Other factors may direct migration to the brain via chemotaxis, or promote adhesion and extravasation through the BBB. Lastly, inside the brain, other factors facilitate several processes that allow the successful colonization of the tissue such as vessel co-option (A), angiogenesis (B), seeding (C), growth (D), survival (E), or invasiveness (F). Although not yet demonstrated for melanoma, it is possible that metastatic spread to the brain may also originate from other visceral metastatic sites such as the lung or the liver ("metastasis of metastasis").

HEPARANASE

The enzyme Heparanase (HPSE) degrades heparan sulfate chains of proteoglycans that are known to have multiple functions including maintaining capillaries support or retaining soluble factors (e.g., chemokines). Using a brain slice model it was shown that higher HPSE levels lead to increased invasion of the brain, that was repressed when specific HPSE inhibitors were used (Murry et al., 2006). In support of the role of HPSE in promoting B-Met is a recent study that reported miR-1258 to be a suppressor of breast cancer B-Met through the direct targeting of HPSE (Zhang et al., 2011). Since HPSE is a potent pro-tumorigenic and pro-metastatic agent, its effects might not be confined to brain-specific processes. Still, HPSE role could be more evident in B-Met models and patients' samples since its activity is essential for the successful extravasation of the blood-borne melanoma metastasis through the heparan sulfate-rich endothelial cell layer. Furthermore, co-incubation of astrocytes with melanoma brain metastatic cells resulted in elevated HPSE activity and markedly increased invasive capacity *in vitro* (Marchetti et al., 2000). This further supports brain-specific activity for this enzyme.

ENDOTHELIN RECEPTOR B

A recent study has implicated Endothelin Receptor B (EDNRB) as a factor that potentially influences brain metastatic potential. Using a pre-clinical model of melanoma B-Met developed by the same group (Cruz-Munoz et al., 2008), the authors showed that EDNRB overexpression enhanced overall metastatic disease, and increased the incidence of spontaneous B-Met. The study showed that the interaction of EDNRB with its ligands caused increased intracranial melanoma growth. Therapeutic treatment by an EDNRB-specific inhibitor translated into improved outcome in mice. This study implicates a protein critical for melanocyte biology in promoting melanoma metastatic potential in general and B-Met in particular (Cruz-Muñoz et al., 2012). Although the pro-metastatic effects were not exclusive to the brain, the authors postulate that the high levels of EDNRB ligands in the brain relative to other organs may explain the overall increased growth within the brain and the increased frequency of B-Met in this study. Importantly, endothelin 3 levels are also high in lung tissue and may be responsible for the increased lung metastasis frequency when EDNRB was ectopically expressed (Fagan et al., 2001). These results are exciting since they exemplify how melanoma metastatic cells are affected by surrounding specific microenvironmental ligands and utilize them for their growth. The successful therapeutic aspect of this study highlights EDNRB as a potential druggable target. Interestingly, EDNRB overexpression was ectopically induced within the implanted tumor in the flank where endothelins are not abundant. Still, EDNRB overexpressing cells metastasized more frequently to the brain. This points to a model in which EDNRB generally facilitates metastatic spread, but its effects are exacerbated in the brain, where its ligands are abundant.

BCL2A1

A second factor implicated in the same study is the anti-apoptotic protein BCL2A1, which did not affect the incidence of B-Met but facilitated intracranial tumor growth, possibly by enhancing cell survival. Since cells were injected intra-cranially in those experiments, this finding needs to be further investigated for its physiological relevance (Cruz-Muñoz et al., 2012).

CONNEXIN 26

Connexins have been lately shown to mediate early events in brain colonization using transparent zebrafish and chicken embryo models of B-Met. One study showed that melanoma cells utilize the gap junction protein Connexin 26 (Cx26) to initiate B-Met formation in association with the vasculature. Cx26 silencing or pharmacological inhibition of connexins blocked cell extravasation and blood vessel co-option (Stoletov et al., 2013). The idea that specific connexins mediate cancer metastasis to the brain by increasing gap junction communication with the BBB is intriguing, particularly in the context of previous observations highlighting vessel co-option among the initial steps of brain colonization unique to melanoma (Kienast et al., 2010). Interestingly, a study by Lin et al., reported that activated astrocytes surrounding melanoma B-Met protect them from chemotherapeutic drugs. This chemoprotection was dependent on physical contact and gap junctional communication between astrocytes and tumor cells (Lin et al., 2010). It will be interesting to examine whether the specific silencing of Cx26 will be sufficient to eliminate these chemoprotective effects.

CCR4

The expression of the chemokine receptor CCR4 was found significantly higher in one melanoma brain metastatic variant compared to the corresponding tumor implanted in the flank (Izraely et al., 2010). The same group has reported that brain-derived soluble factors upregulate the expression of CCR4 in both cutaneous and brain-metastasizing melanoma cells and enhance the migration of the latter, but not that of the cutaneous variants (Klein et al., 2012). These findings support the hypothesis that some alterations may occur early at the primary tumor site where certain clones express molecules that promote spread of melanoma cells to the brain. One can postulate that CCR4 ligands secreted from the brain interact with the CCR4-positive melanoma cells and attract them to the brain. This kind of directed migration was reported previously for breast cancer cells overexpressing CXCR4 that facilitated their transmigration through the brain endothelial cells (Lee et al., 2004).

TGFβ2

Overexpression of TGFβ2 in mouse melanoma cells increased their ability to seed in the brain parenchyma, suggesting a role for this pathway in determining site specificity in the brain microenvironment (Zhang et al., 2009b). This study illustrates how specific factors may be crucial for B-Met growth and potentially be exploited therapeutically to diminish successful seeding.

miR-146a

MicroRNAs (miRs) have demonstrated to play critical roles in cancer metastasis including melanoma [reviewed in (Segura et al., 2012)]. miRs emerge as optimal candidates to regulate such a complex and multi-layered process as the metastatic dissemination within the brain due to their ability to concomitantly control multiple targets and thus impact various molecular processes. A recent

study found miR-146a to be nearly undetectable in melanoma cells selected to metastasize to brain relative to their parental counterparts. Overexpression of miR-146a suppressed the migratory and invasive capacity of those cells possibly by targeting hnRNPC and increasing β-catenin (Hwang et al., 2012). While the clinical relevance of this finding needs to be further elucidated, ongoing studies focusing on the potential roles of miRNAs in the modulation of melanoma B-Met might provide a deeper understanding of the critical pathways that drive or support this condition.

BBB DISRUPTORS

Since melanoma B-Mets are blood-borne, cells must extravasate through the highly restrictive BBB. Thus, the integrity of the BBB is essential for the prevention of metastatic infiltration. An *in vitro* model of the BBB demonstrated how melanoma cells are able to penetrate the BBB disrupting major tight junctions molecules such as ZO-1 and Claudin-5, and reducing transendothelial electrical resistance (TEER), an indicator of junctional integrity (Fazakas et al., 2011; reviewed in Wilhelm et al., 2011). The mechanism by which melanoma cells induce endothelial cells junctional degradation is still unclear but the ability of supernatants of melanoma cells to generate similar effects points to the involvement of secreted soluble factors such as proteolytic enzymes mentioned above.

The development and use of models to study melanoma B-Met is yielding potential candidates as regulators of B-Met. However, the physiological relevance of those factors to human disease should be further confirmed to conclusively establish their clinical impact.

CHALLENGES AND OPPORTUNITIES

In recent years conventional therapeutic regimens are clearing the way for tailored, patient-specific therapy. This approach is aimed to maximize responsiveness to treatment based on the tumor's genetics while indirectly reducing side effects caused by the administration of ineffective treatments, and sparing the normal cells of the body that do not harbor the same genetic alteration. Some case reports and early phase clinical trials show promise for targeted therapies in the treatment of melanoma patients with B-Met. This is encouraging, especially since those patients have been, thus far, systematically excluded from most clinical trials. Still, current therapies improve overall survival only marginally and there is a pressing need for B-Met-specific treatments. The notion that the predilection to metastasize to the brain is present in melanoma cells possibly already at the time of primary diagnosis provides a unique opportunity to use specific adjuvant therapy to prevent or reduce metastatic dissemination in patients at higher risk of developing B-Met. The characterization of mechanisms that endow cells with brain-specific tropism and colonization is incipient and ought to be thoroughly investigated. This might add another layer of specificity to the treatment regimens patients are offered based on their site of metastatic dissemination.

The development of *in vitro* and *in vivo* models of melanoma B-Met to discover the molecular mechanisms underlying melanoma B-Met has progressed significantly. Molecular alterations most often seen in melanoma B-Met are typically those resulting in: (i) increased BBB permeability (via junctional, adhesion, and proteolytic factors), (ii) increased tropism to brain microenvironment (via chemokine and cytokines signaling), (iii) enhanced survival in the brain (through modulation of pro-proliferative and anti-apoptotic factors). Nevertheless, novel imaging techniques such as multiphoton microscopy may provide better resolution, real-time assessment of the metastatic process in the brain and its modulation by certain molecules or therapies. Data accumulated from current and future experimental and pre-clinical models of melanoma B-Met should be used to develop new site-specific therapies to efficiently target melanoma B-Met. One can envision therapies focusing on preventing the arrival and seeding of melanoma cells to the brain by blocking certain cell surface receptors or secretion of specific proteolytic enzymes. Targeting the specific interactions of melanoma B-Met with resident cells in the brain parenchyma is another good example of future site-specific therapy that may be developed relying on data arising from pre-clinical models. The outstanding question of whether melanoma B-Met is indeed a separate molecular entity remains mostly unanswered. In that regard, generalized use of next-generation deep sequencing of clinical specimens should provide new insights and might alter dramatically our perception of this disease.

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