CELLULAR AND PHENOTYPIC PLASTICITY IN CANCER

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CELLULAR AND PHENOTYPIC PLASTICITY IN CANCER

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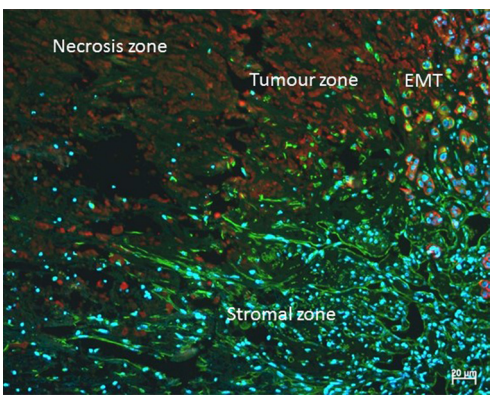

Co-expression of epithelial cytokeratins (pan CK [AE1/AE3]•Flour594-Red) and mesenchymal vimentin (Vim [C-20]-Flour488-Green) in MDA-MB-468 human breast cancer cells growing as xenografts in SCID mice. Cell nuclei are stained with DAPI (blue).

Image courtesy of Dexing Huang and Erik Thompson.

The process of Epithelial-Mesenchymal-Transition (EMT) is known to result in a phenotype change in cells from a proliferative state to a more invasive state. EMT has been reported to drive the metastatic spread of various cancers and has also been associated with drug resistance to cytotoxics and targeted therapeutics. Recently phenotype switching akin to EMT has been reported in non-epithelial cancers such as metastatic melanoma. This process involves changes in EMT-Transcription Factors (EMT-TFs), suggesting that phenotype-switching may be common to several tumour types.

It remains unclear as to whether the presence of both Epilthelial-like and Mesenchymal-like cells are a pre-requisite for phenotype switching within a tumour, how this heterogeneity is regulated, and if alteration of cell phenotype is sufficient to mediate migratory changes, or whether drivers of cell migration result in an associated phenotype switch in cancer cells. Similarly it has yet to be clarified if cells in an altered phenotype can be refractory to drug therapy or whether mediators of drug resistance induce a concurrent phenotypic change. Little is known today about the underlying genetic, epigenetic and transient changes that accompany this phenotypic switch and about the role for the tumor micro-environment in influencing it. Hence this is currently an area of speculation and keen interest in the Oncology field with wide-ranging translational implications.

In this Frontiers Research Topic, we discuss our current understanding of these concepts in various cancer types including breast cancer, colorectal cancer and metastatic melanoma. This topic covers how these processes of cellular and phenotypic plasticity are regulated and how they relate to cancer initiation, progression, dormancy, metastases and response to cytotoxics or targeted therapies.

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EDITORIAL
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Editorial: Cellular and phenotypic plasticity in cancer

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Keywords: cancer, phenotypic plasticity, signaling pathways, drug resistance, epithelial-mesenchymal transition (EMT), cross-talk, exosomes, immune system

Cellular and phenotypic plasticity is a key feature of development and normal function of cells within most multicellular organisms. The ability to respond to various intrinsic and external cues and stimuli in a regulated fashion allows for appropriate cellular adjustments. This plasticity observed in most cell types is retained in cancer and can lead to opportunistic adaptation allowing therapeutic escape and acquisition of motile and invasive abilities that pose ongoing challenges for effective therapy. The dynamic nature of this plasticity and the apparent requirement for widely divergent phenotypes for different aspects of the metastatic cascade (e.g., initial escape – mesenchymal and distant colonization – epithelial) is especially challenging.

The consequences and functional outcomes of this plasticity are well-studied and widely reported in relation to epithelial–mesenchymal transition (EMT). In normal and cancer cells alike, EMT is regulated through signaling pathways (1), the outcome of which is dictated by the balance and cross-talk between the pathways as reviewed within this Research Topic (2). EMT is classically defined as a dynamic, multistep cellular process that allows non-motile, highly organized and polarized epithelial cells to acquire motile and more fibroblast-like, mesenchymal characteristics. It is accompanied by the loss of some epithelial characteristics including specialized cell–cell junctions and apical-basal polarity, and a complex reorganization of the cellular cytoskeleton (3). Functional consequences of EMT, including enhanced or acquired migratory capacity, can result in the release of tumor cells into circulation (4) and ultimately metastasis of the cancer. Not all cells within a tumor undergo EMT, as different signaling inputs at different tumor sites or a variation in genetic drivers in different clones can lead to different cellular states. However, EMT and EMT-like processes contribute greatly to tumor heterogeneity, the challenges of which are highlighted for colorectal cancer within this Research Topic (5). EMT-like phenotype-switching processes have also been described in non-epithelial cancers, such as in melanoma (6). As reviewed in this Research Topic (7), this can involve similar EMT inducers and EMT-transcription factors (EMT-TFs), but variable patterns in terms of expression. Also, unlike the more distinct differences seen after EMT in development, carcinoma systems often exhibit a partial EMT, sometimes called a metastable or hybrid phenotype, reinforcing the concept of dynamic epithelial–mesenchymal plasticity (EMP) (3, 8, 9).

Besides the already mentioned functional changes, tumor cells can undergo changing antigen patterns in a dynamic process parallel to phenotype switching (10), allowing for escape from recognition by already primed cytotoxic T lymphocytes (CTLs) and resulting in immune evasion. Thus, while adaptive as well, the immune system needs to constantly re-adjust, just one of the many obstacles for immune-based tumor control.

Recently, there has been an expanding body of research linking EMT and the mesenchymal-like phenotypic state of cells to therapy resistance. In the case of epithelial cancers, such as breast

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and colorectal cancers, elevated expression of mesenchymal markers or alterations to mesenchymal phenotypes has been shown to be associated with increased metastasis (11) and poor response to treatment (12). Cancer cells in a more mesenchymal-like state are more refractory to conventional cytotoxic therapies, to radiation, and to targeted therapies (13, 14). The exact mechanism of survival in these cells is not yet clear. It is possible that phenotypic mediators can confer survival or anti-apoptotic signals, or possibly the altered phenotypic state usually associated with reduced proliferation renders them refractory to cell death following treatment.

The extent of changes within the proteome and signaling networks of cancer cells, particularly in the context of acquired resistance to targeted therapies (driven by genetic changes), is broad and exemplified in this Research Topic by a phospho-proteomics study of melanoma cells resistant to BRAF inhibitor therapy (15). Experience from the clinic of patients relapsing within months of BRAF-inhibitor treatment demonstrates the challenging clinical implications of this complexity (7).

Cancer cells do not exist in isolation. They are in direct contact with stromal cells and an intense crosstalk between "normal" cells and cancer cells is constantly occurring. The tumor microenvironment is regulated by factors produced by both the tumor and the stromal cells (5) and is not limited to close proximity. In recent years it has been demonstrated that primary tumors can establish favorable conditions for future metastasis in distant organs. The so-called pre-metastatic niche can be formed by non-tumorigenic host cells, cytokines, and tumor-derived exosomes, small extracellular vesicles that transfer information from a tumor to other cells as reviewed by Vella (16), thereby influencing other cellular compartments for promotion of tumor growth and metastasis (17).

Based on these data, it is clear that prevention of a phenotypic switch to this refractory state or reversal to an epithelial-like state (MET) that would restore proliferative capacity may provide a means for maintaining or enhancing drug responsiveness, and possibly even reducing heterogeneity. Hence, identifying the molecular drivers that induce or maintain the mesenchymal-like phenotype, provides an opportunity for "drugging" this phenotypic drug-resistant state, through inhibition of the key signaling pathways that regulate the critical EMT-inducers (12, 18, 19). This approach must be tempered with the risk that reversion to a proliferative MET state will increase tumor burden if the therapies delivered are not sufficiently effective. Our understanding of the balance, regulation, and cross-talk between the pathways intrinsically within the cancer cells, as well as extrinsically in relation to the tumor microenvironment, is revealing opportunities for multi-modality therapies. The identification of potential biomarkers of cell state and drug response will also help to guide the choice of therapy and timing for individualized treatment for each patient. The use of novel, multi-cellular organisms for expanding our understanding of these processes (20) is a necessary path to advance research discoveries in this area.

With a better understanding of the processes implicated in cancer progression, and the key regulatory elements, options for improved therapeutic strategies can be designed to specifically predict and exploit the plasticity of cancer cells. As we move from an era of DNA damaging therapy into an era of combination multi-modality treatments, the focus of the therapeutic target shifts from just the tumor type or the genetic alteration, to the interplay between oncogenic drivers, the vasculature, the microenvironment, and, most promisingly, the immune system. Understanding this complex interplay and the adaptive changes induced by therapy, within the tumor cells as well as within interacting compartments, is an undeniably important aspect of current and future research efforts toward effective treatment to control, and hopefully cure, cancer.

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Crosstalk of oncogenic signaling pathways during epithelial–mesenchymal transition

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Epithelial–mesenchymal transition (EMT) and cell transformation have been well-documented in multiple cancer cell models and are believed to be one of the earliest events in tumor progression. Genetic and epigenetic modifications shift cells toward either end of the EMT spectrum, and can be influenced by the microenvironment surrounding a tumor. EMT and mesenchymal–epithelial transition are critical to normal function and development and an intricate network of transcription factors and transcriptional regulators tightly regulates these processes. As evidenced in normal and transformed cell lines, many signaling pathways trigger EMT during development and differentiation. The signaling pathways include those triggered by different members of the transforming growth factor superfamily, epidermal growth factor, fibroblast growth factor, hepatocyte growth factor, hypoxia-inducible factor, Wnt, Notch, and many others. Functional redundancies allow cells to undergo EMT even if these key transcriptional regulators are lacking, but these same redundancies also make these pathways particularly susceptible to gain-of-function mutations or constitutive signal activation; the "forced" transition toward either a mesenchymal or epithelial phenotype.

Keywords: epithelial–mesenchymal transition, microenvironment, invasion, motility, transforming growth factor-beta

INTRODUCTION

Historically, signaling pathways were studied in isolation and treated as linear entities that never interacted; however, studies in the emerging field of systems biology have provided a growing appreciation of the importance of pathway crosstalk and emphasized the complexity of signaling webs during development and tumor progression. This is especially true for the process known as epithelial–mesenchymal transition (EMT). EMT was first described in the 1980s because of its pivotal role during embryonic development and was later implicated in the physiological response to injury (1). EMT is critically involved in normal embryogenesis and development and epithelial cells have developed an intricate network of signaling pathways that include redundancies that safeguard and ensure proper functioning even in the event of a genetic lesion. However, these functional redundancies also leave the signaling network particularly susceptible to gain-of-function mutations and inappropriate signal amplification, eventually leading to tumor progression. Thus, EMT is not only a key biological process during embryonic morphogenesis but also a defining characteristic. EMT is also one of the earliest steps of solid tumor progression, associated with tumor growth, invasion, and metastasis, and contributes to the conversion of tumors from low- to high-grade malignancy (2, 3).

During EMT, epithelial cells undergo a developmental switch that results in decreased adhesion and loss of cell polarity, increased proliferation, and increased motility and invasiveness (4). Invasion is a key step to progression toward a malignant phenotype, and occurs when tumor cells translocate from the relatively constrained initial neoplastic mass into neighboring host tissues. To accomplish this, cancer cells must somehow detach from the primary tumor and migrate through surrounding tissues, opening up the opportunity to penetrate the basal-membrane surrounding a blood or lymphatic vessel, travel throughout the body via the circulatory system, and colonize distant sites where metastatic foci can be formed. Growing evidence suggests that in order for benign cancer to progress toward malignant disease tumor cells undergo EMT (5). The EMT process is associated with a number of morphological and biochemical changes where polarized and basal-membrane anchored epithelial cells acquire a mesenchymal, fibroblastoid phenotype. Morphologically, during the onset of EMT cells transform from a cuboidal epithelial-like cell to a

Abbreviations: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; CTBP, C-terminal binding protein; CTC, circulating tumor cell; CXCR4, C–X–C, chemokine receptor 4; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GRB2, growth factor receptor-bound protein 2; GSK, glycogen synthase kinase; HDAC, histone deacetylase; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IL-6 interleukin 6; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MET, mesenchymal–epithelial transition; MMP3, matrix metalloproteinase-3; MMSET, multiple myeloma SET domain; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; PTHRP, parathyroid hormone-related protein; RANKL, receptor activator of NF-κB ligand; SDF-1, stromal-derived-factor-1; SOS, son of sevenless; TGF, transforming growth factor; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAF, TNF receptor-associated factor; ZEB, zinc-finger E-box-binding homeobox; ZO, zonula occludens.

spindle-shaped mesenchymal-like cell. These changes are associated with the down-regulation of epithelial cell surface markers and cytoskeleton components [e.g., E-cadherin, zonula occludens (ZO)-1, claudins, occludins, cytokeratins] and the up-regulation of mesenchymal markers (e.g., vimentin and α-smooth muscle actin) and extracellular matrix components (e.g., collagens and fibronectin) (6). The essential features of EMT as it relates to tumor progression are disruption of intercellular contacts and enhanced migration, the capability of matrix remodeling and tumor tissue remodeling, invasion into and migration through the extracellular matrix without the assistance of cell–cell contacts, and apoptotic resistance. Although the molecular basis of EMT have not been completely elucidated, *in vitro* and *in vivo* model systems have identified five main interconnected transduction pathways that lead to EMT and EMT-like phenotypes, many of which connect EMT to the extracellular matrix and the microenvironment surrounding tumors: tyrosine kinase receptors including the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF); nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB); integrins; transforming growth factor (TGF)-β; Wnt; and many others (7). Many of these pathways share common downstream signaling effectors, highlighting the complexity of the signaling networks involved in EMT (8). In this review, we summarize some of the most prominent EMT-inducing networks and the associated molecular events leading to the transition of differentiated, polarized epithelial cells to a fibroblastic, mesenchymal cell.

EMT-RELATED SIGNALING NETWORKS THAT REGULATE E-CADHERIN

Most signaling pathways involved in the initiation of EMT result in the down-regulation of E-cadherin, an epithelial cell adhesion molecule that serves as a "master programmer" of EMT [recently reviewed in Ref. (9)]. A critical mediator of EMT, E-cadherin has often been described as the gatekeeper of EMT (10, 11) and in most cell types, the loss of functional E-cadherin results in loss of cell adhesion, leading to rapid cell growth and metastasis (9). In addition to its role in cell adhesion, E-cadherin is involved in transmitting signals within cells that control cell maturation, differentiation, motility, and growth. E-cadherin also acts as a tumor suppressor protein, preventing cells from growing and dividing too rapidly or in an uncontrolled way; E-cadherin down-regulation has been implicated in cell migration and invasion in murine models of mammary, prostate, and pancreatic cancer (12). Providing further correlative support for a role of E-cadherin during tumor formation, E-cadherin is inactivated in many diffuse-type cancers such as lobular breast carcinoma and gastric carcinoma, in which cells in a tumor mass lose epithelial characteristics and exhibit a highly invasive EMT-derived histological pattern. E-cadherin down-regulation occurs in solid, non-diffuse-type cancers at the tumor-stroma boundary where single EMT-derived tumor cells invade otherwise healthy tissue. In the case of single cell infiltration, E-cadherin loss and subsequent resulting EMT could be a transient, reversible process, possibly regulated by the tumor microenvironment; neoplastic cells that have undergone EMT during invasion seem to regain E-cadherin expression and their epithelial, cohesive characteristics at the secondary foci (13).

Molecular events during EMT result in transcriptional regulation of the transcription factors Snail (Snail1), Slug (Snail2), zinc-finger E-box-binding homeobox (ZEB)1/2, and Twist1/2, leading to a molecular fingerprint that serves as a phenotypic marker during EMT (14). In particular, Snail, Slug, Twist, SIP1/ZEB, and E47 negatively regulate E-cadherin expression (14, 15) and display overlapping functional redundancy, in part through their common recognition of E-box sequences (**Figure 1**). Snail and Slug initiate EMT during development, fibrosis, and the initial invasion of cancer by repressing epithelial genes like E-cadherin by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains (16). While ZEB1/2 also binds to E-box sequences, ZEB-mediated transcriptional repression often involves the recruitment of a C-terminal-binding protein (CTBP) co-repressor (16). Twist1/2 belongs to the basic helix–loop–helix (bHLH) family of transcription factors and represses E-cadherin expression independently of Snail, probably through interactions with co-repressors (16). E47 also binds to the E-cadherin E-box, but appears to independently promote angiogenesis during tumor growth (17). Involved in most physiological EMT situations, overexpression of Snail, Slug, ZEB1/2, or Twist1/2 in epithelial cell lines typically induces EMT (18–20). These transcription factors also regulate genes other than E-cadherin. Twist and Snail have emerged as promising candidates of EMT "master genes" because they regulate genes involved in motility, proliferation, differentiation, and survival, including matrix metalloproteinases, N-cadherin, and E-cadherin in *in vitro* and *in vivo* experiments (19, 21). The signaling pathways involved in EMT should not be viewed in isolation, for evidence of interactions and crosstalk

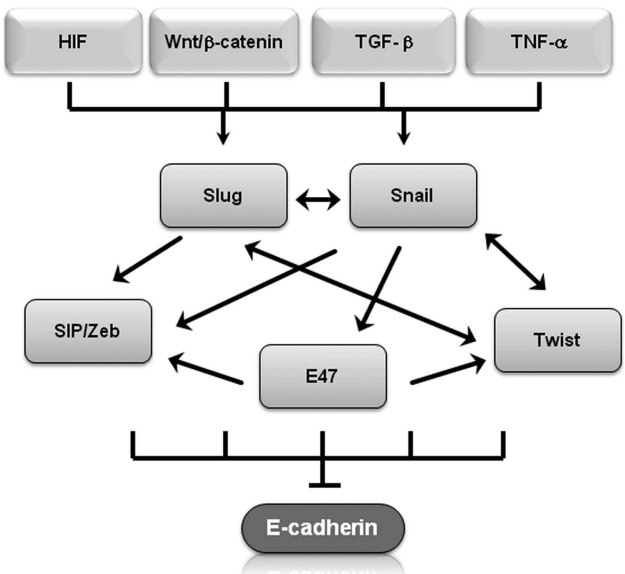

FIGURE 1 | Transcriptional regulators of EMT regulate E-cadherin. Shown is a graphical representation of how several of transcriptional regulators of EMT regulate E-cadherin expression. Arrows represent up-regulation, T-bars represent inhibition.

between multiple pathways exists. For example, Snail and Slug both repress E-cadherin levels and are co-expressed in various carcinomas, including breast and ovarian cancer (22). Nevertheless, by employing multiple signaling cascades, Snail and Slug could have both overlapping and simultaneously distinct roles during tumor progression, similar to what has been described for Dnmt3a/b and Vav1/2 during hematopoiesis (22–25). β-Catenin not only interacts with E-cadherin to maintain cell–cell adhesion but is also shuttled to the nucleus where Wnt serves as the transcription effector of the Wnt signaling pathway to promote proliferation and cell survival (26). This is particularly relevant during EMT because Wnt gene mutations and aberrant activation of β-catenin are considered critical events in tumor cell maintenance and growth (27). Glycogen synthase kinase (GSK)-3β-mediated stabilization of Snail is not only part of the Wnt signaling cascade but is also required in colorectal cancer cells for EMT induced by the pro-inflammatory cytokine tumor necrosis factor (TNF)-α (28). Slug was identified as a downstream Wnt signaling pathway effector in a basal-like carcinoma model that also linked the Wnt pathway to tumor proliferation and self-renewal (29). These finding suggest that Slug and Wnt play important roles in maintaining the stemness of human mammary tumor cells.

EMT AND CIRCULATING CANCER CELLS

It is believed that some cells slough off the outer edges of a tumor as it proliferates and are swept away by the bloodstream or lymphatic system (30). These rare so-called circulating tumor cells (CTCs) from a primary tumor have the capacity to be shed into the vasculature, where they circulate throughout the bloodstream and eventually find a suitable location to colonize and form distant metastases in new tissues (30). One of the key features of the CTC theory of metastasis is that these cells are able to become quiescent until microenvironmental conditions favor growth. Interestingly, two EMT-inducers, Snail and Twist, are upregulated in CTCs and allow cancer cell populations to revert to a stem-cell-like quiescent state (31). Further supporting a link between EMT and CTCs, more than 80% of CTCs collected based on the expression of an epithelial marker (EpCAM) also expressed a mixture of epithelial and mesenchymal markers, suggesting that these CTC were currently transitioning along the EMT axis (32). Indeed, these cells expressed many molecular markers only seen in cells undergoing EMT, including epithelial proteins such as cytokeratin and E-cadherin; mesenchymal proteins including vimentin, N-cadherin, and O-cadherin; and the stem cell marker CD133 (32). Additionally, CTC-like cells increased after immortalized human mammary epithelial cells were transfected with Ras to initiate EMT (33, 34). Further implicating EMT in CTC production, CTCs obtained from metastatic breast cancer patients express a much higher rate of Twist and vimentin, two markers of EMT, than patients with early breast cancer (35). Many of the same microenvironmental cues that regulate EMT also seem to be upregulated in CTCs, especially hypoxia and inflammatory mediators such as NFκB and TGFβ (36). Inflammatory signaling mediators emanating from the microenvironment also play a critical role in the maintenance of CTCs. The receptor for stromal-derived-factor-1 (SDF-1), C–X–C chemokine receptor 4 (CXCR4) enhances the survival of CTCs as they circulate throughout the body (37). Microenvironmental signals also serve as cues to tell CTCs to leave the circulation and to colonize distant organs. In breast cancer, bone marrow cells secrete parathyroid hormone-related protein (PTHRP), TNF-α, interleukin 6 (IL-6), and/or IL-11 stimulate the release of the receptor activator of NF-κB ligand (RANKL) from osteoblasts and suppress the release of the RANKL antagonist osteoprotegerin, allowing for breast tumor CTCs to colonize the bone marrow (37). TNF-related apoptosis-inducing ligand (TRAIL) has also been recently shown to contribute to EMT by miR-221-induced suppression of phosphatase and tensin homolog (PTEN) (38). Similarly, interactions between endothelial selectins and selectin ligands expressed on CTCs, up-regulation of adhesion molecules, and interactions between adherent neutrophils within inflamed sinusoids and CTCs contribute metastatic colonization of the liver (39). Together, these examples paint a complex picture of signaling crosstalk that intersect at the CTC and play a critical role in tumor progression.

MicroRNA REGULATION OF EMT

MicroRNAs (miRNAs) are small non-coding RNA molecules that play key roles in the regulation of transcriptional and post-transcriptional gene expression (40). In addition to their important roles in healthy individuals, miRNAs are important players during EMT and are differentially expressed in a broad range of cancers (41). Because a single miRNA can target several messenger RNAs, dysregulation of miRNAs can influence multiple signaling pathways leading to tumor formation and metastasis. For example, miR-138 controls EMT by targeting at least three genes: vimentin, ZEB2, and enhancer of zeste homolog EZH2 (42). Similarly, the miR-200 micro RNA family targets at least two transcriptional repressors of E-cadherin, ZEB1 and ZEB2; altering miR-200 in transformed cell lines induced changes consistent with either inducing EMT or the reverse process, mesenchymal–epithelial transition (MET) (43). Reduced expression of miR-30a promotes TGF-β-induced EMT by targeting SNAI1 (44). In addition to the control of transcription factors, miRNA also affects multiple aspects of the EMT process, including increased motility and invasiveness, cell adhesion, disassembly of epithelial cell junctions, and destabilization of tight junctions (45).

GENETIC LESIONS AND EPIGENETIC MODIFICATIONS IN EMT

Many signaling pathways associated with EMT result in increased cellular proliferation and create feedback loops, resulting in a perpetual proliferative state during the initial stages of EMT. In normal, healthy cells, genomic integrity during cell division is ensured by DNA repair and cell cycle checkpoints that respond to DNA damage by inhibiting critical cell cycle events (46). However, the increased proliferation rate in cells undergoing EMT provides tumor cells an opportunity to proceed through mitosis without high-fidelity proofreading and/or repair, consequently, resulting in the potential of increased mutation rates (47). However, increased proliferation during EMT is not sufficient for tumor development on its own and additional genetic lesions are required to move past the initial cellular dysplasia toward a malignant tumor. Consistent with this model, the carcinogenic potential of estradiol, for example, is thought to be mediated

by a combination of proliferation and increased mutation rate [reviewed in Ref. (48, 49)].

Epigenetic deregulation of gene expression is involved in the initiation and progression of multiple cancers and an important initiator of EMT. Similar to its role in differentiation, development, and malignant transformation, epigenetic reprograming during EMT is largely mediated by chromatin remodeling (50). DNA methylation patterns are preserved during EMT and sustained EMT activation leads to epigenetic alterations, inducing heritable changes that maintain the mesenchymal phenotype even after EMT-initiating signals are removed. Epigenetic modifications, especially histone and DNA methylation, are critical to gene regulation and establish patterns of gene repression during development and EMT (51). Snail represses E-cadherin expression by forming a co-repressor complex with histone deacetylase HDAC1 and HDAC2, resulting in E-cadherin silencing and *in vivo* pancreatic cancer progression (52). Overexpression of the histone methyltransferase MMSET (multiple myeloma SET domain) in prostate cancer influences histone 3 lysine 36 dimethylation (H3K36me2) and lysine 27 tri-methylation (H3K27me3). MMSET overexpression in immortalized prostatic epithelial cells leads to increased migration, increased invasion, morphological changes, and altered gene expression consistent with transition from an epithelial cell-like state to a mesenchymal cell-like state (53). Mediated by the ability of MMSET-mediated activation of *TWIST1*, a gene implicated in tumor-associated EMT and invasion (19), these data suggest that deregulated MMSET results in aberrant epigenetic gene regulation, leading to tumor progression and metastasis. Genome-wide histone maps focusing on H3 lysine 4 and lysine 27 tri-methylation (H3K4me3 and H3K27me3) identified differentially expressed genes in embryonic stem cells (54–57), hematopoietic stem cells/progenitor cells (58), T cells (59), and in prostate cancer cells (60). Although DNA methylation has been implicated in the transition from EMT to MET, reversible histone modifications are the predominant factors in reactivation of E-cadherin expression during the transition from EMT to MET (61).

Genome-scale mapping revealed that most chromatin changes are heterochromatin K9-modifications, suggesting that EMT is characterized by the epigenetic reprograming of specific, large chromatin domains across the genome (50). Similarly, clustered chromatin profiles using combinatorial patterns of posttranslational histone modifications and covalent changes to genomic DNA discovered a distinct chromatin signature among genes in well-established EMT pathways including the epidermal growth factor receptor (EGFR), suggesting that chromatin remodeling of EGFR plays an important role in EMT (62). Acetylation, regulated mainly through HDACs also affects EGFR expression and downstream signaling. HDAC6 up-regulation slows EGFR endocytic trafficking from early endosomes to late endosomes in renal epithelial cells and HDAC6 inhibition results in decreased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, a downstream target of EGFR (63). Future experiments should determine if these findings are common to EMT and determine if similar epigenetic reprograming occurs in other physiological contexts. Aside from this role in epigenetic reprograming, crosstalk between the more traditionally known EGFR signaling cascade and components of other signaling pathways frequently leads to abnormal activation of pro-proliferative and anti-apoptotic pathways. The most common signaling cascades activated by EGFR are the phosphatidylinositol-3-kinase (PI3K)/Akt, Ras/Raf/Mek/extracellular signal-regulated kinase, and the Jak/Stat pathways (64) that both contribute to the development of malignancies by impacting cell cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility, and metastases (65). Crosstalk between EGFR and other signaling pathways impact cancer treatment as well as the initiation of EMT. For example, one well-known mechanism of resistance to the selective EGFR inhibitor gefitinib/erlotinib is HGF receptor tyrosine kinase gene amplification. HGF receptor tyrosine kinase gene amplification bypasses normal EGFR signaling to instead activate AKT through HER3-mediated activation of PI3K in the presence of EGFR tyrosine kinase inhibitors (66).

CROSSTALK BETWEEN TGFβ AND OTHER SIGNALING PATHWAYS MEDIATING EMT

Signaling pathways are not independent from each other, but rather interact to form complex signaling networks; the TGFβ signaling pathway is no exception. Most likely, due to its involvement during many cellular processes including proliferation, differentiation, apoptosis, and cellular homeostasis, the TGFβ pathway interacts with many other signaling pathways during EMT (**Figure 2**). One mechanism by which TGFβ initiates EMT is by removing β-catenin from adherens junctions in a process that involves TGFβ-dependent PTEN dissociation from β-catenin and Akt activation (67). Depending on the context, Notch can either synergize with TGFβ/bone morphogenetic protein (BMP) signals to induce target genes or inhibit TGFβ/BMP signaling (68–71). In

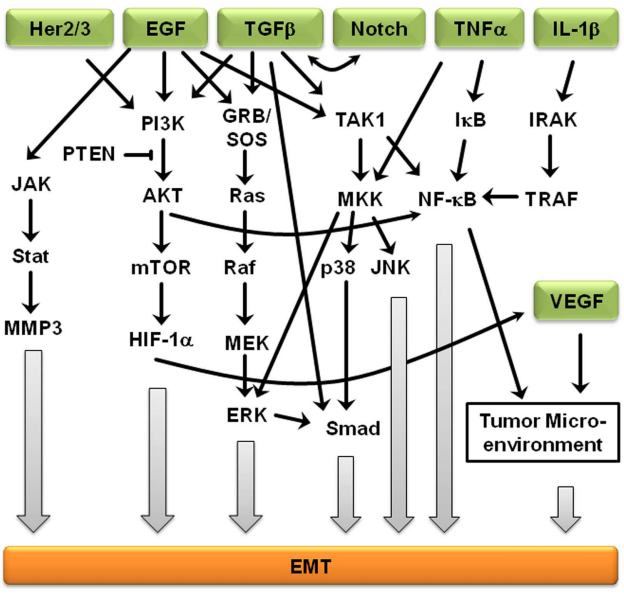

FIGURE 2 | Crosstalk between EMT-inducing signaling pathways. Representation of some of the points of intersection between various EMT-inducing signaling pathways. For clarity, branches that did not result in crosstalk are not shown. Arrows represent up-regulation.

the presence of other growth factors, TGFβ/BMP signaling generally stimulates migration and blocks endothelial cell proliferation, but Notch signaling inhibits the migratory affect of BMP (72). Stimulation of endothelial cells with BMP alone promoted cell migration, but in the presence of Notch signaling, cell migration was inhibited (72). Interestingly, the dominance of Notch signaling over BMP signaling was cell–cell contact-dependent, suggesting that endothelial cells not in contact with surrounding cells are stimulated by TGFβ to migrate until new cell–cell contacts are established, at which point Notch induces gene expression changes and arrests further migration (72). Similarly, Notch signaling is necessary for growth arrest by TGFβ in epithelium; over 30% of TGFβ-induced epithelial genes require Notch signaling for full expression (68). TGFβ leads to increased jagged-1 expression and siRNA-mediated knockdown of jagged-1 leads to reduced TGFβ-induced p21 expression, rescuing TGFβ-inhibited proliferation (68). Therefore, TGFβ induces both c-myc, which stimulates cell cycle progression, and jagged-1, which blocks cell cycle progression through stimulation of Notch and induction of p21. Jagged-1 induction is rapid and transient, so a balance between TGFβ/Notch-induced p21 and TGFβ/Smad-induced c-myc may act as a switch to regulate cell proliferation (72).

Further supporting the importance of crosstalk during EMT, Erk, c-Jun N-terminal kinase (JNK), and p38 indirectly regulate TGFβ signaling, but TGFβ treatment leads to activation of Erk1/2 and mitogen-activated protein kinase (MAPK) signaling (73). Smad-dependent signaling and MAPK-mediated Erk1/2 activation is believed to result in cross-talk between the TGFβ and EGF signaling pathways (74). The MAPK/Erk signaling pathway also mediates Smad2/3 phosphorylation and nuclear exclusion, which is believed to be important for the attenuation of TGFβ-induced nuclear translocation of MAPK (74). MAPK signaling also results in Smad1/5 phosphorylation, leading to an inability to translocate specific Smads into the nucleus (74). Erk-mediated Smad1 phosphorylation creates a docking site for the Smad1/5-specific E3 ubiquitin ligase, Smurf1. Smurf1 binding results in Smad ubiquitination and eventual degradation and blocks Smad interactions with the nuclear pore complex. MAPKs also regulate the protein stability of Smad4 and the inhibitory Smad7 (74), suggesting that Smad3 is indispensable to the mediation of the pro-apoptotic effects of TGFβ, Smad3, but not the closely related Smad2, is the primary target of PI3K/Akt-mediated inhibition (74). In addition, TGFβ regulates Akt activity and phosphatase and tensin homolog (PTEN) function during EMT initiation. In addition to activating the MAPK and PI3K/Akt pathways, ErbB signaling interacts with TGFβ/Smad during development and breast cancer progression (75). The PI3K/Akt pathway is also subjected to TGFβ regulation. Akt activity increases in response to TGFβ treatment, which seems to be required for a variety of TGFβ-induced activities, such as cell migration of HER2-expressing breast cancer cells, EMT of normal mammary epithelial cells, cell survival of mouse hippocampal neurons and mesenchymal cells, as well as growth stimulation of certain fibroblasts (74, 75). EGFR and IL-6R signaling cross-talk through JAK2/STAT3 to mediate EMT in ovarian carcinomas; activated STAT3 in high-grade ovarian carcinomas may occur directly through activation of EGFR/IL-6R or indirectly through induction of IL-6R signaling (76). Another ligand of EGFR, TNF-α, also induces EMT through NF-κB-mediated transcriptional upregulation of Twist1 (77). In breast cancer-related EMT, HER2/Ras antagonizes TGFβ-induced apoptosis and cell cycle arrest while simultaneously enhancing the pro-migratory and pro-invasive functions of TGFβ (78). TGFβ transcriptionally downregulates PTEN in Smad4 null pancreatic cancer cells and relies on the function of the Ras/MAPK pathway (73–75). EMT-related crosstalk is also clinically relevant; pharmacological blockade of IGF-1R fully prevented TGFβ's ability to activate an EMT protein signature (79).

INVOLVEMENT OF THE MICROENVIRONMENT DURING EMT

The tumor microenvironment plays a crucial role in tumor progression and metastasis, and as tumors develop, the integrity of the surrounding basement membrane plays a critical role in invasion and metastasis. The tumor microenvironment is composed of inflammatory and immune cells, physical interactions with neighboring cells, oxygen and nutritional gradients, stromal extracellular matrix, and soluble factors. Cells neighboring the developing tumor secrete growth factors and inhibitory molecules that regulate tumor proliferation and apoptosis, while tumor cells simultaneously secrete factors to neighboring cells that regulate adhesion. The temporal–spatial changes within the microenvironment surrounding tumors and the interaction between tumor cells and their microenvironment are crucial to tumor initiation and development, and are especially critical to cancer cell quiescence, tumor progression, invasion, tumor metastasis, and drug resistance (**Figure 3**) (80).

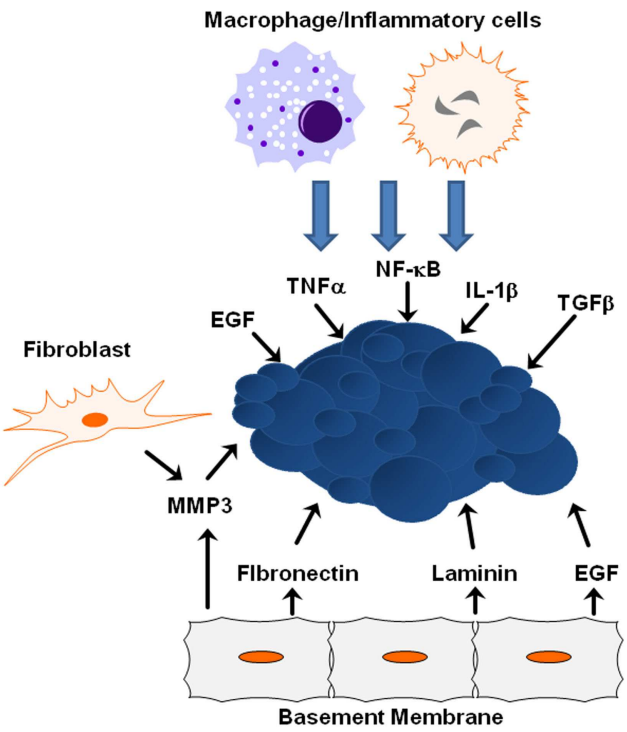

FIGURE 3 | ECM/growth factor involvement in EMT. Shown are several molecular mechanisms by which the cells within the tumor microenvironment can influence tumor progression and EMT initiation.

Many signals received from the tumor microenvironment can initiate EMT including TGFβ, hypoxia-inducible factor (HIF)-1α, EGF, WNTs, and Notch (21). Various signals trigger expression of these transcription factors including heterotypic interactions with neighboring cancer cells and interactions with adjacent tumor-associated stromal cells. Epithelial–mesenchymal interactions within the tumor microenvironment integrate several important signaling molecules that are critical for tumor growth and metastasis, including integrins, cytokines, and growth factors (81). Crosstalk between the TGFβ and HER2/Ras/MAPK signaling pathways often leads to secretion of additional growth factors and cytokines, including TGFβ itself, which in turn promotes EMT and cell invasion, whereas JNK kinases negatively regulate the autocrine expression of TGFβ1 (73, 75). ErbB receptors and their ligands are also involved in cross-talk between cancer cells and the tumor microenvironment. EGFR is activated in tumor-associated endothelial cells, but not in endothelial cells within uninvolved organ regions, suggesting that EGFR activation and expression is partially determined by the tumor microenvironment (82). As time goes on, the importance of the microenvironment to pathogenesis is becoming clearer, from the role the ECM and matrix rigidity plays in determining polarity, to the extracellular metabolism of growth factors and matrix molecules during cancer progression and metastasis.

Extracellular matrix proteins and physical properties within the microenvironment can lead to tumor progression by activating EMT-inducing pathways within tumors. One key extracellular matrix protein is matrix metalloproteinase-3 (MMP3), a matrix-degrading enzyme secreted by stromal fibroblasts known to induce *in vitro* and *in vivo* EMT in mammary epithelial cells (83). When tumor cells are exposed to MMP3, transcription of a splice variant of Rac1 called Rac1b increases stimulating the production of reactive oxygen species and expression of Snail1 (84). EGFR activation in human carcinoma cell lines increases MMP-9 activity and is associated with increased *in vitro* cell invasion (85). Synthetic low-molecular weight or endogenous MMP inhibitors or an anti-catalytic MMP-9 antibody blocked increased invasive activity after EGF-mediated induction, indicating EGFR activation results in enhanced MMP-9 expression and may facilitate the removal of extracellular matrix barriers to tumor invasion. Additional proteins within the basement membrane influence EMT induction from ectopic exposure of MMP3. For example, the laminin suppresses EMT in MMP3-treated cells, while fibronectin promotes EMT due to interactions with specific integrin receptors (7). During this process, α6-integrin sequesters Rac1b from the basement membrane and is required for inhibition of EMT by laminin; α5-integrin maintains Rac1b at the membrane and is required for the promotion of EMT by fibronectin (7). Additionally, matrix rigidity may also play an important role during EMT. The microenvironmental stiffness surrounding cells impacts differentiation and response to external molecular cues, while epithelial cells treated with MMP3 undergo EMT when cultured on plastic or glass, cells cultured on soft matrices do not undergo EMT in response to treatment with MMP3 (7).

INVOLVEMENT OF INFLAMMATORY SIGNALING IN EMT

The microenvironment surrounding a tumor is often dominated by inflammatory cytokines that promote tumor initiation by leading to increased angiogenesis, tumor growth, and tumor progression (86). Tumor-associated macrophages secrete EGF to neighboring cancer cells, which in turn stimulate macrophages to facilitate intravasation and metastatic dissemination of the cancer cells (87, 88). Together, these findings substantiate a role of EGF-mediated signaling not only in EMT and proliferative signaling itself but also in the cross-talk between tumor cells and the microenvironment. The tumor microenvironment is largely orchestrated by inflammatory cells, which facilitate extracellular matrix breakdown, angiogenesis, and tissue remodeling, thus, promoting tumor cell motility (89). Inflammatory cells play a major role in secreting activating factors that lead to NF-κB activation; NF-κB is a key regulator of the inflammatory response shown to regulate Slug and Snail (90). TGF-β activity is deregulated during malignant cancer progression, and plays an important role in EMT (91). Similarly, both TNFα and interleukin-1β (IL-1β) are expressed at low levels in normal breast epithelial cells, but are upregulated in the majority of breast cancer patients, with pronounced expression of both cytokines in over 80% of patients who experience breast tumor relapse (92). *In vivo* murine breast models suggest increased expression and activity of TNFα results in many cancer-promoting functions and that inhibition of TNFα expression leads to reduced breast cancer malignancy (93). Chronic TNFα expression in the tumor microenvironment is correlated with a more aggressive tumor phenotype (93). IL-1β upregulates a variety of processes that contribute to higher angiogenesis, tumor growth, and tumor progression and is considered a strong and causative pro-inflammatory factor whose expression is associated with advanced cancer (94). TNFα impacts cell morphology and may cooperate with TGFβ to lead to EMT in non-transformed breast epithelial cells (95). Sustained co-expression of TNFα and IL-1β acts through the complex regulatory processes of the EMT activators Zeb1, Snail, and Twist to result in morphologic changes including cell spreading, protrusion formation, decreased E-cadherin expression, and increased expression of vimentin, all consistent with EMT (96).

HYPOXIA AND EMT

When microenvironmental cues are favorable for growth, rapid cell growth with a tumor results in local hypoxia and nutrient deficits, regardless of the oxygen tension surrounding the tumor (97). Therefore, sustained tumor growth requires increased local vasculature to provide oxygen and metabolites to feed the growing tumor (98) and the nutritionally impoverished and hypoxic environment within tumors results in local changes in hypoxia-related gene expression, contributing to tumor heterogeneity (99). Tumor cells adjust to hypoxia and lack of nutrients not only by activating specific pathways associated with angiogenesis but also associated with hypermetabolism, glycolysis, and resistance to acidosis-induced toxicity (100). Hypoxia genes, especially HIF-1α, are frequently upregulated within many solid tumors and promote tumor progression (101, 102). HIF-1α induces EMT and self-renewal of cancer stem cells, and facilitates metastasis;

knockdown of HIF-1α inhibits or even reverses the EMT-like phenotype (103, 104). Hypoxia-induced EMT is mediated by HIF-1α via up-regulation of transcription effectors such as Snail, Twist, and ZEB1/2 and results in the suppression of E-cadherin expression (105–107). Several additional signaling pathways that are critical for embryonic development including Notch, Wnt, and TGFβ are also involved in hypoxia-induced EMT. Demonstrating a complex integration of hypoxic signals into EMT, Notch signaling directly upregulates Snail, and potentiates HIF-1α recruitment to the lysyl oxidase promoter, resulting in stabilization of Snail, increased cell motility, and increased invasiveness (108). Similarly, Wnt/β-catenin signaling enhances hypoxia-induced EMT by increasing the EMT-associated activity of HIF-1α and preventing cell death (109). Hypoxia also inhibits Wnt signaling by interfering with β-catenin acetylation (110), blocking secretion of Wnt (111), and activating Siah-1 in a p53-dependent manner (112).

Depending on cell type, Wnt/β-catenin signaling also enhances hypoxia-induced EMT by increasing the EMT-associated activity of HIF-1α and preventing tumor cell death (109). HIF-1α also competes with T-cell factor-4 (TCF-4) to bind β-catenin and form a HIF-1α/β-catenin complex, accompanied by increased HIF-1α transcriptional activity in colorectal tumors (113). TGFβ signaling also integrates hypoxia-related cues, for TGFβ/Smad3 inhibit vascular smooth muscle cell apoptosis through an autocrine signaling mechanism involving VEGF (114). Adding further complexity to the impact of TGFβ signaling, TGFβ not only activates the Notch signaling pathway but Notch signaling also activates TGFβ in rat mesangial cells under high-glucose conditions (68, 115). It is important to note that this example also highlights how signals from the microenvironment can influence signaling outcomes.

CONCLUSION

Epithelial–mesenchymal transition is a key physiological process during normal development and regulated by an intricate network of signaling pathways that allows for the integration of signaling cues during embryonic morphogenesis. While these signaling networks allow for the precise control required for a major switch from a differentiated epithelial cell into mesenchymal cell, it also opens up the possibility of deregulation on multiple levels during pathological processes such as cancer and fibrosis. Owing to the complex interactions between these signaling pathways, activating mutations in signaling molecules can be amplified. Many of these potentially deregulated pathways converge on a few master regulatory molecules or parallel pathways can induce changes on various levels. Thus, it is plausible that EMT contributes to cancer progression in various ways, including tumor growth, invasion, and metastasis. Moreover, depending on the nature of the genetic lesions, EMT can become a very individualized process, adding to the complexity of cancer, while also opening up the possibility of personalized medicine. Thus, our improved understanding of EMT signaling networks and their association with therapeutic resistance is imperative for future development of novel anti-tumor drug and treatment strategies, especially in high-grade tumors and tumors that have developed therapeutic resistance.

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Clinical implications of circulating tumor cells of breast cancer patients: role of epithelial–mesenchymal plasticity

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There is increasing interest in circulating tumor cells (CTCs) due to their purported role in breast cancer metastasis, and their potential as a "liquid biopsy" tool in breast cancer diagnosis and management. There are, however, questions with regards to the reliability and consistency of CTC detection and to the relationship between CTCs and prognosis, which is limiting their clinical utility. There is increasing acceptance that the ability of CTCs to alter from an epithelial to mesenchymal phenotype plays an important role in determining the metastatic potential of these cells. This review examines the phenotypic and genetic variation, which has been reported within CTC populations. Importantly, we discuss how the detection and characterization of CTCs provides additional and often differing information from that obtained from the primary tumor, and how this may be utilized in determining prognosis and treatment options. It has been shown for example that hormone receptor status often differs between the primary tumor and CTCs, which may help to explain failure of endocrine treatment. We examine how CTC status may introduce alternative treatment options and also how they may be used to monitor treatment. Finally, we discuss the most interesting current clinical trials involving CTC analysis and note further research that is required before the breast cancer "liquid biopsy" can be realized.

Keywords: circulating tumor cells, epithelial–mesenchymal transition, breast cancer, clinical application, metastasis

INTRODUCTION

Breast cancer is the most common cause of cancer death among women (1). Prognosis for most patients with early breast cancer (EBC) is generally very good, however, a significant proportion (20–30%) of chemotherapy-treated EBC patients relapse with metastatic disease (2). How to identify those breast cancer patients who will relapse in the future and develop metastatic disease remains elusive. Metastatic disease is initiated by circulating tumor cells (CTCs) that originate from the primary tumor and spread the cancer in the body via the blood circulatory system. These CTCs may migrate to, and remain dormant in, sites such as bone marrow as disseminated tumor cells (DTCs). After variable latency periods, DTCs may develop into overt metastases and although this is not seen in all patients, it is seen more frequently in breast cancer patients with persistent DTCs (3). Although considerable research has been conducted to characterize these cells and their role in dissemination, dormancy, and formation of metastasis, many questions remain. For example, why are CTCs not detectable in some patients with metastases, and why is it that some patients with detectable CTCs never develop metastases?

In a rat model, human mammary tumors have been shown to shed $3.2–4.1 \times 10^6$ cells per day per gram of tissue (4), most of which (~85%) are destroyed within minutes in the circulation (5) by anoikis, a form of apoptosis driven by loss of cell–cell interactions (6). However, some cells are resistant to anoikis (5). In a mouse model, approximately 2.5% of CTCs formed micrometastases (most of which subsequently disappeared over time) and 0.01% of CTCs progressed to form macrometastases (7). Metastatic potential is not only influenced by CTCs resistance to anoikis, but also the ability of CTCs to change their cellular phenotype from epithelial to mesenchymal – termed epithelial–mesenchymal plasticity (8).

Detection of either CTCs or DTCs is commonly associated with an increased risk of metastases and accompanying poor prognosis (9, 10). Researchers have, however, reported considerable variation in CTC detection rates and correlation with prognosis, even in patients with substantial metastatic disease (11). To date, this has prevented the use of CTCs as a routine prognostic clinical tool (12). We focus our review on CTCs, their role in breast cancer progression, and how CTC molecular variation and epithelial–mesenchymal transition (EMT) may explain discrepancies in CTC detection, therapy response, and relationship to prognosis.

CTC CHARACTERISTICS

Circulating tumor cells are extremely rare, with a frequency of typically 1 per $10^{6–7}$ leukocytes (13). Defining characteristics to

delineate CTCs from other blood cells is difficult due to the substantial pleomorphism CTCs exhibit (14). Breast cancer CTCs have a mean diameter of 13.1 μm (15), which is only slightly larger than blood leukocytes measured at 10 μm (16). Accepted CTC characteristics include presence of a nucleus, visible cytoplasm, and the expression of cytokeratin and absence of CD45 expression (17).

Clusters of CTCs, also called tumor microemboli, are found in some patients, comprising 4% of CTCs analyzed in one study (14) and have been demonstrated to form prior to entering the circulation, and to be precursors with more malignant potential than their unicellular counterparts (18). Cluster presence, particularly if sustained through treatment, correlates more strongly with poor prognosis than single CTCs do in metastatic breast cancer (MBC) patients (18).

EPITHELIAL–MESENCHYMAL TRANSITION

Most breast cancers are of epithelial origin (19). Epithelial cells collectively maintain organized tissue architecture through distinct contact between cells facilitated by E-cadherin, a homotypic transmembrane cell–cell adhesion protein (20, 21). A critical step in tumor invasion and metastasis is the phenotypical change known as EMT, normally a highly regulated process involved in embryogenesis and wound healing, and implicated in several disease states including malignancy and fibrosis (22). Physiologically, activation of a range of highly controlled signaling molecules triggers EMT in response to specific stimuli (23). However in cancer cells, activation of this process is dysregulated (22). During EMT, adhesion molecule expression is altered and cells take on mesenchymal characteristics, becoming more elongated, flexible, mobile, and thereby potentially invasive (19). This phenotype also mediates increased resistance to common anti-cancer therapies including taxanes and anthracyclines (24) and is elevated in breast cancer tissues remaining after neoadjuvant therapies (25). Tumor cells surviving in the hostile environment of the blood have undergone demonstrated EMT changes (26, 27), which are considered crucial to the metastatic process (28) and to resistance to anoikis (29). EMT is, for instance, most evident in "triple-negative" tumors [those without estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2)] and HER2 positive (HER2+) tumors, and least frequent in ER positive (ER+) tumors, particularly lobular cancers, mirroring the metastatic potential of these tumor types (27, 30).

Whilst EMT/mesenchymal markers have been demonstrated on CTCs, breast cancer metastases in liver, lung, and brain often express higher levels of E-cadherin and hence are often "more epithelial" than the primary tumor, indicating a reversal of the EMT process (31), termed mesenchymal to epithelial transition (MET). Evidence for the importance of this reverse transition and its role in metastasis is growing rapidly (32–36).

SUB-POPULATIONS OF CTCs

Circulating tumor cells can exist in intermediate states – subpopulations expressing both epithelial and mesenchymal markers to varying degrees (27, 37–40). This is likely to be considerably more common than complete polarization to either state (41). Sub-populations of tumor cells at any point may also acquire cancer "stem-cell" (CSC) attributes such as quiescence, self-renewal, asymmetric division, drug resistance (38, 42), and resistance to radiation (43), facilitating survival in the circulation and resultant metastasis. Breast CSCs are most commonly identified with a CD44+/CD24− phenotype (44) or by the expression of aldehyde dehydrogenase 1 (ALDH1) (45). These CSC markers have been identified in breast cancer CTCs populations by a number of researchers (46–49).

CTC ISOLATION METHODOLOGIES

Current CTC detection methods rely on CTC physical properties (e.g., size, density, electric charge, and cell deformability) or on the retained expression of surface proteins (predominantly epithelial) or messenger RNA. Although there are currently numerous CTC detection methodologies [comprehensive reviews (13, 50–53), the CellSearch system (Veridex, USA)], an immunomagnetic bead capture system based on epithelial cell adhesion molecule (EpCAM), followed by immunofluorescence analysis predominates, as it is the only current method to achieve Federal Drug Administration approval. As malignant cell transcriptional profiles vary, especially during processes such as EMT and CSC formation, expression of identifying proteins may be lost in CTCs as well as being present in non-CTCs, reducing sensitivity and specificity. Barriere et al. (54) reviewed studies exploring CTC isolation, noting their propensity to co-express epithelial, mesenchymal, and CSC markers, and recommended development of a combined isolation method targeting all three phenotypes to avoid missing clinically relevant CTC sub-populations.

CTCs AS PROGNOSTIC TOOLS

Both CTCs and DTCs have been detected in breast cancer patients with disease states ranging from ductal carcinoma *in situ* to MBC (55–60), and their detection is generally associated with a poor prognosis. Although CTCs are not seen in all MBC, this may be due to the inability of current methods to detect EMT subpopulations (54, 61, 62). Extensive studies in MBC show that CTCs associate with disease progression (57, 63, 64) with a meta-analysis by Zhang et al. (10) confirming CTC presence to be an independent prognostic factor for overall survival (OS) in MBC ($HR = 2.33$, $p < 0.005$).

Links with CTC presence and prognosis in EBC are also suggested (65, 66). Confirming this, a defining meta-analysis by Zhang et al. (10) showed the presence of CTCs to be an independent prognostic factor for OS in EBC ($HR = 2.78$, $p < 0.005$).

The association of CTCs and prognosis in EBC appears independent of tumor grade, histological type, degree of nodal involvement, lymphovascular invasion, or Ki-67 (proliferation marker) status (67, 68). Mixed results have been seen when considering receptor-defined breast cancer subtypes. Detection of CTCs is prognostic in EBC patients with "triple-negative" tumors or ER negative (ER−) PR negative (PR−) HER2+ primary tumors, but not in patients with ER+ tumors (69). In contrast, Giordano et al. (70) found CTCs to be prognostic in all MBC disease subtypes except HER2+ tumors, whilst Liu et al. (71) found the contrary.

The prognostic importance of CTCs over long-term follow-up has not been established. CTCs have been detected in patients in prolonged remission with 36% of patients in one study having detectable CTCs 8–22 years out from treatment of EBC, despite no

clinical evidence of disease (72). What proportion of these patients will go on to develop metastatic disease is not known, nor have the beneficial effects of CTC-guided intervention been established (see Monitoring Treatment – Clinical Utility section below).

RECEPTOR DISCORDANCE

Amplification of the HER2/*neu* gene and subsequent HER2 protein overexpression is associated with significantly decreased disease-free survival (DFS) and OS in the absence of HER2-targeted therapy (73, 74). Similarly, patients with HER2+ CTCs have been reported to have worse progression-free survival (PFS) and OS in comparison with patients with HER2− CTCs or any detectable CTCs (75–77). Heterogeneous amplification of HER2 is, however, known to occur within tumors and this serves to confound HER2 diagnostics and studies of receptor discordance (78). Receptor discordance refers to differences in receptors of primary tumor and metastatic tumors or CTCs. Discordance in HER2 status between primary tumor and CTCs reports are variable, in the order of 15–35% in MBC (75, 79, 80). HER2 discordance has also been reported in EBC patients. Wulfing et al. (77) found that, in EBC patients with detectable CTCs, 12 of 24 (50%) patients with HER2− primary tumors had HER2+ CTCs, and 1 of 3 (33%) patients with HER2+ primary tumors had HER2− CTCs. A few studies have shown that trastuzumab treatment is effective in eliminating HER2+ CTCs, including from patients with HER2− primary tumors and significantly reduced the risk of relapse and prolonged the DFS (81, 82).

Clinical trials are underway testing the utility of CTCs as a therapy decision-making tool in such cases of observed discrepancy in HER2 positivity between the primary tumor and CTCs. The DETECT III trial is randomizing HER2− MBC patients with HER2+ CTCs to standard therapy with or without lapatinib, a HER2-targeted therapy. The TREAT-CTC trial is randomizing HER2− EBC patients with detectable HER2+ CTCs post-neoadjuvant therapy (NT) and surgery to either standard care or additional trastuzumab. These studies may provide a foundation for the use of CTCs in standard clinical practice to identify patients who may benefit from the addition of HER2−directed therapy.

Discordance between the ER and/or PR status of primary and metastatic tumors has long been observed (83–85). Given the role of CTC in progression to metastases, it is not surprising that the hormone receptor status of CTCs may also differ from that of the primary tumor. Interestingly, this discordance appears much greater than that seen between primary and metastatic tissue, implying that receptors may be lost then regained once overt metastases form. Aktas et al. (86) found that discordance rates between primary tumor and CTCs for ER and PR in MBC patients were 59% and 55%, respectively, with most CTCs being ER− and PR− (84% and 92%). Fehm et al. found discordance rates between primary tumors and CTCs in EBC for ER and PR to be 71% and 75%, respectively (87) and HER2 discordance rates in MBC patients to be 36% (76). Although this suggests that hormone receptor loss may often be a transient phenomenon connected with the CTC state, this "sanctuary phenotype" could still contribute to endocrine therapy failure.

As with HER2 discordance; there are also implications for treatment of ER+ CTCs where the primary tumor is ER−. It remains to be seen if estrogen-targeted treatments in ER− primary tumors with ER+ CTCs have a therapeutic effect.

MONITORING TREATMENT – CLINICAL UTILITY

Another important clinical area uses CTCs as an early marker of disease progression or treatment failure – potentially giving an indication of a need for change of therapy before conventional imaging and/or tumor markers demonstrate progression. The lack of a reliable method to monitor the effects of adjuvant systemic therapy in particular is a significant area of need. Multiple studies have shown that in EBC, locally advanced breast cancer, and MBC, detection of CTCs after the completion of treatment is a strong prognostic marker (58, 64, 88–90).

Circulating tumor cell changes in MBC response to treatment can yield important prognostic information. For instance, MBC patients in whom initially high CTC counts reduced to low levels after initial therapy, had identical prognosis to CTC-negative patients (64). Correlations between the changes in CTC numbers and an objective response to therapy as assessed by serial imaging were reported by a study conducted by Nakamura et al. (91). Pachmann et al. (92) showed that patients who had higher CTC numbers that declined following treatment had a better prognosis than those whose CTC count did not change. Utilizing this paradigm, the SWOG SO500 trial evaluated switching therapy in MBC patients after one treatment cycle if certain CTC fall thresholds were not met. This trial did not demonstrate that an early switch improved DFS or OS, but presence of CTCs was an adverse prognostic factor (93). It has been suggested that the reason for the failure of this trial to observe a benefit to switching treatment on the basis of CTC levels is due to the fact that breast cancers with acquired chemo-resistance to one agent rarely exhibit high sensitivity to a randomly chosen alternative chemotherapeutic agent (94).

There are a number of ongoing clinical trials examining the utility of CTCs in breast cancer treatment. Details of some of the interventional studies employing CTC assessments, which are currently being run, are shown in **Table 1**. Results are eagerly awaited from the CirCe01 trial, which has similar design to the SWOG SO500 trial but evaluates CTCs serially after each cycle, with patients in the intervention arm changing therapy if CTC counts are adverse (see **Table 1**). Currently, we do not have clinical trial results supporting the use of CTCs to guide clinical decisions. Bardia et al. (94) highlighted the need for future clinical trials to utilize CTC isolation methodologies that are able to isolate CTCs which have undergone EMT, and to genotype CTCs in order to evaluate therapeutic response and guide therapeutic choices.

Circulating tumor cells have been studied with respect to their potential to inform patient therapy. Pierga et al. (95) found a significant correlation between CTC detection before NT and reduced DFS, but no correlation between the persistence of CTCs post-NT and tumor response. Boutrus et al. (96) also found that CTC presence predicted local and distant relapse, but did not correlate with primary tumor volume reduction. Similarly, Riethdorf et al. (68) showed that CTC detection before NT did not correlate with tumor response to treatment, nor did CTC changes necessarily mirror treatment response. This suggests differential responses to treatment between the primary tumor and CTCs.

Table 1 | A selection of current ongoing clinical trials examining the clinical utility of circulating tumor cells in breast cancer treatment.

Trial name (ClinicalTrial.gov registry number)	Rationale	Patient group	Methodology	Estimated accrual completion date
CTC-EMT (NCT02025413)	Evaluating a novel mesenchymal-marker-based ferrofluid (N-cadherin or O-cadherin based) CTC capture method.	Metastatic prostate or MBC patients	Non-randomized study to evaluate novel CTC capture method.	December 2014
STIC CTC METABREAST (NCT01710605)	Evaluating the medico-economic value CTCs provide in deciding on first-line therapy.	HR+, HER2− MBC patients	Randomized study where patients with ≥5 CTC/7.5 ml blood receive chemotherapy and those with <5 CTC/7.5 ml receive endocrine therapy.	February 2015
COMETI P2 (NCT01701050)	Evaluating the algorithm CTC-Endocrine Therapy Index (CTC-ETI) for the identification of patients that will progress.	ER+, HER2− MBC patients	ER, B-cell lymphoma-2 (BCL2), HER2, and Ki-67 markers assessed on isolated CTCs and CTC-ETI determined.	December 2015
Treat-CTC (NCT01548677)	EBC, HER2− primary tumor patients with no overt metastasis having completed (neo) adjuvant chemotherapy and surgery.	HER2−, CTC+ EBC patients	Patients randomized in 1:1 ratio to either the trastuzumab arm or the observation arm.	April 2017
CTC-CEC-AND (NCT02220556)	Evaluation of different analysis methods for CTCs, CECs, and circulating tumor DNA in patient followed for a tumoral pathology.	Patients with solid tumors	Fifteen cohorts. Each cohort will test one analysis method and/or tumoral type. Up to 50 patients in each cohort.	December 2015
CirCe01 (NCT01349842)	Evaluation of the use of CTCs to guide chemotherapy from the third-line of chemotherapy for MBC.	Advanced MBC patients	Patients with ≥5 CTCs/7.5 ml before third-line of chemotherapy randomized between CTC-driven and standard treatment.	January 2018
DETECT III (NCT01619111)	A multicenter, phase III study to compare standard therapy ± Lapatinib in HER2− MBC patients with HER2+ CTCs.	HER2− MBC patients with HER2+ CTCs	Patients randomized between standard therapy ± Lapatinib. Patients with bone metastases treated with denosumab.	March 2018

A large neoadjuvant chemotherapy study conducted by Rack et al. (60) in EBC patients found separate prognostic importance for the presence of CTCs pre- and post-treatment. Interestingly, the initially CTC-negative patients who subsequently developed CTCs fared better than initially CTC positive patients whose CTCs disappeared post-treatment, suggesting CTC clearance does not predict chemotherapy benefit (60).

To date, few studies have examined drug resistance in CTCs. Gradilone et al. (97) evaluated CTC of 42 MBC patients for expression of multi-drug resistance-related proteins (MRPs) and/or ALDH1, a putative tumor-initiating cell/CSC marker that correlates with resistance to some chemotherapeutics. The expression of MRPs on CTCs was found to be predictive of poor response to chemotherapy and significantly correlated with reduced PFS in MBC patients. Patients with CTCs expressing two or more MRPs had shorter PFS than those with CTCs expressing zero or one MRP (7.1 versus 16.4 months; $p = 0.004$). Furthermore, the expression of ALDH1 on CTCs was correlated with MRPs (and the number of MRPs expressed ($p = 0.000$) as well as an increased resistance to chemotherapy. Gazzaniga et al. (98) screened 105 cancer patients (of which 14 had breast cancer) for CTCs and then evaluated the MRP profile of the CTCs, postulating that this could delineate chemotherapy responders from non-responders. Patients were classified as chemotherapy "resistant" or "sensitive" on the basis of their CTC MRP profile, together with the chemotherapy regime the patient had received. This study found that the MRP profiles of patients' CTCs to be highly predictive of response to chemotherapy, independent of tumor type and stage of disease.

CONCLUSION

The presence of CTCs is a powerful independent prognostic factor in both MBC and EBC. However, we increasingly understand that CTCs are heterogeneous, even within an individual patient at different times in the disease trajectory (27, 99). This includes in the receptors that they express, in relation to either the primary tumor or any metastatic disease, as well as in their variable expression of epithelial and mesenchymal markers.

Although CTC count changes are predictive of outcome in MBC, this is largely a disease where serial agents are delivered with palliative intent. Hence, early tailoring of therapies may not greatly impact on outcome. To date, clinical trials have shown that absolute CTC count alterations or CTC persistence do not predict strongly for neoadjuvant response, or improved adjuvant or metastatic outcomes, and hence currently do not provide clinically useful information to drive changes in therapies. With the

maturation of the current clinical trials and further developments in the molecular characterization of CTCs, this information will hopefully become available.

Further work is needed, looking at CTC sub-populations including the presence and importance of EMT and CSC populations, and their alteration with treatment. There may be potential for targeting of otherwise treatment-resistant CTCs through novel targets on such populations. Additionally, the appearance of established therapeutic targets such as HER2 and ER on CTCs not present on the primary tumor is of considerable clinical importance, and the results of ongoing HER2-targeting trials are awaited with interest.

The promise of a "liquid biopsy" to diagnose, characterize, monitor, and influence treatment of cancer is still some way off. However profiling the presence and molecular characteristics of CTCs is very likely to provide important predictive and prognostic information in both early and MBC, and may prove useful in assessing response to treatment and as an early warning system for disease recurrence.

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Implications of epithelial–mesenchymal plasticity for heterogeneity in colorectal cancer

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Colorectal cancer (CRC) is a genetically heterogeneous disease that develops and progresses through several distinct pathways characterized by genomic instability. In recent years, it has emerged that inherent plasticity in some populations of CRC cells can contribute to heterogeneity in differentiation state, metastatic potential, therapeutic response, and disease relapse. Such plasticity is thought to arise through interactions between aberrant signaling events, including persistent activation of the APC/β-catenin and KRAS/BRAF/ERK pathways, and the tumor microenvironment. Here, we highlight key concepts and evidence relating to the role of epithelial–mesenchymal plasticity as a driver of CRC progression and stratification of the disease into distinct molecular and clinicopathological subsets.

Keywords: CRC, epithelial–mesenchymal transition, cancer stem cell, tumor progression, subtypes, serrated

INTRODUCTION

Colorectal cancer (CRC) has provided a paradigm for studying tumorigenesis for the past two decades (1, 2). Despite significant advances in understanding how it develops and progresses, CRC remains a major cause of cancer mortality in the developed world, due largely to its propensity to metastasize (3).

Early models of the molecular genetics underlying sporadic and hereditary CRC suggested that it arises via clonal expansion of crypt cells bearing loss-of-function mutations in *APC* or gain-of-function *CTNNB1* mutations. Such mutations result in persistent activation of the Wnt pathway, a central regulator of stem cell compartments and cell fate along the crypt–villus axis. Aberrant Wnt signaling in CRC is characterized by localization of β-catenin to the nucleus, where it interacts with various transcription factor complexes, including TCF/LEF (4) and YAP/Tead (5), and Rel/NFκB (6). These interactions drive growth, proliferation, or stemness programs contributing to formation and progression of adenomas. Subsequent mutations in oncogenes (e.g., *KRAS*, *BRAF*) and/or tumor suppressors (e.g., *SMAD4*, *TP53*) then drive transition of adenomatous polyps to overt adenocarcinomas and subsequent metastatic disease (1, 2, 7, 8) (**Figure 1**).

The sequential acquisition of mutations within the adenoma-carcinoma axis, coupled with classification of disease stage/grade and histological type has provided an important paradigm to understand the "classic form" of CRC (**Table 1**). However, it has long been recognized that the disease is often associated with considerable heterogeneity in tumor cell phenotype, therapeutic responses, and prognoses (9–11). Indeed, comprehensive genetic and gene expression analyses have revealed variability in the genetic alterations and pathways that underlie CRC, leading to the view that the disease comprises multiple types and subtypes, which evolve through different routes (12–18). Underlying these classifications are concepts of clonal evolution, cancer stem cells (CSC), and reversible epithelial–mesenchymal transitions (EMT), each with the capacity to drive heterogeneity within CRC (6, 19–22).

EMT AND TUMOR CELL PLASTICITY DURING CRC PROGRESSION

That tumor heterogeneity arises through selection and expansion of different cancer cell clones bearing perturbations (e.g., mutations, epigenetic changes) conferring survival and proliferative fitness is widely accepted (1, 2, 8, 12). Heterogeneity can also arise from plasticity in tumor cell behavior, via reversible phenotypic changes driven by micro-environmental, morphogenetic, or therapeutic factors (21). These observations have in part been linked to the cancer stem cell idea, according to which a small but highly tumorigenic population of CSC having the potential to form metastases regenerates itself and progeny exhibiting a cellular hierarchy resembling normal tissue (6, 19–22).

An important source of plasticity in CRC and some other solid cancers is the EMT, which together with its reverse process, a mesenchymal–epithelial transition (MET), is essential for tissue remodeling during embryogenesis and in some pathological contexts (23, 24). Importantly, EMT–MET events also provide a framework through which solid cancers can disseminate and colonize distant sites (21, 25–31). During EMT, hallmarks of differentiated epithelia such as apico-basal polarity and cell–cell adhesions are replaced with mesenchymal traits, including rear-to-front polarity, capacity for individual cell migration, and invasion of basal lamina and blood vessels.

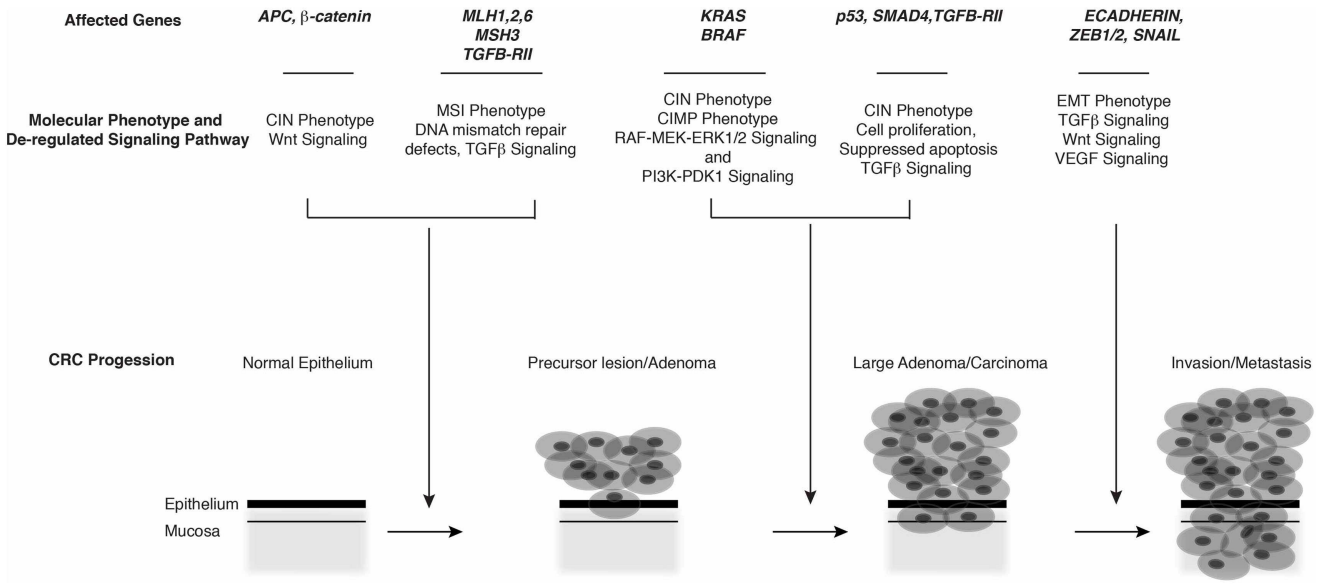

FIGURE 1 | Molecular phenotypes, genetic alterations, and major signaling pathways associated with CRC progression.

Table 1 | Classification of CRC on the basis of the occurrence of genetic lesions, genomic stability, and histopathology.

Genes involved	Molecular defects	Histopathology/molecular characteristics
APC	Point mutation, aneuploidy, polyploidy, LOH,	**Well differentiated tumors/MSS and CIN phenotype**
β-*catein*	Activation of Wnt signaling pathway due to accumulated nuclear	Familial and sporadic CRC
p53	β-*catein*	Predominantly located in distal colon
KRAS	Deregulated TGFβ signaling,	No or low mucin production
SMAD4	Activation of PI3–PDK1 and RAF–MEK–ERK pathways	Low tumor-lymphocyte reactivity
TGFBR	Disruption of cell cycle regulation promoting cell survival and	
PIK3CA	reduced apoptosis	
C-MYC		
MLH1,2,6	DNA single nucleotide mismatch repair defects	**Poor to moderately differentiated tumors/MSI phenotype**
PMS2	Alteration to micro-satellite repeat lengths	Familial and sporadic CRC
MSH3,	Accumulation of oncogenic mutations and tumor suppressor lose	Predominantly located in distal colon
TGF-BRII	Deregulated TGFβ signaling	Mucinous Phenotype
		Tumor-lymphocyte reactivity
		Commonly located in right colon
		Less aggressive/better prognosis
BRAF	BRAF activating point mutations	**Serrated, poor to moderately differentiated tumors/CIMP phenotype**
MLH1	Activation of RAF–MEK–ERK pathway	Sporadic CRC
	Methylation of MLH1 and loss of MLH1 expression that is	Defective mismatch repair
	associated with mismatch repair defects	Commonly located in right colon
		Poor prognosis

Detailed description of the characteristics used for these groupings can be found within the text and references therein.

In addition to providing a mechanism for tumor dissemination, recent studies have identified a further pathological manifestation of EMT – endowing cancer cells with stem-like potential (32, 33) that appears critical for tumor initiation, metastasis, and relapse in CRC (6, 34, 35). The coexistence of mesenchymal and stem-like traits in cancer cells that have undergone EMT has led to the idea

that they constitute "migrating CSC" from which metastases are derived (21, 36). Such cells acquire the capacity to both disseminate and successfully colonize new sites, where they are thought to redifferentiate via an MET and regain the organization of cells present in the primary tumor. This model thus provides a mechanism to explain the observation that CRC metastases often retain a similar degree of differentiation as the primary tumor.

Induction of EMT requires extensive reprograming of gene expression in response to activation of various signaling pathways. Among the best studied are the Wnt, MAPK, TGFβ, and NFκB pathways, which converge on one or more transcription factors (TFs) driving EMT in the embryo, including members of the zinc finger (SNAIL1, SNAIL2/SLUG, ZEB1, ZEB2/SIP1), bHLH (TWIST1, TWIST2), forkhead (FOXC2), or homeobox (Goosecoid, SIX1, PRRX1, PREP1) families (37–39). In CRC, multiple TFs were reported as being aberrantly expressed based on immunohistochemical and transcriptome studies, including ZEB1, ZEB2/SIP1, SNAIL1, SNAIL2/SLUG TWIST, and FOXC2 (21, 40–48). Although these TFs typically function as repressors of epithelial genes, and/or genes required for cell cycle progression, they also activate transcription in some contexts, including that of stemness-promoting genes and cell cycle inhibitors (21, 49, 50).

The effects of EMT-driven TF activation can be antagonized by several species of micro-RNA (miRNA) that in addition to repressing expression of TFs, are themselves repressed by these TFs. Such reciprocal inhibition creates self-enforcing double-negative feedback loops that dictate the epithelial–mesenchymal balance. Two such loops have been well documented to operate in colorectal and other cancer cells – ZEB/miR-200 and SNAIL/mir-34 loops (51–53). In addition to repressing EMT-TFs, the miRNAs also directly target other genes involved in regulating EMT (e.g., cytoskeletal components, Wnt pathway components) and stemness (e.g., BMI1, KLF4, SOX2), underscoring their critical functions in regulating cellular plasticity during cancer progression (26, 51, 54–57). Notably, both miR-200 family members and miR-34 are induced by the tumor suppressor p53 (58–60), whose induction of miR-34 expression was found to reduce levels of several Wnt pathway components, including LEF-1, β-catenin, WNT1, WNT3, LPR6, and AXIN2 (60–62). Reduction in Axin2 via this mechanism was also reported to promote nuclear accumulation of GSK3β, where it can phosphorylate to destabilize SNAIL1 (63).

ASSOCIATION OF EMT WITH CRC PATHOLOGY

The majority of CRCs appear moderately differentiated, with smaller subsets being well or poorly differentiated. The latter cancers are characterized by highly irregular glandular structure, aggressiveness, poor prognosis, and resistance to treatment. However, moderately differentiated tumors can also contain regions of poor differentiation, typically observed at the invasive front (21, 27, 36). Often, these cancers exhibit budding phenotype, in which individual or clusters of tumor cells detach from the tumor mass and invade into the adjacent stroma. This feature is adversely prognostic and linked with enhanced probability of metastasis to the lymph nodes, liver, or lung (36, 64, 65).

Budding tumor cells are thought to have undergone an EMT-like event, losing expression of epithelial differentiation markers while gaining the capacity to express mesenchymal and stemness markers (36, 66). In contrast to central regions of the tumor, budding cells at the invasive front also typically strongly express nuclear β-catenin, which is critical for induction of EMT programs characterized by expression of ZEB1 (42) and altered basement membrane components (67). This intra-tumoral heterogeneity in β-catenin expression is likely to arise from a range of factors, including micro-environmental signals, altered cell–cell and cell–matrix adhesion, and through cross-talk with other signaling pathways such as the ERK module (27, 36, 68, 69).

While EMT–MET events provide a framework for how differentiated CRCs may metastasize, a different model was proposed by Brabletz to account for progression of poorly differentiated cancers (21). Rather than exhibiting high plasticity, these tumors retain a poorly differentiated mesenchymal phenotype that is driven primarily by mutational events. Such cancers may have arisen prior to differentiation of stem or progenitor cells in the crypt, or from cells that have evolved from differentiated tumors but selected for mutations that render them in a stable mesenchymal-like state. A further mechanism through which selection may occur is as a result of therapies, where the relapsing tumors often displaying a mesenchymal, stem-like phenotype (21). Finally, it was suggested that the highly aggressive nature of poorly differentiated tumors may result form their propensity to metastasize through "parallel progression" routes (70), in which tumors and metastasis develop and progress concurrently.

ASSOCIATION OF EMT WITH CRC SUBTYPES

An important question is whether models of tumor cell plasticity involving EMT–MET events and CSC can be incorporated into current approaches for CRC subtyping. Collectively, this approach may help better define the heterogeneity observed in CRC and progress the development of targeted therapies.

CIN, MSS/MSI, CIMP SUBTYPING

Conventional approaches to classify colorectal tumors have centered primarily on molecular [chromosomal instability (CIN); micro-satellite stability/instability (MSS/MSI); CpG island methylator phenotype (CIMP)], and pathological (TNM grade, degree of differentiation, immunohistological markers) characteristics of the tumor (9, 71) (**Table 1**). These classifications recognize the various forms of global genomic and epigenetic alterations that occur during tumorigenesis (**Table 1**). CIN is the most common form of genomic instability in CRC that underlies the sequential deregulation of classical tumor suppressor and oncogenes including *APC*, *KRAS*, and *TP53*. In the MSI classification, genomic instability arises from the mutation or methylation-mediated silencing of genes required for DNA mismatch repair (hMLH1, hMSH2, hMSH6, and hPMS2) and based on the level of MSI, CRCs can be classified as MSI-high (MSI-H), MSI-low, or MSS. MSI tumors have a lower frequency of mutations in *KRAS* and *TP53* compared to MSS cancers, and a higher frequency of mutations in genes harboring repetitive elements in their coding sequence such as *TGFBR2* (72). Recent work indicates that as a result of this loss of TGFβRII function, MSI tumor cells lines fail to undergo EMT in response to TGFβ, which may contribute to their better prognosis (73). In the CIMP classification, tumors harbor aberrant DNA methylation patterns that result in the global epigenetic silencing

of genes. Each of these pathways serves as an important classifier of disease progression and response to therapy (**Table 1**).

INTRINSIC EMT-ASSOCIATED CRC SUBTYPES

While the CIN, MSI, and CIMP are important disease subclassifiers, it is now well-established that tumors defined by these groupings can be additionally stratified into molecularly defined subtypes. Over the past decade, genomic and expression analyses involving large patient cohorts have provided insight into the diversity within CRC. Combined with existing mutational, clinical, and pathological classifiers, these studies have identified several distinct molecularly defined CRC subtypes (e.g., stem-like, mesenchymal, immune, and epithelial/differentiated), each driven by unique and/or overlapping biological pathways and exhibiting differing prognostic and/or therapeutic response (11, 13, 15–17, 41, 74, 75) (**Figure 2**). A unifying feature from each of these studies was the identification of a CRC subtype significantly enriched for genes associated with a poorly differentiated, mesenchymal/invasive phenotype, and that were often co-enriched with genes indicative of a stem-like state (**Figure 2**).

Loboda et al. (41) defined two subsets, epithelial and mesenchymal, where the latter was linked to TGFβ signaling and low expression levels of anti-EMT miRNAs. Examination of the heterogeneity within CRC gene expression profiles also revealed a strong association between EMT gene signatures and subtyping (13). Marisa et al. (17) identified six molecular subtypes (C1–C6) from stage I–IV CRC patients, with two subtypes (C4 and C6) showing a distinct down-regulation of proliferative and upregulation of EMT/motility pathways. Subtype C4 was also characterized by a stem cell-like phenotype. Furthermore, both subtypes were distinct with regard to harboring a serrated tumor signature. Roepman et al. (74) identified three subtypes (A–C) within stages II and III CRC, with C-type tumors featuring an EMT phenotype and low proliferative activity. Two additional studies (15, 16) examined large patient-derived CRC gene expression datasets and defined CRC subtypes characterized by a mesenchymal gene signature. In the study by Sadanandam et al. (16), six subtypes were described on the basis of gene expression signatures associated with their cell of origin within the colon crypt. In this context, a stem cell subgroup was associated with expression of mesenchymal genes. De Sousa et al. (15) described three CRC subtypes (CCS1–3) and in the CCS3 grouping EMT and genes involved with migration, invasion, and TGFβ signaling were elevated. Subsequent analysis suggests that the EMT subgroups identified in both studies show strong overlap (76). Importantly, several of the above studies demonstrated that EMT signature defined tumors consistently display a worse prognosis and were least sensitive to conventional chemotherapy regimes. Thus, a mesenchymal/invasive poor

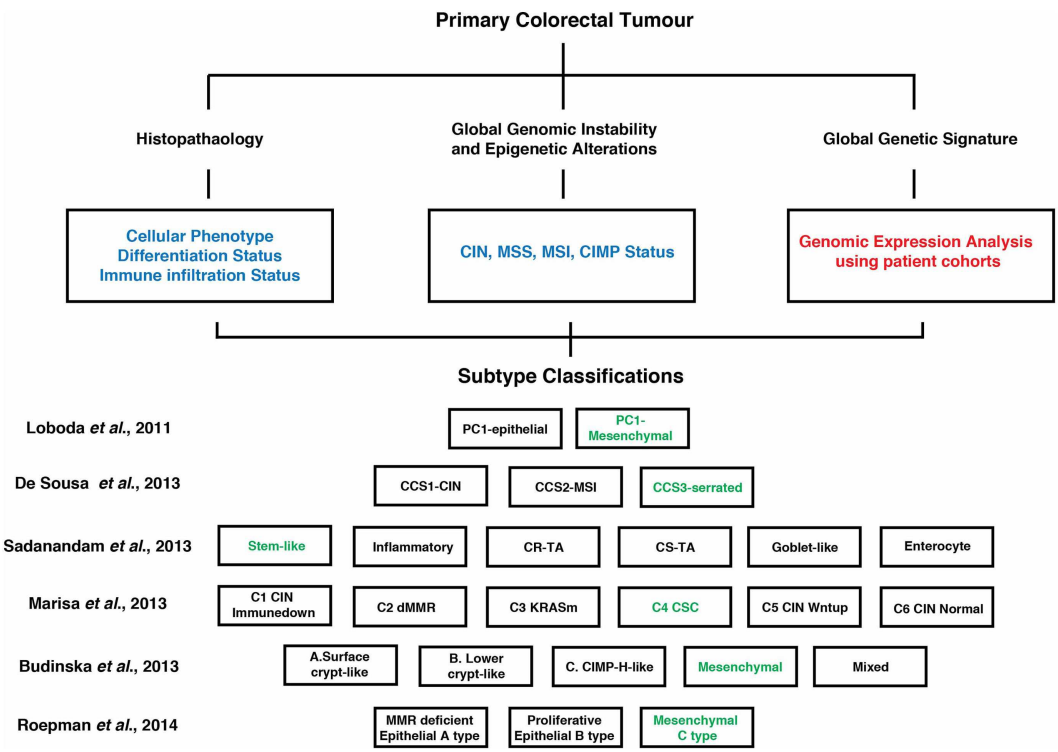

FIGURE 2 | Overview of suggested contemporary subtype classification of CRC. Genomic and expression analyses involving large patient cohorts (highlighted in red) combined with existing mutational, clinical, and pathological classifiers (highlighted in blue) have identified several distinct molecularly defined CRC subtypes as indicated by the various studies. Each of these subtypes is driven by unique and/or overlapping signaling pathways (see **Figure 1**) and exhibit different prognostic and therapeutic responses. A unifying feature is a CRC subtype enriched for genes associated with a poorly differentiated, mesenchymal/invasive phenotype, and often co-enriched for genes indicative of a stem-like state (highlighted in green). A more detailed description of these subtypes and their clinical/therapeutic response can be found within the text (13, 15–17, 41, 74).

differentiation signature is a defining feature of CRC subtyping and clinical response.

An important issue to emerge from the above publications is the extent to which activation of mesenchymal and stem-like programs are linked in CRC subtypes. Consistent with the role that Wnt signaling plays in regulating the fate of stem cells at the base of the crypt (8), Sadanandam et al. (16) found elevated activation of this pathway in stem-like tumors and cell lines, which co-expressed markers of intestinal and colorectal stem cells and EMT genes (34). However, whether Wnt signaling alone is sufficient to drive stem-like/mesenchymal programs expression requires further clarification as Zhu et al. (75) suggest that the pathway is not only active in mesenchymal-type tumors but also in those exhibiting differentiated or proliferative expression signatures. Instead, they found that the context of Wnt activation differed between these cancers, with migratory/EMT subsets also enriched for VEGF signaling, whereas Wnt and Notch were active in differentiated/epithelial-type tumors. Only the proliferative group (enriched for genes involved in early colon development) showed Wnt activation alone. The notion that VEGF signaling may be important for activating EMT/migration programs in the context of Wnt signaling is also supported by the finding that genes associated with sprouting angiogenesis, a process regulated by the VEGF pathway were co-enriched in mesenchymal-type tumors identified by Marisa et al. (17).

A second pathway that appears to be critical for activation of EMT programs in mesenchymal tumors is the TGFβ pathway (77, 78). Transcriptional outputs of this pathway were significantly enriched in several studies and associated with the mesenchymal phenotype (15, 17, 41, 74). Interestingly, in one study (15), TGFβ and EMT programs appeared to be active in the absence of Wnt transcriptional signatures or activation of stem cell programs. One implication of this observation is that Wnt signaling is required for stemness programs but not necessarily required for EMT in poorly differentiated cancers. Interestingly, the CCS3 group (15) enriched for sessile-serrated adenoma (SSA) tumors comprised both differentiated and poorly differentiated tumors, suggesting that further stratification based on differentiation status may be possible.

SESSILE-SERRATED ADENOMA PATHWAY

A distinct feature of CRC that has emerged from recent studies is that groups harboring an EMT gene expression signature may display a pathology related to serrated adenoma (13, 15, 17, 76). As such, the CRC subtype displaying a serrated pathology provides an important context to examine the role of EMT events in driving CRC progression.

In the classical adenoma-carcinoma sequence, tumors are often located in the distal colon or rectum and genetically are defined by CIN. In contrast, the serrated adenoma represents an alternative pathway to tumorigenesis. Typically, the serrated adenoma is located in the proximal or right colon and is characterized by the sawtooth appearance of the crypt epithelium (79). Traditionally viewed to have limited potential to progress to a neoplastic lesion, it is now established that precursor "serrated polyp" can be subdivided into hyperplastic polyp (HP), SSA, and traditional serrated adenoma (TSA) with both the SSA and TSA having significant potential to develop into neoplastic lesions (80, 81).

It has been suggested that up to 30–35% of CRCs evolve through a serrated pathway (82–84). In addition to their distinct morphology, serrated CRCs are also distinct in the genetic background that drives their development. For example, serrated colon tumors predominately display mutations in *BRAF* and *KRAS* rather than APC. With respect to the MSI and CIN classification, serrated tumors usually lack CIN but are often MSI-H and CIMP-H (71, 85, 86). Thus, serrated tumors have been classified in three subtypes: CIMP-low/MSS/MSI-low/KRAS mutant; CIMP-H/MSI-H/BRAF mutant; CIMP-low/MSS/MSI-low/BRAF mutant (9, 87). In the context of EMT-driven cellular plasticity, it is important to note that clinically CIMP-low/MSS/MSI-low/BRAF mutant tumors confer a poor prognosis and display high tumor budding. This observation is consistent with the increased EMT potential associated with wild-type TGFβRII and active TGFβ signaling and MSI-low status. In contrast CIMP-H/MSI-H/BRAF mutant tumors have a more favorable prognosis (86, 88, 89). Here, EMT potential is reduced due to the increased incidence of mutated TGFβRII (72, 73).

CLINICAL IMPLICATIONS AND CONCLUDING COMMENTS

The CRC classifications outlined above may provide new opportunities for the more targeted therapeutic/clinical management of CRC disease progression. This possibility is illustrated in the studies by Sadanandam et al. (16), De Sousa et al. (15), and Roepman et al. (74). Each of these studies revealed subtype-specific responses to therapy that could potentially contribute to more effective manage of disease. In case of the study by De Sousa et al., the CCS3-serrated subtype was reported to be resistant to cetuximab therapy, suggesting that new targeted therapies would be required for this subtype (15). The identification of CCS3 specific elevated TGFβ signaling suggested that this pathway may be an avenue for targeted therapy (15). The six CRC subtypes identified in the study by Sadanandam et al. (16) also displayed subtype-specific responses to therapy. Here, three subtypes, CR-TA, CS-TA, and Goblet were suggested to not respond to FOLFIRI chemotherapy treatment and patients with this form of disease may better spared this therapy in the context of local disease. However, in the context of metastatic disease, the CR-TA and CS-TA subtypes were suggested to respond to cetuximab therapy (16). In contrast, stem cell-like-subtypes and inflammatory subtypes may respond best to FOLFIRI treatment. The specific treatment of a stem cell-like subtype is an important consideration given that such populations of cells are key drivers of the moderately differentiated phenotype that are seen in most CRCs and which due to their stem-like behavior (e.g., low proliferative index) have thus far proved highly resilient to current therapies. Collectively, these studies strongly support the idea that distinct, clinically relevant CRC subtypes can be used as a guide for subtype-specific therapy.

Tumor heterogeneity has posed a major obstacle for the successful treatment of metastatic forms of CRC and several other common cancers. The studies highlighted here have provided a substantial insight into CRC heterogeneity. The identification of various degrees of epithelial–mesenchymal plasticity, acting in concert with clonal evolution and the concept of CSC, have helped

dissect the heterogeneity underlying CRC and resulted in a more detailed classification of CRC into distinct molecularly defined subtypes. These classifications will provide new opportunities for understanding CRC and the key oncogenic pathways and mechanisms required for disease progression. This new information may also be invaluable for re-focusing basic and translational/preclinical studies on identifying and targeting key pathways required for the malignant growth of the most aggressive subtypes.

AUTHOR CONTRIBUTIONS

Lloyd Pereira and Amardeep Singh Dhillon conceived and drafted the manuscript. John M. Mariadason and Ross D. Hannan provided critical intellectual input and assisted with revision of the text. All authors approved the final version to be published and agree to be accountable for all aspects of the work.

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Phenotype switching in melanoma: implications for progression and therapy

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Epithelial–mesenchymal transition (EMT) is a key process associated with the progression of epithelial cancers to metastatic disease. In melanoma, a similar process of phenotype switching has been reported and EMT-related genes have been implicated in promotion to a metastatic state. This review examines recent research on the role of signaling pathways and transcription factors regulating EMT-like processes in melanoma and their association with response to therapy in patients, especially response to BRAF inhibition, which is initially effective but limited by development of resistance and subsequent progression. We highlight studies implicating specific roles of various receptor tyrosine kinases (RTKs) in advancing melanoma progression by conferring a proliferative advantage and through promoting invasive phenotypes and metastasis. We also review the current knowledge of the mechanisms underlying resistance to BRAF inhibition and the potential role of melanoma phenotype switching in this process. In particular, we discuss how these important new insights may significantly enhance our ability to predict patterns of melanoma progression during treatment, and may facilitate rational development of combination therapies in the future.

Keywords: melanoma, phenotype switching, EMT, metastasis, RTK signaling, BRAF inhibition, resistance

INTRODUCTION

Malignant melanoma accounts for 75% of deaths from all skin cancers in the U.S (1). Women have higher survival than men (2) and the Caucasian population has a 10-fold greater risk than ethnic groups with deeply pigmented skin (3). The 5-year survival rate is over 90% for localized melanoma but drops to 16% for distant-stage disease (1), indicating that metastasis is the main reason for poor outcome. The classic Clark model depicts stepwise transformation of melanocytes to malignant melanoma and subsequent development of invasion and metastasis (4), involving tightly regulated switching of cellular phenotypes. This phenotype switch bears resemblance to the epithelial–mesenchymal transition (EMT), a well-characterized process of phenotypic change that is associated with metastatic progression in epithelial cancers. This mini-review will focus mainly on the signaling and molecular events that lead to the invasive and metastatic phenotypes of melanoma, and discuss the implications of phenotype switching on the response to treatment.

Abbreviations: EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; EMT-TF, epithelial–mesenchymal transition-transcription factors; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; KIT, stem cell factor receptor; MAPK, mitogen-activated protein kinases; MET, hepatocyte growth factor receptor; MITF, microphthalmia-associated transcription factor; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ROR, receptor tyrosine kinase-like orphan receptor; RTK, receptor tyrosine kinases; STAT, signal transducer and activator of transcription; TGF, transforming growth factor.

CHARACTERISTICS OF EMT IN EPITHELIAL CANCERS

Epithelial–mesenchymal transition has been suggested to play an important role in conferring metastatic properties in many solid tumors by altering the integrity of cell–cell junctions, promoting loss of polarity and epithelial markers, eventually resulting in loss of contact between neighboring cells. Through this process, tumor cells become more mesenchymal-like, exhibiting higher migratory and invasive properties that allow them to interact with the extracellular matrix and invade surrounding tissues (5). It is generally accepted that the EMT process involves changes in expression of epithelial and mesenchymal markers. The loss of E-cadherin is a characteristic feature during EMT, which is detected in the cells located at the invasive front of many solid tumors (6, 7). The expression of E-cadherin is tightly regulated by multiple transcription factors that bind to and repress the activity of the E-cadherin promoter (8, 9). The first characterized direct repressor of E-cadherin was the zinc-finger transcription factor Snail1 (10, 11), which initiated intense efforts to understand the molecular mechanisms of EMT and subsequently led to the discovery of the E-cadherin repressors SNAI2 (also known as SLUG) (12), ZEB1 and ZEB2 (13, 14). Other repressors of E-cadherin include E47 (TCF3), TCF4 (15), and Twist (16), which participate in both

developmental EMT and tumor progression. Beta-catenin/TCF4 binds directly to the ZEB1 promoter and activates its transcription, conferring invasiveness in colorectal cancer (17).

A common signaling mechanism that induces EMT in a range of cancers is activation of the MAPK/ERK pathway, which can activate SNAI1 to repress E-cadherin expression and the epithelial phenotype (18). In addition, EGF signaling can induce TWIST through a JAK/STAT3 pathway in epithelial cancer cell lines and the EGF–STAT3-positive correlation has been confirmed in primary breast carcinomas (19). Receptor tyrosine kinases (RTK) signaling activated through FGF, HGF, IGF, and other ligands, as well as the serine/threonine kinase TGF-β superfamily, can also initiate EMT and metastasis, through various mechanisms converging on the induction of E-cadherin repressors (20, 21).

EVIDENCE OF EMT-LIKE PHENOTYPE SWITCHING IN MELANOCYTES AND MELANOMA

Epithelial–mesenchymal transition is a critical step for embryonic morphogenesis and a similar process is particularly important for melanocyte lineage differentiation. It involves restructuring of the cytoskeleton, cell membrane, and cell–cell junctions. This developmental plasticity allows melanocytes to emerge from the pluripotent neural crest cells (22). Phenotype switching with similarities to the EMT program operates during development and has a recognized role in acquisition of metastatic properties in the vertical growth phase of melanoma (23). A comparison of the features of primary cutaneous melanomas from the patients who develop metastasis to those who do not, revealed differences in the expression of the epithelial and mesenchymal phenotype markers (24). By gene expression profiling, loss of E-cadherin with gain of N-cadherin and osteonectin (SPARC) was significantly associated with development of metastasis (24). Further evidence comes from the finding that both proliferative and invasive cells are present within heterogeneous metastatic tumors, and the observation of switching between the two phenotypes during melanoma progression *in vivo* (25).

INDUCERS OF EMT-LIKE PHENOTYPE SWITCHING IN MELANOMA

Recently, the concept of an EMT spectrum has been introduced to describe a progressive transition characterized by an intermediate mesenchymal status and fluctuating expression of EMT markers, as reported in carcinomas of the breast, colon, and ovary (26). Given the intermediate mesenchymal nature of melanoma, fluctuating expression of EMT inducers are observed. Therefore, the literature about phenotype switching in melanoma and about EMT in many epithelial cancers is not always consistent.

The role of EMT transcription factors (EMT-TFs) in melanoma phenotype switching and plasticity has recently been reviewed (27). Induction of ZEB1 and SNAIL family members as discussed by Vandamme and Berx, as well as repression of E-cadherin is observed during melanoma progression. The traditional paradigm in epithelial cancers is that the EMT-TFs SNAIL1/2, ZEB1/2, and TWISTS act as repressors of E-cadherin, thereby inducing EMT (9). However, unlike epithelial cancers, in melanoma ZEB1 and ZEB2 are reported to be differentially expressed in alternate phenotypic states (28). Normal epidermal melanocytes from a melanoma patient expressed low ZEB1 and high ZEB2 expression, whereas the melanoma cells at deep sites from the same patient had high ZEB1 and low ZEB2 levels (28). Analysis of a large patient series by immunohistochemistry revealed high expression of ZEB1 and TWIST1, with low expression of ZEB2 corresponded with significantly reduced metastasis-free survival (28). Another recent study analyzing a large cohort of patient samples also confirmed that low expression of ZEB2 corresponded to significantly reduced melanoma recurrence-free survival (29). The study also demonstrated that loss of ZEB2 in melanocytes resulted in dedifferentiation, and in melanoma cells resulted in increased ZEB1expression, repressing E-Cadherin, and contributing to progression and metastasis (29). These studies suggest that ZEB2 could function as a differentiation factor, through maintaining E-Cadherin expression (29). Both studies also reported that the melanoma differentiation marker microphthalmia-associated transcription factor (MITF) was regulated by the switch in ZEB expression. Down-regulation of MITF could lead to an invasive phenotype, consistent with the previous literature on the role of MITF in phenotype switching (25, 27). Gene expression profiling comparing non-metastatic and metastatic patient samples, previously revealed that loss of E-cadherin/gain of N-cadherin was a major determinant of melanoma metastasis (24). The relevance of this cadherin switch was established in early studies on prostate and melanocytic cancers (30, 31), whereas SPARC was found later to drive activation and sustain expression of SLUG to promote melanoma cell invasion (32). SLUG was also identified in melanoma cell lines as a direct transcriptional activator of ZEB1, resulting in repression of E-cadherin (33). Interestingly in contrast, switching to a proliferative state was reported to occur in aggressive uveal melanoma with up-regulation of E-cadherin. However, the study revealed that this phenomenon was caused by the loss of an E-Cadherin suppressor called Id2, and as a result of down-regulation of Id2 there was increased anchorage-independent growth of the cells (34). These studies suggest that the interchange between epithelial-like and mesenchymal-like phenotypes is context dependent in different types of melanoma, but the ability to switch phenotype in various types of melanoma has been implicated in conferring a higher risk of death due to metastasis. The dynamic switch back and forth between proliferative and invasive states is the model that is biologically reflective of melanoma progression (35).

Phenotype switching in melanoma can be initiated by mechanisms other than those characterized in EMT. In epithelial cancer cell lines, increased LEF1 transcription activity by stable nuclear beta-catenin expression can induce EMT, which is reversible by removal of LEF1 (36). In melanoma, the beta-catenin interacting factors LEF1 and TCF4 are both expressed in a phenotype-specific manner and their expression is inversely correlated. Loss of LEF1 and gain of TCF4 expression is associated with tumor progression involving a change from proliferative to an invasive phenotype (37). The beta-catenin/LEF1 complex is regulated by Wnt signaling and activates a melanocyte-specific gene encoding MITF (38). MITF is a master regulator of melanocyte development and has been reported to be critical for melanoma progression (39, 40). MITF can control melanoma cell differentiation and proliferation through cell cycle arrest (41, 42). It also regulates

diaphanous-related formin Dia1, which promotes actin polymerization and coordinates cytoskeletal networks at the cell periphery resulting in morphological changes (43). Expression of MITF has been used as a benchmark to distinguish melanoma cells in the proliferative or invasive state (25). In addition, Wnt activation, rather than acting via the classical Wnt pathway, can signal through the Protein Kinase C pathway to mediate an EMT-like phenotype switch and melanoma migration (44). These studies, as summarized in **Table 1**, indicate that EMT-like phenotype switching can be induced at both transcriptional level and through well-defined canonical signaling pathways.

SIGNALING PATHWAYS INVOLVED IN MELANOMA PHENOTYPE SWITCHING

Wnt and Notch signaling have well-characterized roles in developmental programs of neural crest cells (51, 52). These embryonic signaling pathways are also implicated in tumorigenic functions of melanoma cells (53). Notably, melanoma have a high frequency of activating mutations within the MAPK pathway, as over 50% metastatic melanomas are driven by the oncogenic $BRAF^{V600E}$ mutation (54) and over 15% by the $NRAS^{Q61R}$ mutation (55). The MAPK and the PI3K signaling pathways are known to activate NF-kB, which further induces Snail to mediate a mesenchymal phenotype in epithelial cells (56), but similar evidence for the NF-kB/Snail mechanism in melanoma is lacking (57), although Snail is a demonstrated inducer of the mesenchymal-like phenotype in melanoma (58). However, this study may suggest that RTKs could be a means of mediating NF-kB/Snail activation given that they activate the MAPK and PI3K signaling pathways.

Additionally, there is abundant evidence that RTK signaling can induce migratory, invasive, and metastatic properties in melanoma cells. Knockdown of EGF in EGF over-expressing melanoma cells results in reduced lymph node metastasis, which is considered a key initial step of melanoma progression (59). FGF2 is a growth factor produced by melanoma cells but not by normal melanocytes, that activates the FGFR1 receptor. FGF2 promotes melanoma cell migration via down-regulation of focal adhesion kinase (FAK) and subsequent loss of cellular adhesion (48). As previously discussed, a cadherin switch is an important marker of EMT-like phenotype switching in melanoma. By studying exogenously introduced HGF ligand-induced activation of its receptor MET and pharmacological inhibition of downstream MAPK and AKT signaling, HGF signaling was shown to mediate the cadherin switch through up-regulation of Snail and Twist (60). Additionally, HGF signaling can also induce fibronectin matrix synthesis, which promotes malignant transformation and migration of melanoma cells (49). IGF-1 can also induce migration, through increased production of IL-8 by melanoma cells (61). In patients with uveal melanoma, a significant correlation was found between high expression of IGF-1 receptor and liver metastasis (50).

TGF-β is the most extensively studied inducer of EMT, with established roles in regulating extracellular matrix remodeling and in influencing cell phenotype (62, 63). Moreover, TGF-β can signal through SMAD3 and activate SNAI2/SLUG in a Rho-pathway dependent manner (64). Enhanced TGF-β signaling is implicated in mediating resistance to the inhibition of a range of oncogenic signaling targets. Loss of MED12, a repressor of TGF–βR2 signaling, not only confers a mesenchymal phenotype, but also results in resistance to inhibitors of ALK, EGFR, and BRAF in multiple cancers including melanoma (65).

IMPLICATIONS OF PHENOTYPE SWITCHING ON RESPONSES TO THERAPIES

Uncontrolled proliferation is a cancer hallmark, a result of activation and crosstalk of many signaling pathways. Advances in genomic sequencing technology have enabled the successful identification of the key oncogenic events in melanoma, including identification of the $BRAF^{V600E}$ mutation (54). Subsequent developments of highly selective and efficacious therapies such as vemurafenib and dabrafenib that target mutant BRAF have achieved remarkable responses in patients (66–68). However, ongoing clinical studies have revealed that the therapeutic benefits are often

Table 1 | Inducers of phenotype switching in melanoma.

Phenotype switching inducers	Outcome	Study model[a]	Type of melanoma	Reference
↓ZEB2	↓Metastasis-free survival	Patient, *in vivo* and *in vitro*	multiple	(29)
↑ZEB1&TWIST/↓ZEB2&SLUG	↓Metastasis-free survival	Patient and *in vitro*	multiple	(28)
↑MITF	Differentiation	*In vitro* and *in vivo*	Cutaneous	(45)
EGF/STAT3	Growth and Metastasis	*In vitro* and *in vivo*	Cutaneous	(75)
WNT5A/↑ROR2	Invasion	*In vitro* and *in vivo*	Cutaneous	(46)
MET/Exosome	Metastasis	Patient and *in vivo*	Cutaneous	(47)
↑TCF4/↓LEF1	Invasion	*In vitro*	Cutaneous	(37)
↓MITF	Invasion	*In vivo*	Cutaneous	(25)
WNT5A/PKC	Migration	*In vitro*	Cutaneous	(44)
FGF2/↓FAK	Migration	*In vitro*	Cutaneous	(48)
↓E-Cad/↑N-Cad	Metastasis	Patient	Cutaneous	(24)
↑E-Cad	Invasion	*In vivo*	Uveal	(34)
HGF/Fibronectin	Migration	*In vitro*	Cutaneous	(49)
IGF-1	Migration	Patient and *in vitro*	Uveal	(50)

[a] *In vitro indicates melanoma cell lines in 2D culture, in vivo indicates xenograft models or mouse models and Patient indicates patient samples.*

short-lived with the majority of patients developing resistance and disease progression (66). There are several reports on the mechanisms of resistance to BRAF directed agents as reviewed by Sullivan and Flaherty (69). Besides the intrinsically resistant clones, some of the surviving drug-sensitive melanoma cells are able to adapt to BRAF inhibition. Studies have revealed that the adaptation can involve various phenotype changes including EMT-like processes, altered glycolytic activity (70) and ER stress response-activated cytoprotective autophagy (71). Hypoxia induced switching of the expression of ROR1 and ROR2 through non-canonical WNT5A signaling, resulting in an invasive phenotype of melanoma with reduced sensitivity to BRAF inhibitors (46). Concurrent inhibition of BRAF and glycolysis or autophagy was demonstrated as good methods to induce cell death or tumor regression, respectively, in BRAFi-resistant melanoma (71, 72). However, to target phenotypic-switching through therapeutic intervention remains difficult. Thus, the remainder of this mini-review will emphasize the involvement of phenotype switching in the context of emerging and recently developed therapies.

Using $BRAF^{V600E}$ melanoma lines and BRAF inhibitors, Caramel et al., demonstrated that the ZEB switch described above, can be initiated and sustained by MAPK/ERK signaling through FRA-1, an ERK-regulated component of the AP-1 complex. Accordingly, the expression patterns of ZEB1/2 and TWIST were reversed by pharmacological inhibition of BRAF/ERK signaling (28). Together with the TGF-β/MED12 study that showed changes of expression of phenotype markers concomitant with development of drug resistance (65), these recent discoveries support the emerging understanding that the mechanisms of phenotype switching in melanoma may have broader implications with respect to therapeutic responses in patients.

An important question raised by all the studies described above is whether EMT-like phenotype switching has any value as a therapeutic "target" in the treatment of melanoma. To date, three major strategies have been proposed to address this important question. Considering the aggressiveness of melanoma, the first suggested approach is to directly reduce invasive potential. Compounds such as the potent green tea catechin, Epigallocatechin gallate (EGCG), have been demonstrated to have inhibitory effects on migration and invasion in the BRAF-mutant cell line A375, with a reversal of EMT-like phenotypic changes orchestrated by induction of E-cadherin and suppression of N-cadherin (73). A second reported strategy is to use phenotype switching as a method to induce changes in melanoma to a specific phenotype that reveals a "drug-targetable" state. As previously discussed, high expression of MITF usually associates with a proliferative phenotype in melanoma. The chemotherapeutic agent methotrexate (MTX) causes an increase in MITF and its direct target TYR (tyrosinase) that inhibit invasiveness in melanoma. This can provide an avenue for treatment with a tyrosinase-processed antifolate pro-drug that was shown to mediate apoptosis selectively in the MTX-treated cells with high expression of MITF and tyrosinase (45). The third reported strategy is based on the success of the approved and emerging therapies targeting the BRAF/MAPK signaling in melanoma. Phenotype switching, cell migration, and invasion occur instead of, or concomitantly with,

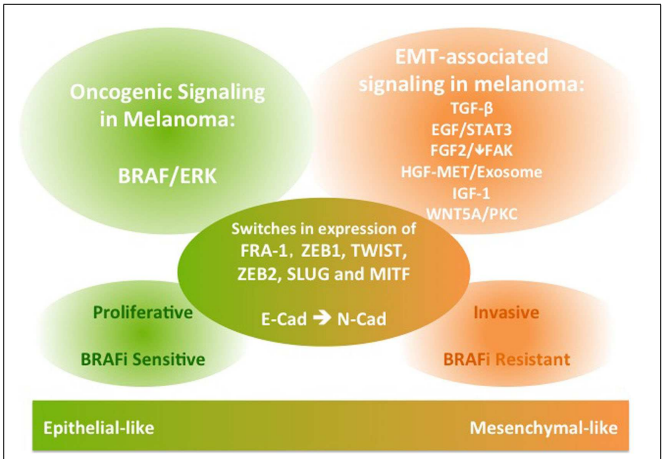

FIGURE 1 | A schematic diagram of the signaling and molecular features of melanoma phenotype switching. The EMT-like phenotype switching confers melanoma invasive functions. The EMT-associated signaling in melanoma is also implicated in conferring resistance to BRAF inhibition therapies in BRAF-mutant metastatic melanoma.

the development of drug resistance (65). Thus, the rationale involves inhibition of phenotype switching and cell migration in conjunction with a therapy such as vemurafenib that targets the oncogenic BRAF signaling that leads to growth arrest or/and cell death. Studies reveal that combination of inhibitors of TGFβR2 with vemurafenib overcomes the TGFβ-mediated resistance to vemurafenib (65). Chronic inhibition of BRAF was also found to result in elevated Wnt signaling and increased expression of the EMT inducer, WNT5A, and knockdown of WNT5A reversed resistance caused by chronic treatment with vemurafenib (74).

Given that signaling by various RTKs can mediate phenotype switching and promote migration through mechanisms distinct from those enhancing BRAF/MAPK-dependent proliferation and regulation of EMT-TFs, co-targeting of selected RTK signaling pathways and oncogenic BRAF appears to be a logical combination. For example, EGF signaling confers resistance to BRAF inhibition and induces melanoma invasion through Src pathways. Inhibition of the EGF receptor and Src re-sensitizes treatment-resistant BRAF-mutant melanoma cells to Vemurafenib and blocks their invasiveness (75). HGF secreted by stromal cells in the tumor microenvironment can activate the HGF receptor MET, initiating MAPK and PI3K signaling to confer resistance to BRAF inhibition. Consistently, dual inhibition of either HGF or MET was found to forestall the resistance (76). This may be of particular importance because melanoma-derived exosomes were able to confer metastatic properties and a pro-vasculogenic phenotype on bone marrow progenitors through MET (47). Exosomes are important export machinery that maintains normal compartmentalization of molecules. In a range of cancers including melanoma, exosomes derived from melanoma cells contain oncogenic drivers influencing EMT and metastasis (77). Interfering with regulators of exosome formation and MET expression can reduce metastasis (47).

CONCLUSION

The EMT process is crucial for normal development and for initiation of malignant transformation and metastasis in a wide range of epithelial cancers. It involves activation of various signaling pathways, as well as repression of E-cadherin through transcription factors. EMT-like phenotype switching is critical for melanocyte lineage differentiation and initiation of melanoma transformation and metastasis. While common EMT-TFs are implicated, their expression during the switch of melanoma to a mesenchymal-like invasive phenotype can differ from the role in classical EMT. In addition to TGFβ and WNT5A signaling, EGF, FGF, MET, and IGF signaling have established roles in conferring migratory and invasive functions in melanoma (**Figure 1**). Importantly, these EMT-associated signaling pathways also have roles in conferring resistance to inhibitors of BRAF/MEK, hindering therapeutic outcomes in patients with metastatic melanoma driven by BRAF mutations. Therefore, integrating insights from this body of literature may aid in the design of studies aiming to predict the patterns of melanoma progression during treatment with targeted therapeutics and may facilitate development of novel combination therapies.

AUTHOR CONTRIBUTIONS

FL wrote the mini-review. PF, AD, GM, and RA provided intellectual input and contributed to editing the manuscript.

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PERSPECTIVE ARTICLE
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Effects of epithelial to mesenchymal transition on T cell targeting of melanoma cells

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Melanoma cells can switch phenotype in a manner similar to epithelial to mesenchymal transition (EMT). In this perspective article, we address the effects of such phenotype switching on T cell targeting of tumor cells. During the EMT-like switch in phenotype, a concomitant change in expression of multiple tumor antigens occurs. Melanoma cells undergoing EMT escape from killing by T cells specific for antigens whose expression is downregulated by this process. We discuss melanoma antigens whose expression is influenced by EMT. We assess the effect of changes in the expressed tumor antigen repertoire on T-cell mediated tumor recognition and killing. In addition to escape from T cell immunity via changes in antigen expression, mesenchymal-like melanoma cells are generally more resistant to classical chemotherapy and radiotherapy. However, we demonstrate that when targeting antigens whose expression is unaltered during EMT, the capacity of T cells to kill melanoma cell lines *in vitro* is not influenced by their phenotype. When considering immune therapies such as cancer vaccination, these data suggest escape from T cell killing due to phenotype switching in melanoma could potentially be avoided by careful selection of target antigen.

Keywords: T-lymphocytes, melanoma, epithelial–mesenchymal transition, tumor antigens, T cell killing, cancer testis antigens

INTRODUCTION

The ability of melanoma cells to switch phenotype from proliferative to more invasive states, in a process similar to classical epithelial-mesenchymal transition (EMT), has been well described (1). Non-motile, polarized, and proliferative epithelial-like (E-like) cells, acquire motile, fibroblast-like mesenchymal characteristics (2). The process contributes to the heterogeneity of the tumor, and may be a factor in disease progression (3, 4). In melanoma, the EMT-like switch in phenotype can be driven by environmental pressures such as changes in the cytokine milieu at the tumor site, e.g., due to inflammation (5), and drug treatments such as inhibition of oncogenic BRAF (6).

We have previously generated a panel of 57 melanoma cell lines derived from patient samples (7). We have characterized these cell lines as E-like or mesenchymal-like (M-like) on the basis of expression levels of E-cadherin or N-cadherin, respectively (8). Between these two cohorts, we documented significant disparity in gene expression levels of many mRNA, reflective of the functional and phenotypic differences between the groups. Included in these differentially expressed genes, were mRNAs encoding melanocytic differentiation proteins, such as Melan-A and tyrosinase. Both of these have demonstrated antigenic epitopes, which have been previously used as targets of cancer vaccines in melanoma clinical trials (9–11), and are currently the target antigens in ongoing trials (NCT01331915, NCT01748747). Expression of these genes, as well as the master regulator of differentiation, MITF (Microphthalmia-associated transcription factor), was significantly downregulated in M-like cell lines compared to E-like (8). A decrease in expression of the melanoma differentiation antigens (MDA) under the control of MITF during EMT has also been reported by others, e.g., gp100.

The cancer testis group of antigens (CTAg) have been identified as important immune targets in melanoma, as well as other cancer types, and have been studied extensively (12, 13). Expression of CT genes is generally restricted to immune-privileged body sites, yet is frequently and selectively re-activated in a broad variety of human cancers (12). Almost 150 families have been defined (http://www.cta.lncc.br) (14). Their immunogenicity and selective expression in cancer makes them attractive targets for therapeutic cancer vaccines, and many have been used for this purpose over the past ~2 decades, with limited success (15). In parallel with EMT-associated downregulation of certain melanoma antigens, particularly the differentiation antigens, which are under the control of MITF, we have also observed upregulation in expression of a number of cancer testis antigens (CTAg). In particular, we showed that expression of the SPANX family of CTAg was enhanced in M-like compared to E-like cell lines (8). This group of CTAg has documented immunogenicity (16) and their expression is found at higher levels in more aggressive tumors (17). A range of CTAg (including SPANX and a number of MAGE family members) were also recently shown to be upregulated following EMT in a colorectal cancer cell line (18).

Independent of the well-documented functional differences between E- and M-like cells, the changes in expression of immunogenic antigens between melanoma phenotypic subsets has profound implications for immune-based recognition and clearance of tumor. For example, based on our studies, immune responses induced against antigens such as Melan-A and tyrosinase would potentially only be effective in clearing differentiated E-like tumor cells. Indeed, a previous study highlighted the EMT-like phenotypic switch in melanoma as a method of escape from adoptive T cell therapy with T-lymphocytes targeting Melan-A (19). This study also showed that M-like cells were poorly recognized by T-lymphocytes that were specific for melanocytic antigens, whereas recognition by T-lymphocytes specific for non-melanocytic antigens was unaffected or even increased *in vivo*.

Taken together, the studies discussed here highlight the disparity of the immunogenic profile of E-like versus M-like tumor cells, and emphasize the importance of appropriate target antigen selection for cancer vaccine/T cell targeting approaches.

The M-like phenotype has been associated with tumor progression, invasion, and metastasis in melanoma (20, 21). This generally more aggressive phenotype is accompanied by acquisition of resistance to drug treatment and radiotherapy (22). Furthermore, this phenotype also emerges after the development of resistance against BRAF inhibitors (6). Thus, M-like cells are generally considered more difficult to eradicate, by multiple mechanisms, compared with their E-like counterparts.

In this article, we ask whether M-like melanoma cells are inherently resistant to killing by T-lymphocytes at a functional level, reflective of the mesenchymal state, or whether an escape from T cell-mediated killing via phenotype switching is simply reflective of the decrease in expression of many target tumor antigens. We show that expression of the prototypic cancer testis antigen NY-ESO-1 is unaltered between melanoma phenotypic states. We compare the ability of NY-ESO-1 or Melan-A specific T cell clones to recognize and kill melanoma cell lines, which were phenotypically either E-like or switched to M-like following incubation with TGFβ (23).

MATERIALS AND METHODS

MELANOMA CELL LINES AND CULTURE

Establishment and characterization of the melanoma cell lines used has been previously described (7). Cells were cultured in RPMI 1640, 2 mM Glutamax, 100 IU/ml Penicillin, 100 μg/ml Streptomycin, and 10% fetal calf serum (RF10) (all Invitrogen). To induce EMT, subconfluent cell lines were incubated with 5 ng/ml TGF-beta 1 (Pepro Tech) for 72 h.

GENE EXPRESSION ARRAY

The gene expression array method and analysis have been previously described (24). Briefly, genomic DNA was purified from melanoma cell lines (Qiagen AllPrep kits) and assayed using Illumina standard protocols.

QUANTITATIVE REAL-TIME POLYMERASE CELL REACTION (QRT-PCR)

cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed using the QuantiFast SYBR Green PCR Kit (Qiagen). Primer sequences were as follows: NY-ESO-1 (forward) 5′-gagccgcctgcttgagtt-3′ and (reverse) 5′-agcactgcgtgatccacatc-3′; Melan-A (forward) 5′-gaga aaaactgtgaacctgtggt-3′ and (reverse) 5′-gactgttctgcagagagtttctcat-3′; N-cadherin (forward) 5′-ctccatgtgccggatagc-3′ and (reverse) 5′-cgatttcaccagaagcctctac-3′ β-actin (forward) 5′-ctggaacggtgaagg tgaca-3′ and (reverse) 5′-cggccacattgtgaactttg-3′.

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Melanoma cell lines were treated with 5 ng/ml TGF-β1 for 72 h and fixed with 4% paraformaldehyde. Mouse anti-E-cadherin antibody (HECD1, Invitrogen) and rabbit anti-N-cadherin antibody (AB18203, Abcam) were applied at 2 and 1 μg/ml concentration, respectively, overnight at 4°C. Cells were subsequently treated with Alexa flour 488 (anti-mouse) and 555 (anti-rabbit) conjugated secondary antibodies for 45 min at room temperature (Molecular probes, USA). Cells were counter stained with DAPI for 10 min.

GENERATION OF ANTIGEN SPECIFIC CD8$^+$ T CELL CLONES

CD8$^+$ T cell clones specific for the NY-ESO-1 HLA-A*0201 restricted peptide 157–165, or the Melan-A HLA-A*0201 restricted peptide 26–35, were generated from patients who consented to participate in our cancer research protocol (approved by Austin Health Human Research Ethics Committee). PBMC (peripheral blood mononuclear cells) were stimulated with 1×10^{-6} M peptide, then cultured for 10 days in the presence of 25 IU/ml IL-2 (Miltenyi biotech). Specific cells were labeled with a fluorescent tetramer, comprising the relevant peptide and HLA (TCMetrix) and single-cell sorted using a MoFlow cytometer. Clones were expanded with pooled, autologous healthy donor PBMC as feeder cells, PHA-L (Sigma), and 600 IU/ml IL-2. After approximately 20 days, $1–10 \times 10^3$ clones were restimulated in the presence of autologous PBMC as feeder cells, PHA-L, and 600 IU/ml IL-2, as described above. Clone specificity was confirmed by tetramer staining.

T cell clones/lines were cultured in RPMI 1640 media supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 60 mg/L penicillin, 12.5 mg/L streptomycin, 2 mM L-glutamine, 1% non-essential amino acids, and 10% human serum (TCRPMI).

T CELL KILLING ASSAYS

Twenty thousand melanoma cells were plated in wells of a flat bottom 96-well plate. T cells were added to selected wells as appropriate to give the effector to target (E:T) ratios shown in the text. Samples were incubated overnight (~16–24 h) at 37°C. The following day, T cells were resuspended by gentle pipetting and then removed. The plate was washed once with PBS. MTS reagent (CellTiter 96® AQueous One Solution Cell, Promega) was added and samples incubated for 1–2 h at 37°C. Absorbance at 490 nm was measured, and normalized to melanoma samples incubated in absence of T cells for each cell line to give percentage of viable cells.

RESULTS

We assessed gene expression in our panel of 57 early passage melanoma cell lines (7, 24). Cell lines were classified as E-like or M-like on the basis of E-cadherin or N-cadherin expression, respectively. We have previously reported widespread variation in

antigen expression between E- or M-like phenotypes (8). Here, we focus on the difference in expression of a range of immunogenic antigens, including MDA and CTAg, shown in **Figure 1**. As previously reported by ourselves and others, expression of MDA (e.g., Melan-A and tyrosinase) and MITF was largely lower in M-like, compared to E-like, cells. In contrast, a number of CTAg (e.g., SPANX family members) were upregulated in M-like cells. The expression of some antigens (e.g., MAGED1) was unaffected by the differences in phenotype between our cell lines. These data highlight the immunogenic heterogeneity of melanoma.

Based on our previous studies (7, 8), we selected four E-like melanoma cell lines, which were HLA-A*0201^{+}, and which were also positive for expression of either Melan-A, or NY-ESO-1, or both. As a control, we also obtained an M-like cell line (LM-Mel 53). The selected lines were treated with TGFβ for 72 h to induce EMT, and successful switch to an M-like phenotype was confirmed by demonstrating upregulation of N-cadherin gene expression by qPCR, or protein expression by immunofluorescence (**Figures 2A,B**). In all four E-like cell lines, upregulation of N-cadherin expression was observed following TGFβ incubation,

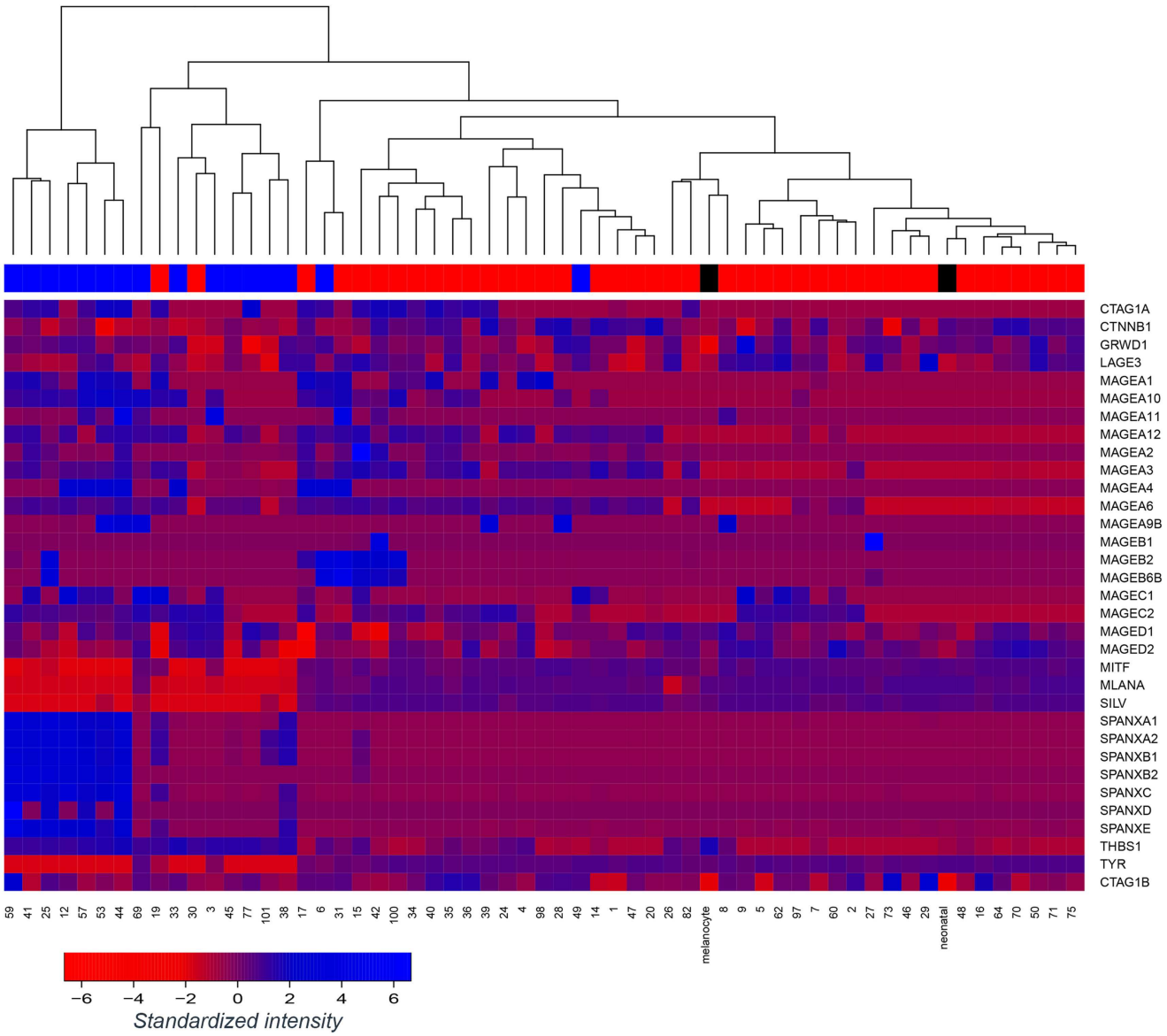

FIGURE 1 | Tumor antigen expression in melanoma epithelial or mesenchymal-like cell lines. Microarray-based gene expression of a range of melanoma antigens, including several melanoma differentiation antigens and cancer-testis antigens across a panel of 57 early passage melanoma cell lines and 2 controls (normal melanocytes; "melanocyte" and neonatal melanocytes; "neonatal"). Melanoma cell lines were classified as epithelial-like (top bar; red) or mesenchymal-like (top bar; blue) on the basis of dominant E-cadherin or N-cadherin expression, respectively, however, unsupervised clustering of the cell lines was performed without reference to this annotation. Control melanocytes have been labeled in black.

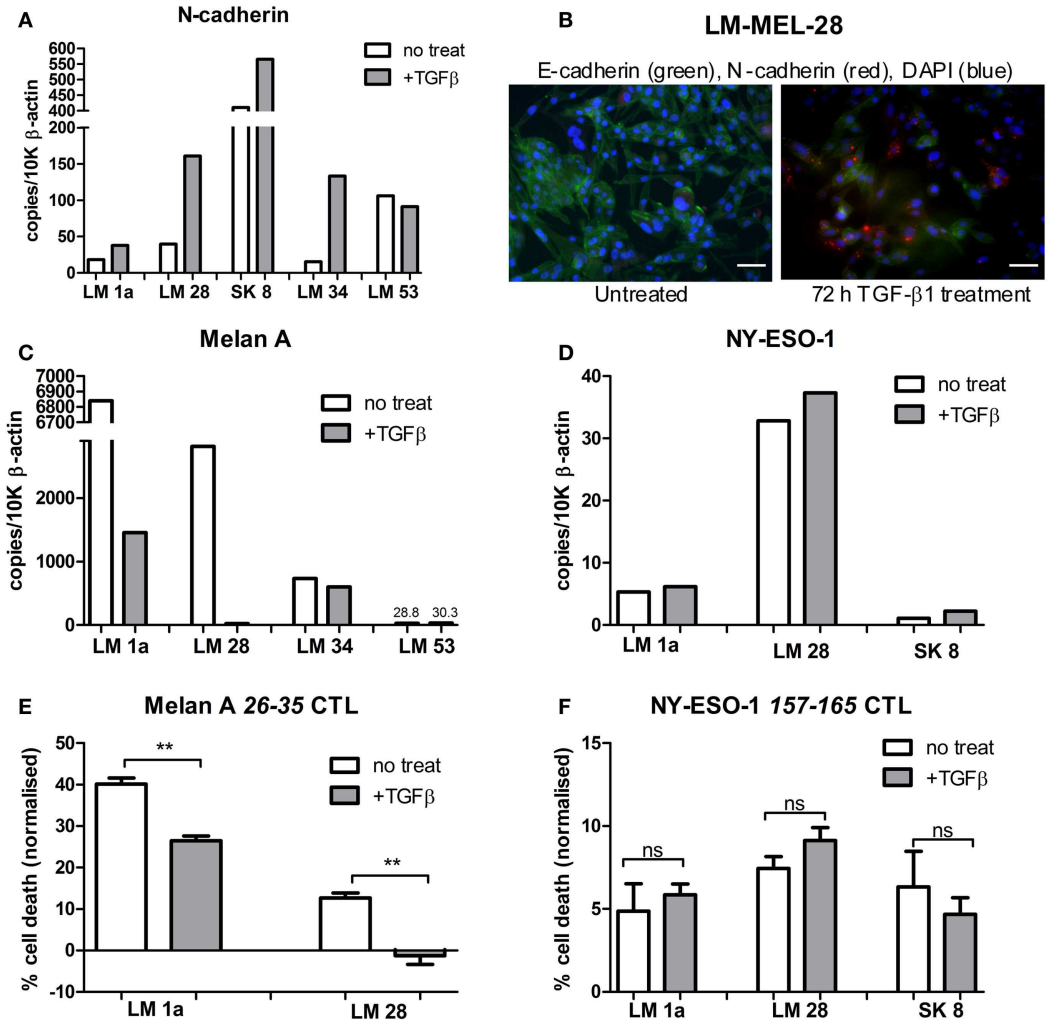

FIGURE 2 | Antigen expression and T cell-mediated lysis of melanoma cell lines following TGFβ induced EMT. Epithelial-like melanoma cells lines, LM Mel 1a, LM Mel 28, SK Mel 8, and LM Mel 34, and mesenchymal-like cell line LM Mel 53, were incubated with 5 ng/ml TGFβ for 72 h to induce EMT. Cells were lysed, and cDNA was generated (as in Section "Materials and Methods"). **(A)** Expression levels of N-cadherin in treated and untreated cell lines were determined by qPCR, in order to assess switching to the mesenchymal-like phenotype, and expressed relative to 10,000 copies of β-actin. **(B)** Immunofluorescence confirmed increase in N-cadherin protein expression (Red) and concomitant reduction in E-cadherin (Green) after 72 h of TGF-β1 treatment, shown here for a representative cell line (LM-Mel 28). Scale bar = 100 μM. Expression levels of **(C)** Melan-A and **(D)** NY-ESO-1 were compared between TGFβ treated/untreated melanoma cell lines expressing these antigens. Melanoma cell lines expressing Melan-A, and/or NY-ESO-1 were treated with/without 5 ng/ml TGFβ for 72 h. Specific T cell clones, which recognized the HLA-A*0201 epitopes **(E)** Melan-A 26–35 or **(F)** NY-ESO-1 *157–165* were incubated with 2×10^4 treated/untreated melanoma cells at a 1:1 effector to target ratio. Cells were incubated for 24 h at 37°C. T cells were washed off, followed by addition of MTS reagent (Promega) and 1 h incubation at 37°C. Absorbance was read at 490 nm, and normalized to untreated control cells for each treatment condition. Each measurement was in triplicate, and treated/untreated samples were compared using a paired *T*-test. ns = not significant. $^{**}P \leq 0.01$.

whereas there was no significant change in N-cadherin expression by the M-like cell line.

Epithelial-like cell lines LM-Mel 1a, LM-Mel 28, and LM-Mel 34 express Melan-A under steady state conditions, while the M-like cell line LM-Mel 53 expresses low levels of this antigen. In our heatmap (**Figure 1**), no cell line demonstrated expression of NY-ESO-1/CTAG1B, which is likely due to inefficiency of the probe (probes to CTAg are traditionally inferior), since we have shown by qPCR (**Figure 2D**) and IHC (not shown) that several of our cell lines express NY-ESO-1. The effect of phenotypic switching by EMT on the expression of either Melan-A or NY-ESO-1 was assessed in the relevant cell lines. qPCR demonstrated that while gene expression levels of NY-ESO-1 did not alter significantly following EMT, the expression levels of Melan-A decreased in all E-like, but not M-like cell lines expressing this antigen (**Figures 2C,D**). This result is in line with previous studies from our group and others, which demonstrated downregulation of several MDA including

Melan-A and related antigens under the transcriptional control of MITF.

Previous studies have shown that TGFβ treatment results in downregulation of MHC class II molecules, such as HLA-DR; however, no significant effect of TGFβ treatment on HLA class I levels has been demonstrated (25). We assessed HLA Class I expression in our cell lines before and after 72 h TGFβ treatment, and confirmed that no significant change in HLA Class I levels occurred (data not shown). This result is important for our subsequent experiments, which assess T cell killing of TGFβ-treated/untreated melanoma cell lines. In these studies, we can confirm that HLA does not play a limiting role for either melanoma phenotype.

Since M-like cells are generally more resistant to various treatment modalities, we tested whether T cell-mediated lysis of these cells was also impeded. T cells, which recognized the HLA-A*0201 restricted epitope *157–165* from NY-ESO-1 or *26–35* from Melan-A were incubated with melanoma cell lines expressing their respective target antigen, at a 1:1 effector:target ratio. Cells were incubated for 24 h before viability of melanoma cells was assessed using an MTS assay (methods), and normalized to untreated control cells (**Figures 2E,F**). In all cell lines tested, NY-ESO-1 specific T cells killed melanoma cell lines comparably both before and after TGFβ treatment. In contrast, Melan-A specific T cells were significantly more effective at killing E-like melanoma cells prior to induction of EMT. Following TGFβ treatment, cell lines were less efficiently cleared by Melan-A specific T cells in all cases tested.

Our results indicate that the ability of M-like cells to escape from T cell-meditated lysis was not due to any inherent functional characteristic of these cells, but rather due to changes in expression of target antigen. Melan-A expression decreased following EMT, leading to corresponding diminishing ability of antigen specific T-lymphocytes to kill target cells. In contrast, the expression of NY-ESO-1 was unchanged following EMT, and in parallel the ability of specific T cells to recognize and kill melanoma cells in an antigen specific manner was unaffected.

DISCUSSION

Contemporary melanoma therapy is undergoing a revolution due to the unprecedented success of immune checkpoint inhibitors; agents that re-activate anti-cancer immunity within treated patients (26). These successes are a culmination of what scientific researchers have long striven to achieve using methods such as cancer vaccination and adoptive cell transfer, that is, appropriate immune targeting in a manner which results in tumor clearance. Phenotype switching by melanoma cells to an M-like phenotype has been considered an inherent mechanism of tumor escape from therapy and resistance to various treatment modalities. Set against the back drop of recent immune-based treatment successes, we find here that immune-mediated clearance of melanoma cells is unaffected by EMT under the appropriate settings.

Previous treatment strategies have been hampered by the heterogeneity of the disease – escape of residual cells from radiotherapy, emergence of resistance to BRAF inhibition, etc – and a strategy which could target the tumor as a whole has eluded researchers. Patients successfully treated by immune checkpoint inhibition can continue to remain disease free long-term [4 years at the most recent follow up for anti-CTLA4 therapy (27), and ~25 months for anti-PD-1 treatment (28)]. It is therefore clear that appropriate immune control can achieve clearance of melanoma.

Despite the relative success of immune checkpoint inhibition, many patients are still not rendered disease free with these agents. In this cohort, rational combination therapies are likely to be beneficial and extensive studies are currently underway to ascertain which kinds of combination will exert broadest patient benefit and disease control. These combination therapies will include targeting melanoma specific antigens by cancer vaccination or adoptive cell therapy, in conjunction with immune checkpoint inhibition, and indeed this is the focus of several current clinical trials (NCT01176474, NCT02077114, NCT01176461, NCT01988077, NCT01701674), including our own trial combining vaccination with NY-ESO-1 and ipilimumab (NCT01810016).

Our study here, and others (8, 19, 29) highlight not only functional but also immune heterogeneity of melanoma cells. We demonstrate that EMT in melanoma can result in escape from T cell targeting. Importantly, we have shown that the means of tumor escape from immune-mediated killing is not due to an intrinsic functional resistance mechanism of cells that display the M-like phenotype, but rather is due to decrease in the expression of target antigen. T lymphocyte targeting of selected antigens with constant expression during EMT results in comparable killing of melanoma cells *in vitro* independent of their phenotype. Since M-like cells have traditionally been considered more difficult to kill than their E-like counterparts, this finding is significant. The ability of M-like and E-like tumor cells to be equally killed by T cells recognizing appropriate targets confirms the potential of combination treatments using appropriately selected antigens as targets.

These data further highlight the importance of antigen selection for cancer vaccination or adoptive cell transfer, since even if completely effective, therapeutic options which target antigens preferentially expressed by E-like cells will be incapable of clearing the tumor as a whole. Indeed such therapies could potentially exert selective pressure for tumor cells with the more aggressive M-like phenotype and thereby promote cell populations that carry a poorer prognosis for the patient.

To develop this rationale further, antigen specific immunotherapy strategies should ideally target antigens which are not only expressed in differentiated E-like cells, but also those which are either induced when the phenotype changes during EMT, or which are consistently expressed in both E-like and M-like cells. The former have been little examined in melanoma to date, most studies focusing on antigen loss leading to immune escape during EMT. However, **Figure 1** demonstrates that a subset of MAGE antigens and the SPANX family of CTAgs are upregulated in M-like melanoma cell lines. Furthermore, Boisgerault et al. demonstrated that in prostate cancer, topoisomerase IIα and CD44 are antigens which are specific to M-like cells, and further showed that a subset of melanoma cells also expressed topoisomerase IIα (29, 30).

Hopefully, combination treatment strategies with appropriately selected target antigens as discussed here will result in enhanced tumor clearance irrespective of tumor heterogeneity. Indeed, we are currently assessing the efficacy of this approach using the CTAgs SPANX, expression of which is induced during EMT,

and Ropporin, whose expression in melanoma is widespread and constant during EMT (manuscript in preparation).

AUTHOR CONTRIBUTIONS

Katherine Woods designed the work, acquired and analyzed data, and wrote the manuscript; Anupama Pasam, Aparna Jayachandran, and Miles C. Andrews acquired and analyzed data and revised the manuscript; Jonathan Cebon contributed to the concept of the work, revised, and approved the manuscript for publication.

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Androgen-targeted therapy-induced epithelial mesenchymal plasticity and neuroendocrine transdifferentiation in prostate cancer: an opportunity for intervention

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Androgens regulate biological pathways to promote proliferation, differentiation, and survival of benign and malignant prostate tissue. Androgen receptor (AR) targeted therapies exploit this dependence and are used in advanced prostate cancer to control disease progression. Contemporary treatment regimens involve sequential use of inhibitors of androgen synthesis or AR function. Although targeting the androgen axis has clear therapeutic benefit, its effectiveness is temporary, as prostate tumor cells adapt to survive and grow. The removal of androgens (androgen deprivation) has been shown to activate both epithelial-to-mesenchymal transition (EMT) and neuroendocrine transdifferentiation (NEtD) programs. EMT has established roles in promoting biological phenotypes associated with tumor progression (migration/invasion, tumor cell survival, cancer stem cell-like properties, resistance to radiation and chemotherapy) in multiple human cancer types. NEtD in prostate cancer is associated with resistance to therapy, visceral metastasis, and aggressive disease. Thus, activation of these programs via inhibition of the androgen axis provides a mechanism by which tumor cells can adapt to promote disease recurrence and progression. Brachyury, Axl, MEK, and Aurora kinase A are molecular drivers of these programs, and inhibitors are currently in clinical trials to determine therapeutic applications. Understanding tumor cell plasticity will be important in further defining the rational use of androgen-targeted therapies clinically and provides an opportunity for intervention to prolong survival of men with metastatic prostate cancer.

Keywords: prostate cancer, epithelial-to-mesenchymal transition, neuroendocrine, androgen deprivation therapy, castrate resistant, tumor cell plasticity, brachyury, Axl

INTRODUCTION

Prostate cancer is the most prevalent malignancy in men, and ranks second as the cause of cancer-related deaths in the developed world (1, 2). Advanced prostate cancer is initially treated with androgen deprivation therapy (ADT) and subsequently with newer generation androgen-targeted therapies (ATT), approaches which rely on the central role of androgens in tumor development and growth. In the majority of patients, castrate resistant prostate cancer (CRPC) develops and tumor progression occurs despite treatment. The development of agents that more effectively block androgen receptor (AR) activity, such as enzalutamide and abiraterone, has greatly enhanced the clinical armamentarium and extended survival (3–6). Nonetheless, advanced prostate cancer remains incurable. Tumor cell plasticity induced by androgen deprivation may play a critical role in disease progression, and potentially provides an additional opportunity to further improve cancer control.

PROGRESSION TO CASTRATE RESISTANCE

While the exact mechanisms underlying the development of CRPC are not yet known, it arises when cancer cells can either maintain AR signaling in the absence of normal levels of ligand or no longer require activation of this pathway for survival and proliferation. There are a number of mechanisms that can produce this outcome, including altered functionality of the AR due to genomic events, resulting in either promiscuous (7, 8), constitutively activated (9, 10), or hypersensitive (11, 12) states; intraprostatic production of androgens by tumor cells themselves (13); and

altered growth factor and/or microenvironment signaling (14–18). Despite the development of multiple strategies that effectively target the androgen axis, disease progression is inevitable. This is underpinned by the accumulation of further genomic abnormalities, outgrowth of different clonal populations of tumor cells, and the adaptive response of cancer cells to therapy. In this review, we focus on adaptive changes induced by therapy, specifically epithelial-to-mesenchymal plasticity (EMP) and neuroendocrine transdifferentiation (NEtD), which may contribute to the development of advanced disease (**Figure 1**). A better understanding of these processes will contribute to the development of new therapeutic strategies that may potentially enhance the efficacy of androgen-targeted agents and delay disease progression.

EPITHELIAL-TO-MESENCHYMAL PLASTICITY

Epithelial-to-mesenchymal transition (EMT) is a process by which adherent, polar cells with an otherwise epithelial phenotype develop more migratory and invasive properties through altered gene expression (19–23). Both EMT and the related process mesenchymal-to-epithelial transition are physiological mechanisms important in development and tissue repair. However, when differentiated epithelium begins to display mesenchymal characteristics it is often a sign of disease progression in cancers (19, 24–27). EMT is commonly characterized by the loss of epithelial markers (typically E-cadherin, epithelial cytokeratins, and desmosomes), and gain of mesenchymal markers (such as N-cadherin, vimentin, and fibronectin) and transcriptional repressors of E-cadherin (Twist1, Snai1, Snai2, Zeb1, Zeb2) (20, 21). EMT has been associated with advanced prostate cancer, and correlated with aggressive behavior and therapy resistance in primary tumors (17, 28–30).

NEUROENDOCRINE TRANSDIFFERENTIATION

While men may present with prostate cancer demonstrating various neuroendocrine features (31), the prevalence of neuroendocrine differentiation increases following ADT and in CRPC (32–37). These cells not only express neuropeptides, reminiscent of the normal NE cells of the prostate, but also proteins that are characteristic of prostate epithelial cells [such as prostatic acid phosphatase cytokeratin 8/18 and/or epithelial adhesion molecules and proliferation markers (38, 39)], while AR expression is typically absent or low (40). Importantly, the number of NE-like prostate cancer cells is positively associated with the duration of hormone deprivation therapy (32–34). There are several hypotheses for the origin of NE-like prostate cancer cells. It has been postulated that NE-like cancer cells can arise during disease progression from NE cells of the prostate (41). However, the observation that genetic aberrations are common to both the adenocarcinoma and NE-like cells (42–45) suggests that this is not likely to be a common mechanism. An alternative explanation is that a common progenitor prostate cancer stem cell gives rise to both the NE-like and adenocarcinoma components and both these components continue to evolve and respond to selective pressures in parallel (42, 44, 46, 47). In contrast, NEtD is a process that can enable prostatic adenocarcinoma cells to gain NE characteristics without relying on genetic divergence. NEtD can occur after prolonged androgen deprivation, and has recently been reported in a patient derived xenograft (48). This mechanism would potentially enable tumor cells to reduce ATT-induced apoptosis and thus provide an adaptive pathway that would contribute to the development of CRPC (41).

ANDROGENS SUPPRESS NEUROENDOCRINE TRANSDIFFERENTIATION

Evidence of NEtD has been observed in both *in vitro* and *in vivo* studies. LNCaP cells, an androgen dependent prostate cancer cell line derived from a lymph node metastasis, undergo NEtD when exposed to media lacking androgens (39, 49–51). In low-androgen conditions, LNCaP cells take on an altered elongated neuron-like

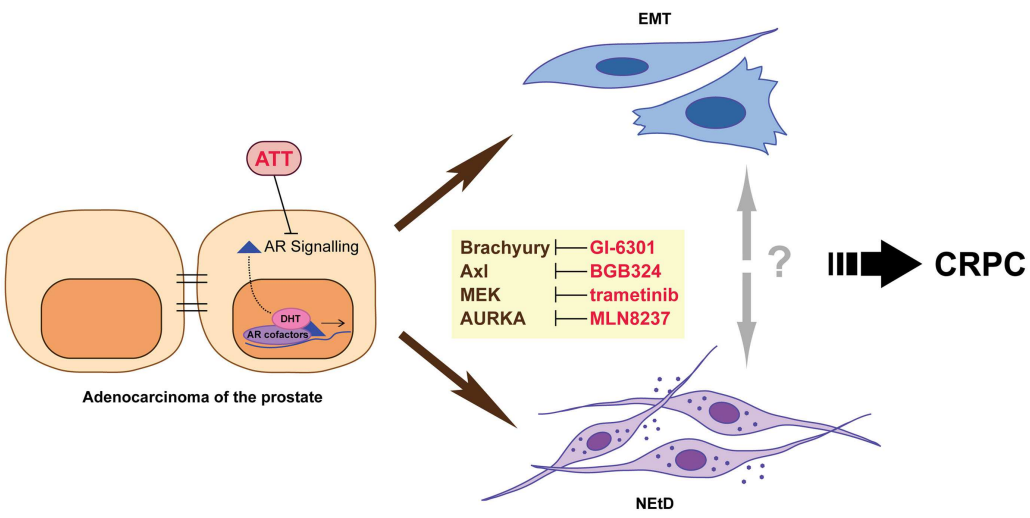

FIGURE 1 | Inhibition of androgen receptor (AR) signaling using androgen-targeted therapies (ATT) induces adaptive responses including epithelial–mesenchymal transition (EMT) and neuroendocrine transdifferentiation (NEtD) in prostate cancer cells. These phenotypes are associated with CRPC (castrate resistance prostate cancer). Inhibition of plasticity drivers Brachyury, Axl, MEK, and Aurora kinase A provide potential mechanisms to reduce the induction of the EMT and/or NEtD phenotypes.

phenotype, gain cytoplasmic secretory granules, and undergo growth arrest. This is accompanied by an increase in expression of NE markers and a decrease in AR and PSA levels. This transdifferentiation is reversible with the addition of androgens (DHT) to the media, an observation consistent with the identical allelic profiles of NEtD LNCaP and parental LNCaP cells. Silencing of the AR using siRNA also induces NEtD in LNCaP cells, suggesting that AR signaling suppresses NEtD (52). *In vivo* studies also provide support for the NEtD model. Castration of nude mice bearing prostate cancer xenografts LNCaP, PC-295, CWR22, and PC-310 increased the number of tumor cells expressing NE markers, consistent with induction of NEtD (53–56). Furthermore, implantation of primary patient tumor tissues from a population of adenocarcinoma cells implanted under the renal capsule of castrated mice appear to undergo an NEtD en masse as an adaptive response (48).

THERAPY-INDUCED EMT

There is accumulating evidence supporting that ADT may induce an EMT, and that this is particularly prominent with the newer generation ATT. ADT has been associated with an increase in the expression of mesenchymal markers N-cadherin, vimentin, Zeb1, Twist1, and Snai2, with a concomitant loss of E-cadherin in patient derived xenografts and clinical prostate tumors (17, 57–59). Traditionally, investigations have primarily focused on the effects of targeting AR signaling in prostate cancer cells; however, ADT/ATT is not specific to tumor cells alone. Recent reports demonstrate significant effects of ADT/ATT on the tumor microenvironment, including stromal and immune cells (15, 18, 60). For instance, elevated numbers of tumor associated macrophages have been reported in men undergoing ADT (60), and these cells have been shown to promote local invasion and metastatic dissemination of tumor cells in response to ADT (18, 57–60). Hence, the implications of targeting the androgen axis and its effect on the multiple cell types comprising the tumor microenvironment needs to be assessed when considering therapeutic interventions.

THERAPY-INDUCED EMT AND NEUROENDOCRINE TRANSDIFFERENTIATION AS CLINICAL TARGETS

Therapeutically targeting regulators of EMP/NEtD is an attractive concept that has recently matured to clinical trials (**Figure 1**). Brachyury is a transcription factor required for the developmental EMT that generates mesoderm by converting epithelial cells into migratory mesenchymal cells (61). In tumor cells, including prostate cancer, Brachyury also induces EMT and an invasive phenotype (62–65). Furthermore, Brachyury is overexpressed at both the transcript and protein level in clinical prostate cancer specimens, and nuclear expression is associated with metastasis (66). While the regulation of Brachyury by androgen-targeted therapies has not been addressed, Brachyury motifs were highly enriched in AR bound promoters when LAPC-4 cells were grown in the presence of AR antagonist flutamide (67). Furthermore, *in silico* bioinformatic analysis using transcriptional profiles from clinical prostate cancer specimens and clustering Brachyury co-expressed genes by functional role/signaling pathways demonstrated an enrichment for regulation of neuron differentiation and nervous system development (68). An inverse relationship between Brachyury and E-cadherin expression, with a concomitant positive correlation of Brachyury with EMT promoting genes FN1, Snai1, IL8, and TGF-β was also observed. Thus, we hypothesize that targeting Brachyury in the context of ATT may modulate the emergence of both a neuroendocrine phenotype and EMP by preventing, for example, the induction of Brachyury mediated release of migration/invasion promoting soluble factors into the tumor microenvironment (62, 68, 69). GI-6301 (Tarmogen) is a Brachyury vaccine (70) currently in Phase I clinical trial in patients with metastatic or unresectable locally recurrent cancers who have failed previous therapy or have no further therapeutic options (NCT01519817). Recent assessment of data from patients with advanced chordoma in this trial demonstrated safety and a confirmed partial response (71), and data from the larger cohort are eagerly awaited.

The receptor tyrosine kinase Axl is implicated in the Snai1-, Snai2-, IL6-, and STAT3-mediated activation of EMT (72, 73) as well as the metastasis promoting AKT/NF-κB and AKT/Snai2 pathways (73, 74) in multiple cancer types. Targeting Axl has shown promise in preclinical models of cancer progression (75–77), and clinical trials are currently underway. BGB324 is a small molecule inhibitor of the Axl receptor tyrosine kinase developed to block EMT with the goal of inhibiting drug-resistance and metastasis. Recent Phase Ia data have demonstrated BGB324 to be safe and well tolerated, and Phase Ib studies commenced in non-small cell lung cancer and acute myeloid leukemia in 2014. Cabozantinib is another tyrosine kinase inhibitor targeting Axl, as well as EMT promoting kinases VEGFR2, RET, KIT, FLT-1/3/4, c-MET, and Tie-2 (78–80). Clinically significant regression of metastatic tumors in CRPC patients was achieved with cabozantinib treatment in a Phase II trial (81). Of course the precise molecular mechanism underpinning this efficacy is not clear and likely involves inhibition of multiple tyrosine kinases in several cell types. Trials investigating whether cabozantinib is a useful addition to ADT in the control of prostate cancer are currently underway (NCT01630590).

MEK inhibitors may also be useful in managing therapy-induced EMP/NEtD. *In vitro*, MEK inhibitor PD98059 blocked the acquisition of NE-like morphology and prevented the increase in NSE levels usually observed in LNCaP-C33 cells induced to undergo NEtD by androgen-depletion (82). Ectopic expression of constitutively active AR in LNCaP cells inhibited RAF/MEK/ERK-induced NSE expression (83), demonstrating the central regulatory role of AR in constraining the emergence of this phenotype. Furthermore, the RAF/MEK/ERK pathway has been shown to be necessary for the induction of Twist1, Snai1, and N-cadherin in multiple cancer models (84, 85). A neoadjuvant trial examining the effect of short-term MEK inhibition (trametinib) prior to radical prostatectomy in the context of ADT on markers of EMT (N-cadherin, vimentin) has recently commenced (NCT01990196).

Finally, Aurora kinase A (AURKA) inhibitors may also be effective in inhibiting ATT-induced EMP/NEtD as they suppress both EMT and NEtD *in vitro* and *in vivo* (86, 87). In cancer cells, AURKA has been demonstrated to play an important role in the genesis of a more mesenchymal phenotype via down-regulation of E-cadherin and up-regulation of vimentin (88). Clinical trials

examining the role of the inhibitors in prostate cancer are currently ongoing (NCT01799278, NCT01094288).

Despite independent lines of evidence implicating key factors in both EMT and NEtD, the functional and molecular relationship between these states in prostate cancer has not been extensively explored. McKeithen et al. (89) have demonstrated that the well-established EMT-inducing transcription factor Snai1 induced both EMT and NEtD in LNCaP cells as defined by morphology and marker expression. However, as the data are mostly presented as analyses of bulk populations of cells, it is not possible to determine whether EMT and NEtD phenotypes are co-expressed within individual cells, and are thus intimately linked, or whether these transdifferentiation processes are independent of each other and become activated by influences such as neighboring cells, local microenvironmental cues, or cell intrinsic factors.

CONCLUDING REMARKS

Multiple factors are clearly involved in the progression to CRPC during treatment with ATT. Studies over the past two decades have associated blockade of the androgen axis with the increased prevalence of neuroendocrine prostate cancer. These observations, in combination with recent reports of androgen deprivation modulating EMP, suggest novel strategies for therapeutic intervention. Further studies will be required to determine whether these adaptive response pathways have a functional role in the progression to CRPC or are simply a consequence of removing the differentiation pressure imposed by active androgen signaling on prostate cells. Moreover, revealing if and how these plasticity pathways intersect in the androgen-targeted environment will be an intriguing area for future research. Improved understanding of the molecular pathways underlying the adaptive responses to ATT provides opportunities to investigate whether targeted inhibition of these pathways will delay tumor progression and thus improve outcomes for men with prostate cancer.

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Phosphoproteomic analysis of cell-based resistance to BRAF inhibitor therapy in melanoma

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The treatment of melanoma by targeted inhibition of the mutated kinase BRAF with small molecules only temporarily suppresses metastatic disease. In the face of chemical inhibition tumor plasticity, both innate and adaptive, promotes survival through the biochemical and genetic reconfiguration of cellular pathways that can engage proliferative and migratory systems. To investigate this process, high-resolution mass spectrometry was used to characterize the phosphoproteome of this transition *in vitro*. A simple and accurate, label-free quantitative method was used to localize and quantitate thousands of phosphorylation events. We also correlated changes in the phosphoproteome with the proteome to more accurately determine changes in the activity of regulatory kinases determined by kinase landscape profiling. The abundance of phosphopeptides with sites that function in cytoskeletal regulation, GTP/GDP exchange, protein kinase C, IGF signaling, and melanosome maturation were highly divergent after transition to a drug resistant phenotype.

Keywords: phosphoproteomics, BRAF, drug resistance, vemurafenib, kinases, label-free quantitation, mass spectrometry

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Introduction

In melanoma, coding mutations in the mitogen-activated kinase pathway (MAPK) (e.g., BRAF and RAS) are common and contribute to disease severity (1). In cutaneous melanoma, BRAF is mutated in ~70% of cases and correlates with poorer prognosis and aggressive disease (2, 3). The mutant BRAF protein is a hyperactive serine/threonine-protein kinase that directs signaling through MEK1/2 to phosphorylate the MAPK ERK1/2 and drive cell proliferation and tumor growth. In recent years, a high-therapeutic value has been attained by targeted inhibition of the mutated BRAF protein with selective inhibitors (e.g., vemurafenib and dabrafenib) (4–6). Vemurafenib and dabrafenib effectively reduce signaling through the MAPK pathway, resulting in disease regression (~85%) and progression free survival for ~5–6 months [reviewed in Ref. (7)], after which almost all treated patients develop drug resistant, progressive disease (5).

Several mechanisms for intrinsic and acquired resistance have been detected *in vivo* and *in vitro* and this has been extensively reviewed (7–9). Relapses in melanoma involve mechanisms that reprogram signaling pathways to bypass inhibition and reactivate the ERK1/2 signaling hub (10). For example, the up-regulation of receptor tyrosine kinases (RTKs), platelet derived growth factor (PDGF), epidermal growth factor receptor (EGFR), and insulin-like growth factor 1 receptor

(IGF-1R) can drive cell survival signals through the PI3K/AKT pathway (11–13). Alternative pathways that reactivate ERK during targeted therapy utilize the multimeric properties of RAF signaling complexes and also occur in BRAF wild-type cells. BRAF inhibitors have been shown to drive the formation of alternative RAF dimers able to phosphorylate MEK and induce ERK signaling (14–17). In drug resistant patients, up-regulation and splicing of MAPK signaling components [CRAF, BRAF, or the MAP3K8 (COT)] provide alternative mechanisms for the reactivation of ERK1/2 signaling (18–20). In response to the microenvironment, phenotypic switching can also occur based upon intrinsic tumor heterogeneity and lead to resistance to therapy (21). For example, paracrine signaling from stromal cells that secrete hepatocyte growth factor (HGF) reestablish the MAPK pathway in BRAF mutated cells by activating the RTK MET (22). Independent of the MAPK pathway, low expression of the melanocyte transcriptional network driver microphthalmia-associated transcription factor (MITF) associates with drug resistance and a more invasive, less proliferative phenotype (23, 24). This and the fact that MAPK inhibitors can themselves drive an invasive phenotype (25) indicate that inter-tumor plasticity allows melanoma to evade complete growth arrest in the face of MAPK inhibition.

The discovery of these mechanisms and others [reviewed in Ref. (8)] has established opportunities for novel melanoma treatment. The design of more effective co-inhibitory-based therapies could represent an improved strategy to prevent the acquired resistant phenotypes currently found in the clinic. In most cases, combination therapies in which BRAF inhibition is paired with inhibitors of the well establish mediators of resistance (PI3K, MEK, HGF, and IGF-R1) is showing promise (12, 26, 27). Because kinases (ERK1/2, IGF-R1, MEK, PI3K) provide key signaling hubs that orchestrate biochemical processes in drug resistant melanoma, characterizing their global activity profiles will aid the design of novel therapies. Kinase activity can be mapped by measuring the abundance of substrates using phosphoproteomic methods that combine phosphopeptide enrichment with high-resolution mass spectrometry (28). A quantitative phospho-site (P-site) analysis has the potential to provide a direct readout of kinase activity, elucidate novel mechanisms driving resistance, and guide the selection of therapies for validation in cell and animal models (29, 30). Previously, Old et al. reported ~90 P-sites that were regulated in a melanoma cell line in response to short-term MKK1/2 inhibition and Girotti et al. screened the phosphoproteome of A375 cells in a model for *in vitro* acquired drug resistance (31, 32). Both studies identify changes in the intensity of P-sites in signaling and cytoskeletal regulators and support the co-inhibition of specific kinase signaling (EGFR-SRC and SFK-STAT3) to provide therapeutic efficacy in drug resistance (32). To add to this work, we have developed and applied a simple and reproducible label-free quantitative phosphoproteomic procedure and analyzed an *in vitro* model of acquired drug resistance in melanoma cell line LM-MEL-28. The abundance of 2230 P-sites was measured by high-resolution mass spectrometry and correlated with the abundance of 3556 unmodified proteins to provide a more accurate determination of kinase activity. Kinase-substrate databases (Phosphosite.org, cell signaling) and motif analysis (NetworKIN) of the flanking linear amino acid sequences predicted several regulatory kinases that are most likely to be responsible for differential phosphorylation detected during long-term exposure to BRAF inhibition in LM-MEL-28. Key regulatory sites that control actin and microtubule-based cytoskeleton and cellular GTP/GDP ratio exhibited large changes in phosphorylation. Phosphorylation of the melanosome G-protein coupled receptor (GPCR) OA1 (GP143) indicated a direct role for the melanocyte maturation pathway. While sites of phosphorylation of the insulin receptor substrate IRS-2 and IGFR2 indicated novel points of regulation in the IGF-1R pathway previously identified to mediate drug resistance in melanoma.

Materials and Methods

Cell Culture and Protein Preparation

The melanoma cell line LM-MEL-28 was selected from the Ludwig Institute for Cancer Research Melbourne Melanoma Cell Line Panel (33). LM-MEL-28 was cultured in RPMI 1640 medium supplemented with 10% (v/v) bovine serum (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2. Cells were treated with PLX4720 (Selleck Chemicals) for a 1-month period in 5 μM PLX4720 to generate a drug resistant line referred to LM-MEL-28R1. Cells were tested for authenticity by short tandem repeat profiling according to the ANSI/ATCC ASN-0002-2011 standards. For phosphoproteomic analysis, six biological replicates were generated by sub-culture and cells were grown to 80–90% confluence with the LM-MEL-28-R1 continuously cultured in the presence of 5 μM PLX4720 and LM-MEL-28 in the presence of vehicle. Cells were washed three times in PBS and harvested by gentle enzyme-based release (TrypLE), pooled and centrifuged at $400 \times g$, cell pellets frozen on dry ice and stored at −70°C. Cells were lysed by boiling for 5 min in 1% (w/v) sodium deoxycholate (Sigma), 50 mM triethylammonium bicarbonate (TEAB) (Sigma), and 1 mM $MgCl_2$ (Sigma). Lysates were cooled to 4°C, sonicated to complete lysis, and DNA was digested by incubation with benzonase (Sigma) (10,000 units). Lysate were centrifuged at $20,000 \times g$ for 10 min and protein amounts determined by the micro-BCA assay (Pierce). Samples were stored at −80°C. Mutational testing was performed by MelCarta assay and all cell lines were tested for mycoplasma and appropriate consent from all patients had been obtained.

Protein Digestion and Phosphopeptide Enrichment

Five hundred milligrams of total protein lysate were reduced with 5 mM DTT (Sigma) for 30 min at 60°C and alkylated with 10 mM of iodoacetamide (Sigma) in the dark at room temperature for 30 min. Trypsin (Promega) was added at ratio of 1:50 ratio for 18 h at 37°C. Samples were adjusted to 1% (v/v) trifluoroacetic acid (TFA) (Sigma), 80 mg/ml glycolic acid (Sigma), and the precipitated deoxycholate was removed by centrifugation. Five milligrams of TiO_2 beads (Titanisphere, 10 μm) were washed once in 0.1% (v/v) TFA, 70% (v/v) acetonitrile (ACN), and 80 mg/ml glycolic acid, added directly to the sample and incubated with shaking for 1 h. A C8 stage-tip was prepared and washed with methanol (Sigma), then 0.1% (v/v) TFA, 70% (v/v) ACN, and 80 mg/ml glycolic acid (40 μl). TiO_2 beads were added to the

C8 stage-tip and tips were centrifuged 1000 × *g* until all liquid was dispensed. Beads were washed on tip with 300 µl of 0.1% TFA, 70% ACN, 80 mg/ml glycolic acid (300 µl) twice then thrice with 0.1% (v/v) TFA and 70% (v/v) ACN. Phosphopeptides were eluted from TiO_2 tip with consecutive 100 µl additions of 1% (v/v) ammonia (Sigma) with 0, 30, and 50% (v/v) ACN. Samples were immediately dried and resuspended in 1% (v/v) TFA and 5% (v/v) ACN for LC-MS/MS.

Isobaric Labeling by Reductive Dimethylation and Peptide Separation

Proteolytic digestion of 100 µg total protein was carried out as described above and samples were labeled by reductive dimethylation using formaldehyde isotopologues (34) with slight modifications (35). After labeling, each sample was pooled and 40 µg separated into six fractions using pH-based strong anion exchange (SAX) STAGE tips (36) described in Ref. (37).

Mass Spectrometry (LC-MS/MS)

Samples were loaded onto a self-packed 100 µm × 3.5 cm reversed phase peptide trap (Solid core Halo® 2.7 µm 160 Å ES-C18, Advanced Materials Technology) and desalted for 10 min with buffer A [0.1% (v/v) formic acid], peptide separation was carried out using a self-packed 75 µm × 10 cm (Solid core Halo® 2.7 µm 160 Å ES-C18, Advanced Materials Technology) column. A buffer B [100% (v/v) ACN, 0.1% (v/v) formic acid] gradient (5–40% in 120 min) was used to elute peptides. Phosphopeptides were ionized by electrospray ionization and data-dependent MS/MS acquisition carried out using a Q-Exactive consisting of 1 full MS1 ($R = 70$ K) scan acquisition from 350 to 1500 m/z, and 10 HCD type MS2 scans ($R = 15$ K). Dimethylated peptides were analyzed on an Orbitrap Elite (Thermo Fisher Scientific) consisting of 1 full MS^1 ($R = 120$ K) scan acquired from 350 to 1500 m/z, and 10 CID type MS^2 scans. On both instruments, monoisotopic precursor selection, charge state screening, and dynamic exclusion were enabled, charge states of +1, >4, and unassigned charge states were not subjected to MS^2 fragmentation. Raw mass spectra were identified using Maxquant 1.3 using a 1% peptide and protein FDR. Searches were conducted against the uniprot complete proteome reference database downloaded on June 06, 2014. The database was supplemented with common contaminants often found in cell culture and proteomics experiments these were later removed. Searches specified for tryptic peptides with four missed cleavages, 7 ppm precursor ion mass tolerance, 0.05 Da fragment ion mass tolerance, fixed modifications of carbamidomethylation (C), and variable modification of oxidation (M), acetylation (N-term, protein), and phosphorylation (STY). For phosphopeptides, quantitation was performed using peptide intensity for modified (STY) P-sites and for proteins using the protein intensity ratio from the protein groups detected in the dimethylated data-set generated by Maxquant (38). Statistical analysis was carried out using Perseus 1.5.0 (39). Intensities were pre-processed by $\log_2$ transformation and checked for normality. To identify differentially expressed peptides, the Student's *t*-test were applied to compare groups, *P* values were filtered for the effect of multiple hypothesis testing using the FDR method (<5%). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (40) via the PRIDE partner repository with the dataset identifier PXD002079.

Phosphosite Localization and Kinase Assignment

To localize modifications search results were processed using Maxquant that generates a score and probability function to assign confidence to amino acid modification location based on available peak depth present in MS/MS spectra. Upstream kinases were putatively assigned using the NetworKIN algorithm (41) and Phosphosite database (42).

Viability Assays

Cell lines were seeded in 96-well plates at 5000 cells/well in triplicate for each drug treatment and time point. After 2 h, cells were treated with dilutions 5 µM for vemurafenib (PLX4072) alone or in combination with 1.25 or 2.5 µM of the CK2 inhibitor (CX-4945). After 72 h, cell viability for each cell line was assessed by Presto Blue Assay (Life Technologies).

Results

Phosphoproteome Analysis of *In vitro* Drug Resistance

Figure 1A outlines the methodology taken to investigate the phosphoproteome of drug-exposed melanoma cells. To model drug resistance, a *BRAF(V600E)* mutant cell line (LM-MEL-28) was cultured in media containing 5 µM of the selective BRAF inhibitor PLX-4720 for 1 month to generate the stable cell line, LM-MEL-28R. The resistant line LM-MEL-28R was threefold less sensitive to the growth inhibitory effects of PLX4720 than the parental line LM-MEL-28 as shown in a viability assay (**Figure 1B**). For phosphoproteomic and proteomic analysis, protein extracts were generated, digested with trypsin, and then phosphopeptides were enriched by micro-column based TiO_2 chromatography analyzed by LC-MS and total peptides labeled by reductive dimethylation using light and heavy isotopes, mixed, separated by SAX chromatography and analyzed by LC-MS; all steps were performed in triplicate. LC-MS identified 3162 unique phosphopeptides (S,T,Y) sequences, mapping to 1164 distinct protein groups and 16,713 none-phosphorylated peptide spectral matches mapping to 3556 protein groups at a FDR of 1% using Maxquant; 836 phosphoproteins (72%) had dual P-site and protein quant estimates providing added confidence in this dataset for detecting changes in phosphorylation occupancy (**Figure 1C**; Tables S1–S3 in Supplementary Material). The intensity of all phosphopeptides within replicates exhibited a strong positive correlation and low variance (R^2 of 0.75–0.84 and CV 27.26–28.28%). Using a probability function, 76% (2395 of 3162) p[S], p[T], p[Y] sites could be localized with high confidence (>75%) by MS/MS spectra (**Figure 1D**; Table S3 in Supplementary Material). The intensity of all peptides containing P-sites was used to compare cell populations initially, while Class 1 (>75%) was used for assigning kinase–substrate relationships.

Phosphorylation of the MAPK1 Pathway

Because the MAPK1 pathway is often at the center of acquired drug resistance to BRAFi, we first examined the relative abundance of P-sites in MAPK1, RB1, and CDK1/2, which can provide

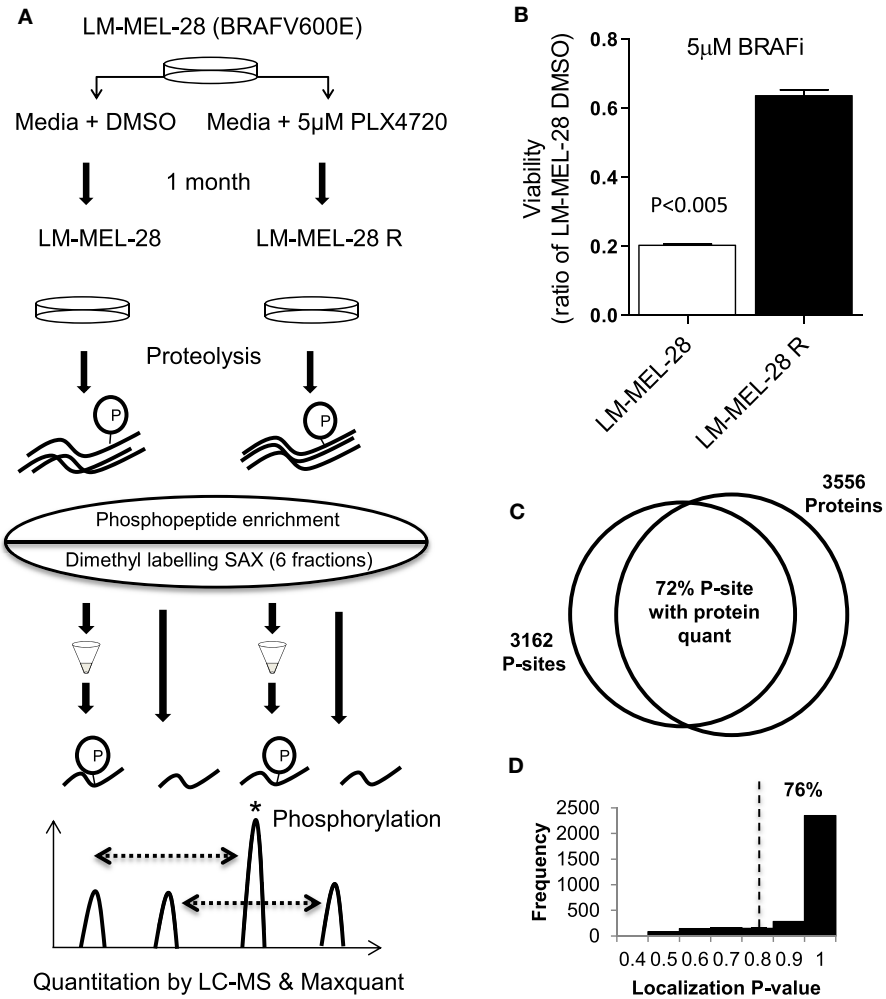

FIGURE 1 | Phosphoproteomic analysis of *in vitro* drug resistance in melanoma cells. **(A)** The melanoma cell line LM-MEL-28 *BRAF(V600E)* was exposed to BRAF inhibitor PLX4072 for 30 days to generate LM-MEL28R cell population. Proteins were extracted, digested, and $\pm TiO_2$ enrichment (for phosphopeptides) or labeled by reductive dimethylation, separated by tip-based strong anion exchange (SAX) chromatography, and analyzed by LC-MS and Maxqaunt. **(B)** The viability of LM-MEL-28R and LM-MEL-28 cells when grown in BRAFi was compared after 3 days (error bars are SD). **(C)** Venn diagram giving the number of P-sites and proteins identified by LC-MS, overlap is calculated where quantitation for P-sites and protein has been determined. **(D)** Histogram of probability values obtained from Maxquant for P-site localization accuracy, dotted line indicates the >0.75 cut-off used for kinase enrichment analysis.

a measure of MAPK1 signaling (**Figures 2A–C**). Regulatory P-sites [MAPK1 (T185), RB1 (T821)] and the inhibitory site in CDK1/2 (T/14Y15) were quantified and increased in abundance in LM-MEL-28-R, indicating reactivation/modulation of MAPK1 signaling had taken place in LM-MEL-28R despite continued BRAFi (**Figures 2A–C**). LC-MS data also provided a site-specific quantitative measure of protein phosphorylation for MAPK1 and RB1 and can thus indicate the activity of the regulatory kinases. To demonstrate this further, we selected the heavily phosphorylated protein, sequestosome-1 (SQSTM1), a known substrate of CDK1 (S269, S272) and demonstrate divergent site-specific protein phosphorylation is detectable. P-site intensity decreased at sites T269, S272 and increased at site S361 providing a snap shot of the activity of multiple kinase and/or phosphatases that target this protein (**Figure 2D**).

Proteome of Drug Resistance

To more accurately determine change in phosphorylation after BRAF drug resistance the proteome of LM-MEL-28 and LM-MEL-28R1 cell populations was compared using isotope coded quantitative proteomics and LC-MS (Table S3 in Supplementary Material). Analysis of these data alone indicated widespread regulation of protein biosynthesis occurs during the development of resistance to BRAFi. Using a twofold cut-off, the majority of proteins were found down-regulated (317) and fewer (151) up-regulated. 1-D gene set enrichment analysis using the $\log_2$ ratio and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database reflects this and is reported in **Table 1** and Table S4 in Supplementary Material). Down-regulated processes indicate major reprograming of metabolic pathways for amino acid metabolism and energy transducing systems (TCA

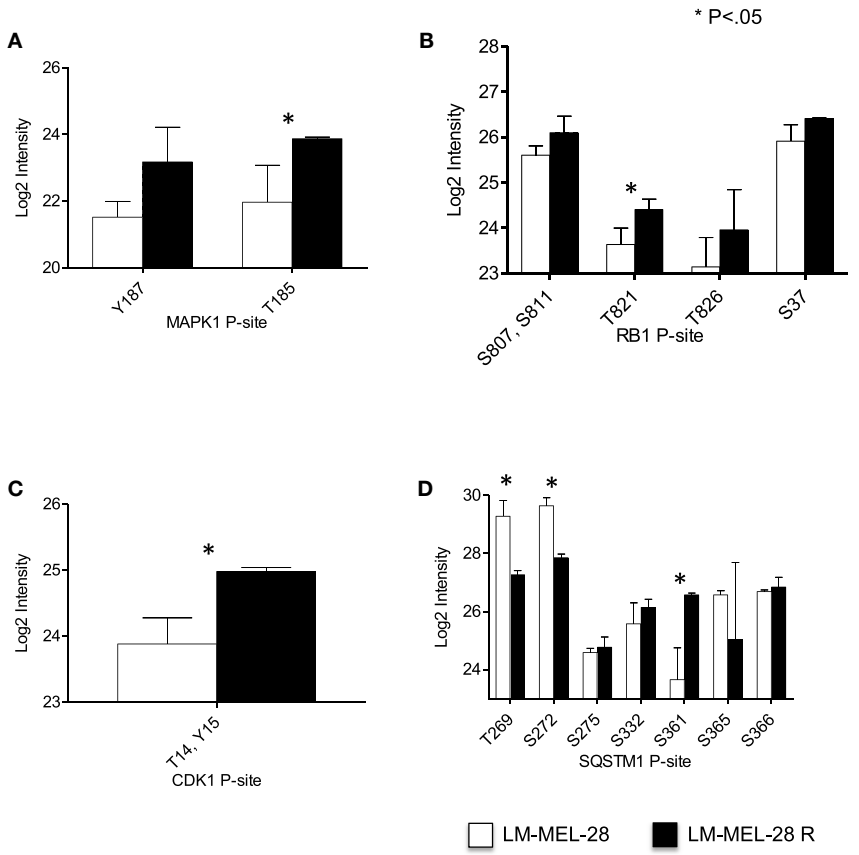

FIGURE 2 | Phospho-site analysis of MAP kinase pathway output. (A–D) The $\log_2$ intensity of key P-sites from proteins that function in and downstream of the MAPK01 (ERK1/2) pathway signaling are plotted and analyzed using a Student's *t*-test (error bars are SD).

and Glycolysis). Up-regulated processes indicate changes in processes controlling DNA metabolism and the cell cycle.

Phosphorylation Dynamics in Drug Resistance

With estimates of both P-site and protein effect for drug sensitive and resistant populations of LM-MEL-28, we subtracted the protein effect to determine more accurately changes in the rate of phosphorylation at significant sites. Protein quantitative estimates for 2895 sites (~72%) were available and using the following equation (Phospho rate = $\log_2$ Phospho − $\log_2$ Protein) specific post-translational activity (kinase or phosphatase) was inferred. **Figure 3** is an x/y scatter plot of the significant P-sites ($n = 148$, Student's t-test FDR corrected $P < 0.05$) where x is $\log_2$ P-site ratio (R1/S1) and y is $\log_2$ protein ratio (R1/S1). Data were well correlated indicating a large effect of protein abundance on P-site abundance (Pearson's $R^2 = 0.6$, $P < 0.0001$) (**Figure 3A**). Forty-seven accurately localized ($P < 0.75$) P-sites were differentially regulated by a minimum of twofold after protein abundance was subtracted, and these were selected for kinome analysis using Phosphosite.org and NetworKIN databases (41, 42) (**Table 2**). Seventeen sites originated from singly phosphorylated peptides, 30 sites were from 15 doubly phosphorylated peptides, of which 5 had a second site where the P-site localization was ambiguous (Class 2) (Table S5 in Supplementary Material). All accurately localized sites (Class 1) were used for further analysis, and it was accepted that for sites originating from the same peptide the quantitative value would amount to the sum of regulation at each site.

Kinase Enrichment Analysis

Using the phosphosite.org and NetworKIN databases, regulatory kinases for 29/46 P-sites could be assigned and are reported in **Table 2** and Tables S3 and S5 in Supplementary Material. Of the 46 P-sites, 11 were in key cytoskeletal regulators and kinase predictions were available for 7 of these sites. For example, myosin regulatory light chain, MLC12A/B/9 (T18 and S19) destrin/cofilin (S3) predicts the activity of ROCK1 and LIMK1/2 protein kinases (**Figure 3B**). P-sites in three distinct guanidine exchange factors (GEF's 11, 40, and 1) were also regulated by phosphorylation and S35 in GEF40 is putative substrate for the p21-associated kinase PAK4 (**Figure 3C**; **Table 2**). Phosphorylation of two microtubule-associated proteins (MAPs) increased at six sites (**Figure 3D**). Here, sites S2019, S2022 predicted the activity of CK1A on MAP1A and S1793, S1797 are putative substrates for GSK3β on MAP1B. Other sites of note for which kinase–substrate predictions were determined included two MAPK1 substrates TPR (S2155) and PPP2R2A (S692). Sites in the key signaling molecules insulin receptor substrate 1 (IRS1, S736), the insulin-like growth factor 2 receptor/cation-independent mannose-6-phosphate receptor (IGF2R/CI-MPR, S2484), and protein kinase

TABLE 1 | 1-D gene enrichment analysis.

KEGG pathway name	Proteins[a]	Median[b]	Benj. Hoch. FDR
Mismatch repair	14	0.50	3.4E-03
DNA replication	24	0.34	1.3E-04
Nucleotide excision repair	21	0.26	4.6E-04
Cell cycle	40	0.22	3.5E-04
Huntington's disease	97	−0.37	3.3E-03
Oxidative phosphorylation	73	−0.40	7.2E-04
Alzheimer's disease	87	−0.41	3.3E-03
Parkinson's disease	76	−0.41	3.0E-04
Glycolysis/gluconeogenesis	34	−0.48	4.6E-03
Ribosome	72	−0.52	1.2E-08
Cardiac muscle contraction	25	−0.55	6.6E-04
Peroxisome	30	−0.59	2.9E-03
Aminoacyl-tRNA biosynthesis	30	−0.60	3.5E-04
Fatty acid metabolism	24	−0.60	1.6E-03
Citrate cycle (TCA cycle)	27	−0.61	2.2E-03
Valine, leucine, and isoleucine degradation	26	−0.62	4.1E-04
Pyruvate metabolism	27	−0.63	1.4E-03
PPAR signaling pathway	22	−0.67	2.6E-03
Tryptophan metabolism	14	−0.75	3.3E-04

[a] *Number of proteins annotated with the KEGG pathway.*
[b] *Median log_2 fold change for proteins annotated within the KEGG pathway.*

C (PKC, S497) indicated activity of GSK3α/β, CK2A, and PDHK1, respectively. Nestin (S680) and sequestosome-1 (T269, S272) decreased in phosphorylation and are predicted to be substrates of CDK1, consistent with the increase in inhibitory phosphorylation of CDK1 (Y15/T14) measured. Finally, Casein kinase 2 alpha (CK2A) was predicted to regulate six sites, four of which increased in abundance for proteins that function in core processes of DNA replication and damage responses [MCM3 (S711, S672) and HERC2 S2928] and protein translation (RPLP1/2, S104/105) (**Table 2**).

Meta-Analysis

Recently, Girotti et al. identified major regulation of phosphoproteins involved in cytoskeletal and cell invasion gene ontology and interaction modules occurs in melanoma cells with acquired BRAFi resistant *in vitro* (32). To investigate our results in the context of this and other data-sets a meta-analysis of datasets including Girotti et al. and Old et al. (a measure of short-term BRAFi in melanoma) was completed Table S7 in Supplementary Material (31, 32). Several sites in cytoskeletal proteins [e.g., Nestin (S680/768), Cortactin (S405) MAPB1 (S1793)] were commonly regulated in both our and the Girotti et al. (32) datasets. Less overlap is observed with the Old et al. screen, with only Cortactin (S405/S401) and NES (S768) regulated in all three data-sets. Additionally, we compared our data to an shRNA screen by Sun et al. for factors that influence the expression of EGFR in acquired drug resistance (13). Both SOX-10 and MTA2 were identified in the screen and both are measured in our proteomic data. We observed no change in SOX-10 protein expression, but an increase in MTA2 expression in drug resistance cells (Figure S1 in Supplementary Material).

Drug Resistant Cells are Sensitive to CK2 Co-Inhibition

Based on the measured increase in phosphorylation of several putative CK2 sites in LM-MEL-28R and our previous finding that CK2 inhibition is synergistic with BRAF inhibition in *BRAF(V600E)* mutated cells (43), we tested whether LM-MEL-28R was sensitive to CK2 inhibition. **Figure 4** demonstrates that the resistant line was sensitive to co-inhibition with CK2 inhibitor CX-4595 and that this inhibition was beyond what was observed for CK2 alone in the drug sensitive LM-MEL-28. A quantitative reduction in cell growth over several concentrations of inhibitor (<50% at 2.5 μM and <90% at 5 μM) was observed in LM-MEL-28R.

Discussion

Changes in the phosphoproteome of a *BRAF(V600E)* mutant melanoma cell line that occur after the development of drug (PLX4720) resistance *in vitro* are described here. Using a single step phosphopeptide enrichment followed by LC-MS analysis and label-free quantitation using the freeware Maxquant, we accurately detected and measured ~2700 phosphorylation events and 3556 proteins. Initially, we quantitated the viability of both unexposed and drug resistant populations in the presence of BRAFi. We observed that although viability was reduced after drug adaption, stable growth was maintained and cells were able to propagate in the presence of 5 μM PLX4720. Our *in vitro* system provided a suitable model to measure phosphorylation in drug resistance *in vitro*; and through kinases landscape analysis the activity of several kinases regulating these events was predicted.

Drug resistance in melanoma often occurs through reactivation of MAPK signaling despite continued exposure to the inhibitor (10). Through selective analysis of regulatory P-sites in the MAPK1 signaling pathway (ERK1/2) and downstream cell cycle regulators (RB1 and CDK1), our cell model was consistent with MAPK reactivation in resistant cells despite exposure to BRAFi. With this data set, we next investigated the relationship between protein phosphorylation and protein abundance. A measure for protein abundance was available for ~72% of measured P-sites. The measure for protein was based upon all identified unmodified peptides mapping to the same protein group as the P-site-derived peptide spectral match. This measure could account for the dominant effects of protein metabolism (synthesis and degradation) often observed in cells during long-term adaptive responses. As expected, the abundance of the majority of P-sites closely followed that of protein expression, with only a small subset (46 P-sites) exhibiting changes in abundance that could not be accounted for by changes in the rate of protein turnover. The most likely explanation for this divergence is the activity of kinase(s) or phosphatase(s) with specific regulatory functions in cellular adaption to BRAF inhibition. Focusing on kinases where the most probable enzyme–substrate relationship(s) can be mapped, potential regulatory mechanisms were identified and are discussed below and summarized in **Figure 5**.

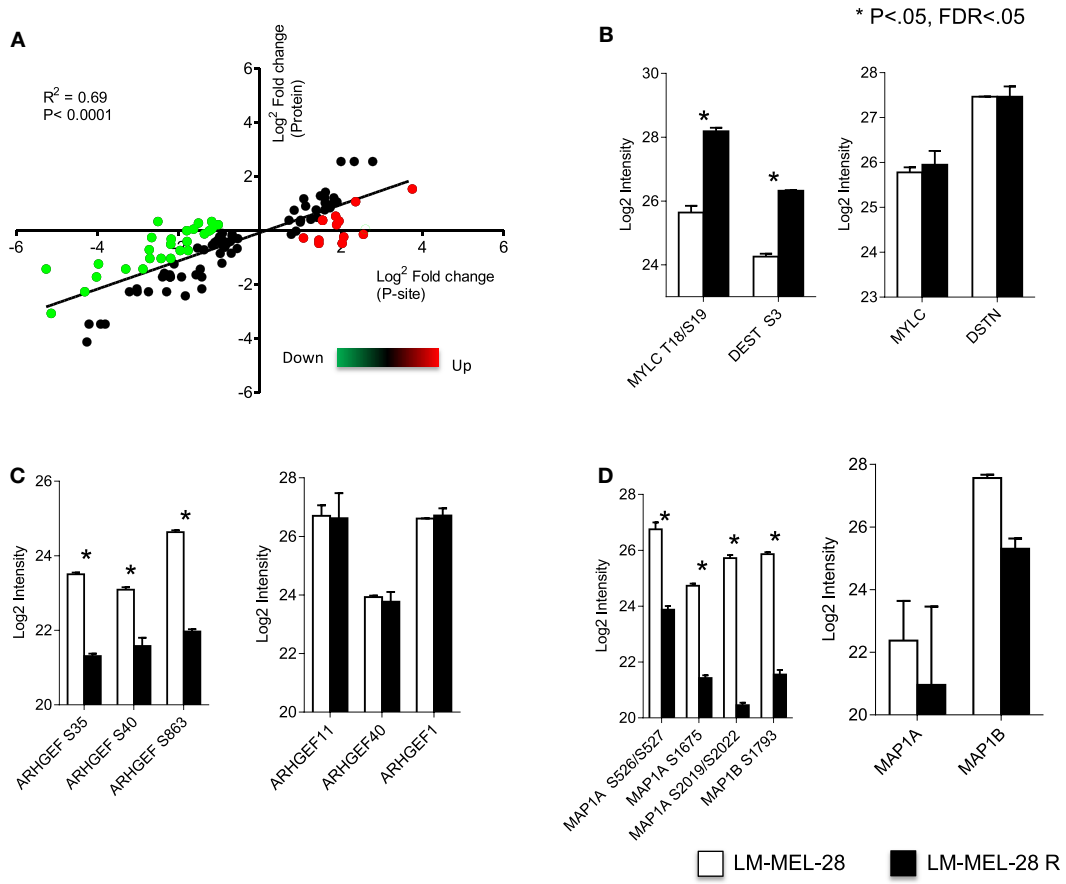

FIGURE 3 | Phosphorylation rate analysis. **(A)** Correlation of metrics for P-site effect with protein abundance effect measured between drug sensitive and resistant cell populations. Green = P-site effect twofold > protein effect. Red = P-site effect twofold < protein effect. Log_2 intensity of P-sites and total protein for key cytoskeletal regulators **(B)** myosin and destrin, **(C)** guanidine exchange factors (11, 40, and 1), and **(D)** microtubule-associated proteins 1A and 1B analyzed using a Student's *t*-test (error bars are SD).

Drug Resistance Induces De/Phosphorylation of the Cytoskeleton Regulators

Cytoskeletal changes are central to the phenotypic transitions that occur in tumor progression, altering invasiveness, metastasis, and resistance to therapy and this was reflected in our data. P-sites that are key regulatory residues in proteins controlling both actin and microtubule-based filaments were altered beyond protein metabolic control. Destrin (actin-depolymerization factor, ADF) is responsible for actin stability (44) and Ser-3 can be phosphorylated by LIM domain kinase 1 and 2 (LIMK1/2) to induce cytoskeletal reorganization to form stress fibers, membrane blebs and alter cell adhesion through the formation of F-actin in non-muscle tissue (45, 46). Myosin light chain (12A/B/9) phosphorylation of S19 and T18 provide evidence for increased activity of several up-stream kinases [myosin light chain kinase (MLCK), Rho-associated kinase (ROCK), citron kinase, leucine zipper interacting kinase ZIPK/DAPK3, and CDC42 binding kinase]. Functionally, phosphorylation of S19/T18 alleviates auto-inhibition of the MYLC globular heads and promotes interaction with actin to form bipolar filaments (47). The activating signals for this are diverse, ROCK2 is activated by the small GTP-binding protein RhoA, which is dependent on the activity of GEFs (48, 49). In the resistant cell population, a drop in phosphorylation of three different GEF's, including S35 in GEF11 was measured. Phosphorylation of GEF11 by the Cdc42 effector kinase PAK4 and p38 MAPK both lead to a drop in GEF activity (50, 51). Kinase enrichment analysis predicted a C-terminal site S35 to be a target for PAK4, indicating a novel site where PAK4 may regulate GEF activity in drug resistant cells. In the microtubule-based cytoskeleton, reduced phosphorylation of MAPs indicated altered tubule stabilization and several sites near microtubule binding domains could influence the tethering of cargo for transportation (52). P-site S1793 and S1797 in MAP2A are putative sites for GSK3-β. GSK3-β activity couples extra-cellular matrix (ECM) signaling to the actin/microtubule cytoskeleton during cell migration (53).

The regulation of cytoskeletal dynamics by Rho/ROCK and GEF signaling is a key driver of the phenotypic transitions or switching that can change the migratory phenotype of cells (54). In melanoma, transcriptional networks that have roles in mesenchymal and amoeboid transitions alter during metastasis and response to therapy (55). These changes may contribute to the intrinsic invasive phenotypes that have been observed in response

TABLE 2 | Regulated phosphosites in drug resistant cells and the prediction of putative regulatory kinases.

Protein names	Gene names	P-site	Diff[a]	*R*	PhosphoSitePlus kinase	NetworKIN
Protein kinase C alpha type	PRKCA	T497	2.21	+		PDHK1
Serine/threonine-protein phosphatase 2A regulatory subunit B" subunit alpha	PPP2R3A	S692	1.41			MAPK3, MAPK1, CDK1
Myosin regulatory light chain 12B	MYL12B	S25	2.74	+	ILK; DLK; DAPK1; ROCK1; AurB; smMLCK; DAPK3; CAMK1A; CRIK; MRCKA; PKCA; PAK1	
Myosin regulatory light chain 12B	MYL12B	T24	2.74	+	ILK; DLK; ROCK1; smMLCK; DAPK3; CRIK	ROCK2
G-protein coupled receptor 143	GPR143	S343	1.28			
Destrin	DSTN	S3	2.29	+	LIMK2; LIMK1; TESK1	
40S ribosomal protein S6	RPS6	S236	2.49	+	PKCD; p90RSK; p70S6K; RSK2	p70S6K
Nucleoprotein TPR	TPR	S2155	1.59			MAPK1
Choline-phosphate cytidylyltransferase A	PCYT1A	S347	1.66			
E3 ubiquitin-protein ligase HERC2	HERC2	S2928	1.32			CK2alpha
Choline-phosphate cytidylyltransferase A	PCYT1A	S343	1.59			
DNA replication licensing factor MCM3	MCM3	S756	1.16			CK2alpha
DNA replication licensing factor MCM3	MCM3	S717	1.15			CK2alpha
60S acidic ribosomal protein P1	RPLP1	S104	1.92			CK2alpha
60S acidic ribosomal protein P2	RPLP2	S105	1.80			GRK2, CK2alpha
Insulin receptor substrate 2	IRS2	S736	1.34			GSK3alpha, GSK3beta
Choline-phosphate cytidylyltransferase A	PCYT1A	S331	−1.25			
ATP-dependent RNA helicase DDX24	DDX24	S82	−1.31		Chk1	
Ras-related GTP-binding protein C	RRAGC	S95	−1.25			
Ankyrin repeat and SAM domain-containing protein 1A	ANKS1A	S663	−1.53			
Septin-9	SEPT09	S85	−1.24			CK1delta
CLIP-associating protein 1	CLASP1	S415	−1.75			NEK2, CaMKIIalpha
C-Jun-amino-terminal kinase-interacting protein 4	SPAG9	S730	−1.25			
C-Jun-amino-terminal kinase-interacting protein 4	SPAG9	S733	−1.25			
Rho guanine nucleotide exchange factor 40	ARHGEF40	S262	−1.80			
MAP7 domain-containing protein 1	MAP7D1	S113	−1.69			
Niban-like protein 1	FAM129B	S646	−1.04			
Sequestosome-1	SQSTM1	S272	−1.40	+	CDK1	MAPK3
60S ribosomal export protein NMD3	NMD3	T470	−1.81			
Niban-like protein 1	FAM129B	S641	−1.23			
Sequestosome-1	SQSTM1	T269	−1.62	+	CDK1	MAPK3
Syntaxin-12	STX12	S142	−1.87			
E3 ubiquitin-protein ligase	NEDD4L	S308	−1.06			PDHK1, GSK3beta
Rho guanine nucleotide exchange factor 11	ARHGEF11	S35	−1.70			PAK4
E3 ubiquitin-protein ligase	NEDD4L	S307	−1.30	+	PKACA; SGK1	TGFbR2
Rho guanine nucleotide exchange factor 1	ARHGEF1	S919	−2.42			
Microtubule-associated protein 1A	MAP1A	S764	−1.46			
Microtubule-associated protein 1A	MAP1A	S765	−1.46			
Microtubule-associated protein 1A	MAP1A	S1913	−1.89			
Cation-independent mannose-6-phosphate receptor	IGF2R	S2484	−2.74		CK2A1	CK2alpha
Nestin	NES	S680	−2.31			CDK1, CDK5
Microtubule-associated protein 1B	MAP1B	S1793	−2.05			GSK3beta
Microtubule-associated protein 1B	MAP1B	S1797	−2.05			GSK3beta
PDZ and LIM domain protein 4	PDLIM4	S112	−2.08			
Microtubule-associated protein 1A	MAP1A	S2257	−3.85			CK1alpha, CK1delta
Microtubule-associated protein 1A	MAP1A	S2260	−3.85			CK1alpha, CK1delta

[a]*Log_2 fold change corrected for protein effect (see text), R (known regulatory site).*

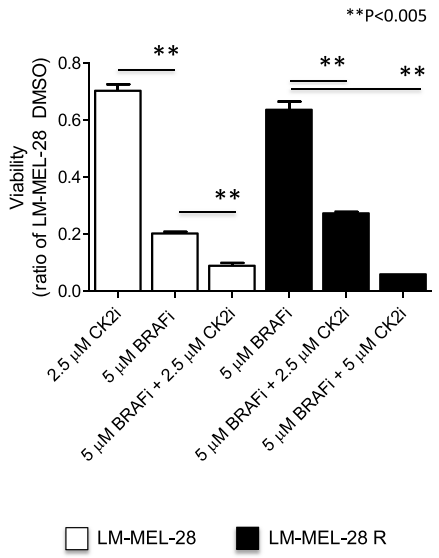

FIGURE 4 | CK2i of the drug resistant cell line (LM-MEL-28-R1). The effects of co-inhibition with the CK2 inhibitor CX-4945 and BRAFi (PLX4072) on proliferation of the drug resistant cell population LM-MEL-28-R1 were quantitated by viability assay, analyzed using a Student's *t*-test (error bars are SD).

to inhibitor therapy (25, 32). ROCK1 and 2 promote myosin phosphorylation and actin fiber formation to drive amoeboid movement, where cell membranes undergo extensive blebbing allowing cells to deform and pass through voids in the surrounding matrix (56). Co-inhibition of ROCK signaling has recently been shown to enhance the anti-proliferative effect of the BRAFi PLX4720 and supports our observation that drug resistant populations utilize ROCK signaling as a pro-survival mechanism (57). Through a meta-analysis of our results with other drug-exposed cell models (31, 32), a clear functional role can now be confirmed for the phosphorylation of proteins that function in the cytoskeleton. However, this analysis revealed that only 20/145 P-sites were shared between our data and that of Girotti et al. (32). This discrepancy could be explained by the myriad of possible mechanisms that can mediate drug resistance and is likely to depend on tumor genotype, heterogeneity, and locale; where each generates a unique cytoskeletal organization of maximum fitness. Outside of biological variation, technical differences in data generation and analysis may underlie the inconsistency in the P-sites identified. However, in Girotti et al., despite differences in methodology and only a twofold cut-off being applied to assign significance, of the sites that do overlap the majority of P-sites (13) exhibit a similar direction of regulation. While carrying out this meta-analysis, we also compared the proteomic data to the results of an shRNA screen for mechanisms of EGFR-based drug resistance in melanoma. Here, we identified MTA2 but not SOX-10 protein expression as altered in drug resistant cells. In Sun et al. (13), MTA2 is ruled out as a false-positive mediator by a targeted approach. These data provide an indication that LM-MEL-28, does not acquire BRAFi resistant through expression of EGFR receptor via SOX-10 attenuation; and could further explain the discrepancy in regulated P-sites with Girotti et al., where EGFR signaling is required for growth of the resistant cell population (32).

Melanosome Signaling Through G-Protein Couple Receptor-143 (OA1)

G-protein-coupled receptors can generate signals key to the development of resistance to BRAF inhibitor therapy (58). Here, we identified novel sites of phosphorylation (S331/S343) in the GPCR 143, also known as ocular albinism type 1 (GPR143/OA1), that increased in BRAFi resistant cells (**Table 1**; **Figure 5**). OA1 is a pigment-cell specific G-protein receptor for tyrosine, L-DOPA, and dopamine it also localizes to intracellular melanosomes and forms a key component of melanosome biogenesis and transport (59–61). OA1 regulates expression of the MITF, sustaining its expression and promoting melanocyte differentiation (62). Oncogenic BRAF can suppress MITF expression preventing normal melanocyte differentiation and promoting transformation to a de-differentiated proliferative state (63). In GPR143/OA1, S331/S343 reside in the C-terminal cytoplasmic domain and while no kinase prediction was assigned, phosphorylation here could drive the classical recruitment of beta-arrestins and lead to inactivation of G-protein signaling by OA1 leading to further de-differentiation observed in drug resistant tumors (64). OA1 also signals through the actin/microtubule cytoskeleton to regulate the transport of melanosomes from the perinuclear region to the cell periphery and could in-part drive phosphorylation dynamics of the cytoskeleton indicated above (59). Finally, S331 is directly adjacent to a two amino acid "WE" domain vital for the correct localization of OA1 protein to the melanosome (65). Mutation of WE > AA redirects OA1 to the plasma membrane (65). The role of OA1 phosphorylation in protein localization, melanosome and cytoskeleton signaling and how this facilitates drug resistance remain to be tested.

Key P-Sites in Known Signaling Nodes Reflect MAPK1 Reactivation

T497 in protein kinase C alpha (PKCα) increased in expression and phosphorylation in drug resistant cells. T497 in PKCα is located in the activation loop and phosphorylation is essential for full catalytic activity of PKCα (66). Phosphorylation of T497 by PDPK1 (PDK1) is classically dependent on phosphatidylinositol metabolism and PI3K activation induced by GPCR or TRK signaling (**Figure 5**). PKCα activity in melanoma is highly context dependent with roles in both oncogenesis and growth suppression (67). PKCα can contribute to activation of the MAPK pathway through direct phosphorylation of RAF substrates to activate ERK, or promote the c-Jun N-terminal kinase (JNK) MAP kinase pathway through association with RACK1 (68, 69). RACK1 shuttles PKCα to target the stress-related MAPK JNK for phosphorylation leading to constitutive activation of p38 MAPK signaling (69). Interestingly, we measured dephosphorylation of S730/733 in SPAG9 (JIP4), a scaffold protein involved in the spatial organization of MAP kinases and a mediator of c-Jun N-terminal kinase. The meta-analysis of Girotti et al. (32) supported this finding and while kinases/phosphatases able to regulate SPAG9 S730/733 phosphorylation remain unreported our data indicates that regulation may be key to the rewiring of MAPK

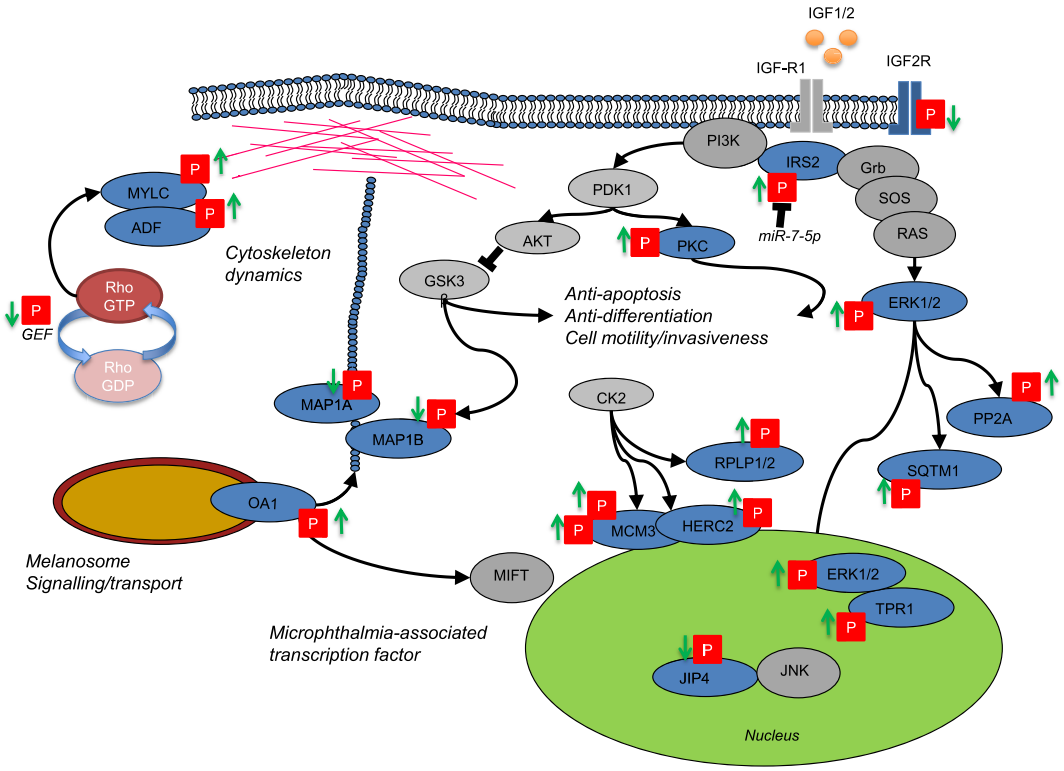

FIGURE 5 | Network cartoon summarizing the prominent differences in phosphorylation measured between BRAFi sensitive and resistant cell populations.

signaling in cells adapted to BRAFi. A further mechanism able to reactivate ERK1/2 signaling in BRAFi resistance was indicated by the increased phosphorylation of the ERK/1/2 substrate TPR at S2155 (**Table 1**). TPR is a nuclear pore complex protein and chromatin regulator that in response to ERK1/2 phosphorylation can bind and localize ERK1/2 to chromatin (70). During short-term exposure to BRAF and MEK inhibitors phosphorylation of TPR at S2155 reduces in *BRAF(V600E)* mutant cell lines (43). The recovery of TPR phosphorylation in the face of chronic BRAFi appears to be associated with the re-establishment of MAPK nuclear signaling in drug adapted cells.

Evidence for potential upstream mechanisms for ERK reactivation is provided by a change in phosphorylation of insulin receptor substrate (IRS2), a downstream effector of insulin-like growth factor receptor 1 (IGF-1R). IGF-1R signaling in cancer cells results from up-regulation of the receptor or its ligands (IGF-I and IGF-II) and contributes to the emergence of chemotherapeutic resistance. Insulin receptor substrate (IRS1/2) proteins transmit oncogenic signals through PI3K and ERK signaling modules (**Figure 5**). IRS1/2 also mediate the termination of IGF-IR signaling and resistance to PI3K inhibitors occurs through a reduction in this feedback inhibition [reviewed in Ref. (71)]. We measured phosphorylation of IRS2 at two sites, (i) S736 confidently localized and predicted to be regulated by GSK-3α/β and (ii) an ambiguous P-site (either S730/731/735/740 or Y742) in the same peptide. A lack of clarity for the position of the second site makes it difficult to predict, which kinase(s) may be responsible for the regulation that we observed. However, phosphorylation of IRS2 represents a key signaling process where cells become reprogramed through PI3K to activate PDK1-PKC/PKB(AKT) or through GRB2-SOS to activate the Ras-MAPK pathway directly (**Figure 5**) (72). IGF-1R has been shown to be up-regulated in drug resistant melanoma cell lines previously (12). Recently, IRS2 was also found up-regulated in BRAFi (PLX4032) resistant tumors and blocking or eliminating IRS or subsequent PI3K-mediated signaling may provide therapeutic potential (12, 73). More specifically, IRS-2 is a target of miR-7-5p found down-regulated in melanoma (74). miR-7-5p down-regulation is associated with increased cell migration and metastasis, and using RNA interference (RNAi) IRS-2 was shown to regulate this phenotype through the PKB/AKT signaling node (74, 75). In support of a role for IGF signaling, a decrease in the phosphorylation of a CK2 site (S2484) in the cytoplasmic domain of the insulin-like growth factor receptor II (IGFR2) known also as the CI-MPR receptor was detected in BRAFi drug resistant cells. This protein acts as both the receptor for IGF2 and mannose-6-phosphate and is implicated in both G-protein signaling and the targeting of lysosomal enzymes. In CHO cells, phosphorylation of this site regulates changes in the trafficking of the receptor in the Golgi-network (76) and down-regulation of plasma membrane IGFR2 is associated with increased signaling through IGF-R1 (77).

This study demonstrated a simple and effective approach to detect kinase activity important in the transition of cells from a BRAF sensitive to BRAF resistant phenotype. Once detected these kinase present themselves as potential targets for future

co-therapies. During our analysis, we detected increases in the phosphorylation and abundance of proteins involved in processes related to DNA metabolism. Several of these sites were substrates for CK2A, and we tested if long-term exposure to BRAFi provided protection from the synergistic inhibitory effects of protein kinase CK2A–BRAF co-inhibition previously observed in *BRAF(V600E)* mutant melanoma (43). This was not the case with an additive effect (>50%) being observed in both parental and resistant populations, suggesting that this drug combination could be effective in reducing the emergence of resistant cell populations. We have previously demonstrated that CK2 plays an important role in priming the activity of Akt through phosphorylation at S129, and that controlling CK2 activity is an effective strategy in preventing cell growth in BRAF melanoma and BRAF thyroid carcinoma (43). Notwithstanding the importance of Akt-driven growth pathway, CK2 is a ubiquitous serine/threonine kinase and in the nucleus plays an important role in modulating DNA-damage and repair machinery (78, 79); it is likely that the inhibitory effect of blocking CK2 leads to wide-spread modulation in numerous other pathways that support cell proliferation. Understanding the mechanistic significance of how CK2 regulates these other pathways in melanoma needs ongoing research.

Conclusion

A central paradigm of acquired drug resistance in BRAF mutant melanomas is the reactivation of MAPK signaling (10). In this work, a quantitative MS method measuring both the phosphoproteome and proteome was developed and implemented to describe novel phosphorylation-based signaling events in cells after this transition *in vitro*. We identified increased MAPK01 phosphorylation alongside well-known and novel protein phosphorylation events driven by this and other kinases. Regulation of key substrates in Rho/ROCK signaling axis provided evidence for cytoskeletal rearrangements able to facilitate a phenotypic switch in cell motility that evolve during BRAFi therapy. Importantly, our study provided evidence for signaling events in several proteins (IGFR2, IRS1, PKC, and GEFs) associated with established pathways of drug resistance in melanoma and other cancers (12, 80). Phosphorylation of IRS1 re-enforces the importance of IGF signaling in drug resistant melanoma as a valid target for co-therapy. Novel sites identified indicate new and untested mechanisms able to promote cell survival and these require confirmation *in vivo.* The diversity of drug resistance mechanisms discovered in melanoma so far indicates a need to develop an individualized approach to multi-targeted cancer treatment. The MS-driven phosphoproteomic method described here can be readily applied to the analysis of tumors biopsied before, during, and after treatment to provide a direct readout for kinases that are drug-able targets in relapsed patients.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fonc.2015.00095

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The emerging role of exosomes in epithelial–mesenchymal-transition in cancer

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Metastasis in cancer consists of multiple steps, including epithelial–mesenchymal-transition (EMT), which is characterized by the loss of epithelial-like characteristics and the gain of mesenchymal-like attributes including cell migration and invasion. It is clear that the tumor microenvironment can promote the metastatic cascade and that intercellular communication is necessary for this to occur. Exosomes are small membranous vesicles secreted by most cell types into the extracellular environment and they are important communicators in the tumor microenvironment. They promote angiogenesis, invasion, and proliferation in recipient cells to support tumor growth and a prometastatic phenotype. Although it is clear that exosomes contribute to cancer cell plasticity, experimental evidence to define exosome induced plasticity as EMT is only just coming to light. This review will discuss recent research on exosomal regulation of the EMT process in the tumor microenvironment.

Keywords: exosomes, cancer, extracellular vesicles, intercellular signaling

INTRODUCTION

Epithelial–mesenchymal-transition (EMT) is a process whereby epithelial cells undergo a shift in plasticity and acquire the ability to disseminate, invade, and cause metastasis. Established as a central process during the early stages of development, it is now clear that EMT has implications on cancer progression by triggering the loss of cell–cell adhesion to facilitate tumor cell invasion and remodeling of the extracellular matrix. While epithelial cells express high levels of E-cadherin and are closely connected to each other by tight junctions, mesenchymal cells express N-cadherin, fibronectin, and vimentin, have a spindle-shaped morphology and less tight junctions.

Intercellular crosstalk between neighboring and distant tumor cells and immune and stromal cells in the tumor microenvironment plays a large role in cancer development, the establishment of the mesenchymal state, and metastasis. Intercellular crosstalk can occur by direct cell to cell contact or via factors secreted into the extracellular environment. Extracellular vesicles, called exosomes, have become recognized as important in cellular communication (1). Unlike soluble factors secreted by cells, exosomes carry a concentrated group of functional molecules, provide protection to the transported molecules and serve as intercellular communicators not only locally but also systemically.

Exosomes are formed from inward budding of the limiting membrane of multi-vesicular bodies (MVB) and are released from the cell into the extracellular environment upon fusion of the MVB with the plasma membrane. Most prokaryotic and eukaryotic cells release exosomes, including cancer cells such as colorectal (2), lung, breast, glioblastoma (GBM), ovarian, and melanoma (3). Exosomes from different cellular types contain a common set of molecules, as well as cell type-specific components. For example, exosomes derived from cancer cells contain proteins that reflect the endosomal origin of exosomes as well as cellular oncogenic drivers including receptor tyrosine kinases (RTKs), oncoproteins, phosphorylated proteins, and miRNA (2, 4–6). After release into the extracellular environment, exosomes act as discrete vesicles trafficking to distant and proximal recipient cells where they alter cell signaling and phenotype by transfer of bioactive molecules. Exosomes transfer their messages in different ways. Firstly, they can activate target cells through the transfer of ligands such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) (7, 8), and epidermal growth factor (EGF) (9). Secondly, they can transfer receptors such as mutant EGFR (10) and HGFR (11) from one cell to another by fusion with the plasma membrane of recipient cells (10). This results in transfer of oncogenic activity via activation of growth factor signaling pathways in recipient cells (11, 12). The third mechanism of action involves endocytosis of the exosome and subsequent transfer of molecules directly into the cytosol of the recipient cell. These can include phosphorylated P13K, AKT, mTOR, cyclins, and cyclin-dependent kinases (13, 14) and miRNA, which can functionally repress target genes in the recipient cell (15).

Over the last decade, a number of studies have demonstrated that exosomes are mediators of the metastatic process. Exosomes derived from both normal and cancer cells can promote angiogenesis (16–19), invasion (20–23), and proliferation (24–26) in recipient cells to support tumor growth.

CHANGES IN EXOSOME COMPOSITION ACCOMPANY THE TRANSITION TO A MESENCHYMAL STATE

Epithelial–mesenchymal-transition entails morphological and phenotypic changes to a cell. To assess the composition of exosomes released from cells following these changes, several

groups have induced EMT via transformation with oncogenic proteins such as Ras or EGFR (27–29). Exosomes released from Madin–Darby canine kidney (MDCK) cells transformed with oncogenic H-Ras contained the EMT marker vimentin, in addition to matrix metalloproteases (MMPs), integrins, and key and core splicing complex components (29). Epithelial markers including E-cadherin and EpCAM were downregulated relative to exosomes from untransformed cells. It was postulated that exosomes from the transformed cells were capable of inducing EMT in recipient cells although no functional experiments were performed to validate this. Proteomic studies on EGFR (coupled with blockade of E-cadherin) induced EMT in A431 and DLD-1 epithelial cancer cells, revealed coordinated loss of EGFR and tissue factor (TF) from the cells (27). This coincided with an increase in exosome release, selective upregulation of TF in exosomes, and expression of 30 additional proteins unique to the mesenchymal cell-derived exosomes (28). The mesenchymal-like cells transferred TF to recipient endothelial cells via exosomes rendering the recipient cells procoagulant, suggesting EMT promotes exosome release and shedding of TF from cells via exosomes (27).

Jeppesen et al. studied the protein content of exosomes derived from a human bladder carcinoma cell line without metastatic capacity relatively to two isogenic derivate metastatic cell lines formed in the lung and liver of mice. Although proteins associated with EMT were found in exosomes derived from the metastatic cells (30), no functional studies correlating changes in protein content with alterations in exosome function were carried out, so it is unclear in this case if exosomes from the metastatic cell line had an increased metastatic potential. With that said, exosomes from a range of mesenchymal-like breast and ovarian cancer cell lines differentially impacted on recipient cells compared to epithelial-like cell lines (31). Exosomes from the mesenchymal-like cell lines contained increased angiogenic molecules including PDGF, IL-8, and angiogenin suggested to promote AKT phosphorylation and subsequent activation of recipient endothelial cells (31).

EMT INDUCERS ARE ASSOCIATED WITH EXOSOMES

The protein composition of exosomes has been analyzed extensively, predominantly by mass spectrometry to reveal a defined subset of cellular proteins common to exosomes originating from a variety sources and species (32–35). Inducers of EMT have been found in association with exosomes including TGFβ (36), TNFα, IL-6, TSG101, AKT, ILK1, β-catenin (37, 38), hepatoma-derived growth factor, casein kinase II (CK2), annexin A2 (30), integrin 3 (39), caveolin-1 (40), and matrix metalloproteinases (41–44). Functional studies to demonstrate that exosome associated EMT inducers promote a prometastatic phenotype are outlined below.

The WNT signaling pathway participates in EMT by inhibiting glycogen synthase kinase-3β (GSK3β) to stabilize β-catenin, promoting a gene expression program that favors EMT (45). Exosomes released from human and *Drosophila* cells contain WNT (46, 47), which can be transferred and activate WNT signaling in recipient cells (48–50). Luga et al. observed that WNT containing exosomes derived from cancer associated fibroblasts (CAFS) promoted motility and metastasis by activating autocrine WNT-planar cell polarity signaling in recipient breast cancer cells (48). Similarly, mesenchymal stem cell (MSC) and macrophage-derived exosomes (51) promoted migration and/or invasion of breast cancer via activation of WNT signaling (49). In melanoma, recombinant WNT5A induces the release of soluble mediators including IL-6, IL-8, VEGF, and MMP2 in association with exosomes (52) suggesting that not only does exosomal WNT promote EMT in recipient cells but it changes the composition of the released exosome to promote further EMT. Kock et al. examined the contribution of exosomes to cancer population equilibrium and tumor heterogeneity (53). They showed that diffuse large B-cell lymphomas possess a self-organized infrastructure comprising two populations of cells, where transitions between clonogenic states could be modulated by exosome-mediated WNT signaling (53). This study goes some way in broadening our understanding of the complex processes that maintain tumor cell heterogeneity and highlights exosomes as key players in this process.

Hypoxia in the tumor environment can promote EMT and several studies have provided evidence that hypoxia promotes the release of exosomes from different tumor cell types including breast, glioma, leukemia, and prostate (38, 54–57). Exosomes released by prostate cancer cells under hypoxic conditions contain more TGFβ IL-6, TNFα, and MMP, TSG101, AKT, ILK1, and β-catenin (38), suggesting that they could differentially modulate recipient cells compared to exosomes from normal cells. Indeed, exosomes released from A431 carcinoma (58), glioma cells (55), and leukemia cells (54) promoted angiogenesis in recipient cells (16, 55). Similarly, exosomes derived from hypoxic GBM cells promoted tumor cell survival by inducing angiogenesis both *in vitro* and *ex vivo* through phenotypic modulation of endothelial cells and increased autocrine, promigratory activation of GBM cells (57).

Latent membrane protein 1 (LMP) of Epstein–Barr virus (EBV) contributes to the metastatic phenotype of nasopharyngeal carcinoma (NPC) by inducing EMT. Aga et al. (22) investigated if LMP1-positive exosomes could mediate EMT. They demonstrated that LMP1 positive exosomes and exosomal HIF1α modulate expression of EMT markers in recipient cells (22). Following treatment with LMP1-positive exosomes, recipient cells expressed less E-cadherin and more N-cadherin along with morphological spindle-like changes in cell shape indicative of EMT (22). Although exosome concentration was not reported and downstream signaling pathways associated with EMT were not examined, it is clear that LMP1-positive exosomal transmission of HIF1α correlates with EMT-associated changes in the cadherin expression profile in recipient cells.

A growing number of miRNAs have been implicated in the regulation of EMT-related pathways in cancer (59) and in recent years exosomes have been reported to contain nucleic acid such as DNA, RNA, non-coding RNA, and miRNA (60–62). MiR-223, a miRNA specific for IL-4-activated macrophages, could be transported from macrophages to breast cancer cells via exosomes (63) to promote breast cancer cell invasion via modulation of the β-catenin pathway. Similarly, exosomes released from bone marrow-derived mesenchymal cells promoted multiple myeloma (MM) formation in an animal model by transfer of exosomal miR-15a (64). Josson et al. recently performed one of the first studies to show that transfer of stromal-derived exosomal miRNA results

in morphologically and biochemically defined EMT in cancer cells (65). Exosomes were isolated from normal prostate stromal cells overexpressing miR-409. Exosome associated miR-409-3p and -5p decreased the expression of target genes in prostate cancer cells and increased proliferation. Interestingly, 6 weeks after maintaining the prostate cancer cells in stromal cell media, the prostate cancer cells underwent EMT, which was biochemically defined by decreased E-cadherin and increased vimentin mRNA expression. *In vivo,* co-injection of prostate cancer cells and miR-409-overexpressing stromal fibroblasts resulted in tumor cells expressing miR-409 and enhance tumor growth suggesting that miR-409 was secreted out of stromal fibroblasts and taken up by the adjacent tumor. Further *in vivo* modeling however is required to conclude that stromal-derived exosomes were responsible for transfer of miR-409 to surrounding cancer epithelial cells and subsequent tumor growth.

EXOSOMES RELEASED FROM TUMOR CELLS PROMOTE PHENOTYPE CHANGE IN STROMAL CELLS

The tumor microenvironment consists of a complex network consisting of an extracellular matrix populated by CAFs, endothelial cells, and immune cells. Exosomes derived from tumor cells communicate with stromal cells and vice-versa to promote tumor growth. MSCs have multi-lineage potential and can differentiate into a variety of cell types including tumor stromal cells, which are pro-tumorigenic. One way they do this is by promoting differentiation of MSCs, in some cases via mesenchymal-to-epithelial transition (MET).

Ovarian cancer cell-derived exosomes can induce adipose tissue-derived MSCs (ADSC) to exhibit the characteristics of CAFs, by increasing expression of TGFβ and activation of Smad-dependent and -independent pathways (9). Similarly, gastric cancer exosomes trigger differentiation of umbilical cord-derived MSCs to CAFs through the TGFβ/Smad pathway (66) and breast and prostate cancer-derived exosomes can induce a myofibroblastic phenotype (67, 68). Together, these studies show that via activation of both Smad-dependent and -independent pathways, tumor-derived exosomes can hijack MSCs to promote a prometastatic environment. In some cases, this process appears dependent on TGFβ1 expressed at the exosome surface in association with the transmembrane proteoglycan betaglycan (67). Although existing in a latent state, this complex was fully functional in eliciting Smad-dependent signaling in recipient cells. Interestingly, myofibroblasts generated using soluble TGFβ1 were not pro-angiogenic or tumor-promoting, suggesting that exosomal TGFβ1 is required for the formation of tumor-promoting stroma (36).

In an elegant series of experiments, Abd Elmageed et al. demonstrated that tumor-tropic patient-derived ADSCs primed with prostate cancer cell-derived exosomes undergo genetic instability, MET, oncogenic transformation, and develop prostate tumors *in vivo* (69). Oncogenic transformation was associated with down-regulation of tumor suppressors upon delivery of prostate cancer-derived exosomal oncogenic H-ras and N-ras transcripts, Rab proteins, and oncogenic miRNA.

CONCLUSION

Exosomes play an important role in the development and progression of cancer. The studies outlined above highlight their role in the regulation of EMT-related pathways and suggest that tumor and stromal cells can regulate the invasiveness of cancer cells through exosome-mediated delivery of protein and miRNA. In the last decade, there has been an exponential increase in the number of studies aiming to understand the biology and composition of exosomes. These studies established that exosome composition changes upon transition to a mesenchymal state and that EMT inducers are associated with exosomes. In the last 2 years, experimental evidence has come to light defining exosome induced plasticity in recipient cells as EMT. Future investigations should further reveal how multiple cellular populations communicate via exosomes to promote a premetastatic phenotype and how exosomes can be employed for diagnostic and prognostic purposes to improve patient outcome.

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Embryonic chicken transplantation is a promising model for studying the invasive behavior of melanoma cells

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Epithelial-to-mesenchymal transition is a hallmark event in the metastatic cascade conferring invasive ability to tumor cells. There are ongoing efforts to replicate the physiological events occurring during mobilization of tumor cells in model systems. However, few systems are able to capture these complex *in vivo* events. The embryonic chicken transplantation model has emerged as a useful system to assess melanoma cells including functions that are relevant to the metastatic process, namely invasion and plasticity. The chicken embryo represents an accessible and economical 3-dimensional *in vivo* model for investigating melanoma cell invasion as it exploits the ancestral relationship between melanoma and its precursor neural crest cells. We describe a methodology that enables the interrogation of melanoma cell motility within the developing avian embryo. This model involves the injection of melanoma cells into the neural tube of chicken embryos. Melanoma cells are labeled using fluorescent tracker dye, Vybrant DiO, then cultured as hanging drops for 24 h to aggregate the cells. Groups of approximately 700 cells are placed into the neural tube of chicken embryos prior to the onset of neural crest migration at the hindbrain level (embryonic day 1.5) or trunk level (embryonic day 2.5). Chick embryos are reincubated and analyzed after 48 h for the location of melanoma cells using fluorescent microscopy on whole mounts and cross-sections of the embryos. Using this system, we compared the *in vivo* invasive behavior of epithelial-like and mesenchymal-like melanoma cells. We report that the developing embryonic microenvironment confers motile abilities to both types of melanoma cells. Hence, the embryonic chicken transplantation model has the potential to become a valuable tool for *in vivo* melanoma invasion studies. Importantly, it may provide novel insights into and reveal previously unknown mediators of the metastatic steps of invasion and dissemination in melanoma.

Keywords: embryonic chicken transplantation, melanoma, epithelial-to-mesenchymal transition, invasion, neural crest cells

INTRODUCTION

Melanoma is a frequent malignant neoplasm, and metastasis of melanoma is the main cause of death in these patients (1–3). Metastasis is a complicated, multi-step process that is still poorly understood. Model systems have been developed to recapitulate cellular invasion, which is the early crucial step of the metastatic cascade (4, 5). Transwell invasion assays using the reconstituted Matrigel in Boyden chamber inserts have been utilized to study melanoma cell invasion *in vitro* (6, 7). However, the lack of *in vivo* microenvironmental factors may confound the results. Due to the transient and rare nature of the invasive process, there is a paucity of techniques for studying and visualizing motile melanoma cells *in vivo*.

Here, we describe a model using transplantation of melanoma cells into the neural tube of the embryonic chicken that is gaining traction for melanoma tumor invasion studies *in vivo*. This model was originally reported by Drews et al. for assessing melanoma cell behavior *in vivo* (8) and has been subsequently used by several other groups (9–13). It involves injecting melanoma cells into a microenvironment that is populated with neural crest cells that undergo an epithelial-to-mesenchymal transition (EMT) to exit from the neural tube and undergo extensive migration, to eventually populate a great diversity of areas in the embryo (14, 15). The developing chicken has been used extensively as a model to study developmental EMT and neural crest biology since the neural crest cells give rise to a wide variety of cell types including melanocytes, peripheral neurons and glia, secretory cells of the medulla, and bone and cartilage cells in the head (16–19).

Recently, this model has been adapted for studying cancer cell invasion since cancer cells use molecular programs, which are comparable to those utilized by migrating neural crest cells in the embryo (16, 20). Indeed, the molecular mechanisms for tissue interaction, penetration, and remodeling that are seen during

EMT in melanoma appear to have much in common with those seen with their ancestral cells undergoing similar processes in the neural crest. For instance, over 50 percent of genes associated with EMT and cell migration were induced in melanoma cells following exposure to the neural crest microenvironment (21, 22). Furthermore, transplanted melanoma cells respond to cues within the host embryonic microenvironment and subsequently mimic many aspects of neural crest cell motility without forming tumors (11, 22).

The key advantages of using this model are, first, the easy access to the developing embryo to visualize *in vivo* tumor cell behavior and its ability to respond to microenvironmental cues (23). Second, to clarify which specific factors and signaling pathways in embryonic development also participate in maintenance of tumor cell plasticity. Third, the legal and ethical restrictions are limited with early developmental stages of the chick embryo before hatching. Fourth, the transplants are not rejected (13). Finally, the short time frame required from start of the experiment to readout, it's relative affordability and the lack of need for a specialized housing facility renders the chick embryo a suitable model system.

We and others have used this model to investigate the role of candidate genes in invasion *in vivo* by perturbing gene expression with morpholino or siRNA approach (10–12, 24). The ease of integrating this model with intravital imaging techniques and laser capture microdissection assisted gene profiling strategy has enabled the examination of dynamic temporal and spatial gene regulation exhibited by motile cells *in vivo* (22). Alternatively, invasive behavior of other tumor cells that share ancestral relationship with neural crest cells could be studied using this method (25). Although the avian embryo offers many advantages, it may be preferable to compare the results obtained in mammalian embryos, which are presumably more closely related to the environment found in human embryos (26).

We have previously reported the classification of metastatic human melanoma cell lines into epithelial- and mesenchymal-like cells based on gene expression profiling and functional assays (12). Herein, we have compared the behavior of epithelial- and mesenchymal-like melanoma cells *in vivo*. LM-MEL-8 is an epithelial-like cell line that lacks invasive ability *in vitro,* whereas LM-MEL-3 is a mesenchymal-like melanoma cell line with high invasive ability *in vitro*. Both cells were chosen for further study following transplantation into the chick neural tube where invasive ability was assessed *in vivo*. Although previous studies have transplanted melanoma cells at the head level of chick neural tube, we primarily performed transplantation at the trunk level of the chick embryos as the melanocyte progenitors arise predominantly from neural crest cells at this location.

MATERIALS

REAGENTS

- Melanoma cell lines LM-MEL-3 and LM-MEL-8 were established from resected melanoma metastases. All tissue donors provided written informed consent for tissue collection and research, which was covered by protocols approved by the Austin Health Human Research Ethics Committee, Melbourne, Australia (approval number H2012/04446). All cell lines were matched with their donors by HLA-typing. Cells were cultured in RF-10 media (RPMI1640 supplemented with 10% fetal calf serum) as described previously (27).
- Adult normal human melanocytes (Lonza Australia).
- 1× Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Life Technologies).
- TrypLE^{TM} (Life Technologies).
- Vybrant labeling DiO dye (Life Technologies).
- Fertilized chicken embryos (Research Poultry Farm, Australia).
- 70% ethanol.
- Sterile phosphate buffered saline and 1% penicillin/streptomycin (PBS-Pen/Strep).
- India ink (Pelikan Fount).
- Paraformaldehyde, 4% (wt/vol): add 30 ml of 1× DPBS to 10 ml of 16% (wt/vol) paraformaldehye (ProSciTech).
- 30% sucrose solution in DPBS.
- Tissue-Tek OCT solution (Olympus).
- Liquid nitrogen.
- Fluorescent mounting media (Dako).

EQUIPMENT

- Egg incubator (Bell South).
- Dissection microscope.
- Fluorescent stereomicroscope with epi-illuminaton (SteREO Lumar V12 Carl Zeiss).
- Forceps size 3 and size 5.
- Scissors.
- Microscissors.
- Syringe (5 cc with a 18G needle and 1 cc with a 27G needle).
- Thin-walled glass capillaries (Harvard apparatus GC150T) pulled to generate a fine tip.
- Sharpened tungsten wire needles.
- Transparent tape.
- Sylgard petridish.

METHODS

The experimental design of the protocol is depicted in **Figures 1** and **2**.

Incubation of fertilized eggs:

1. Place fertilized chicken eggs at 38°C in a humidified incubator for 48 h to allow the embryos to reach the desired stage of development (28).
2. Turn the eggs after 24 h to ensure that the yolk does not attach to the eggshell.

Labeling and hanging drop culture of melanoma cells:

3. Cells can be pre-treated with siRNA or other factors prior to culturing as a hanging drop (11–13).
4. Aspirate media and wash cell monolayer with 1× DPBS. Add 3 ml of TrypLE^{TM} to the flask and incubate at 37°C until cells detach.
5. Add 3 ml of media to the flask and transfer cells into a 15 ml conical tube. Centrifuge at 1800 rpm for 5 min.
6. Aspirate the media and suspend cells at a density of 1×10^6 in 1 ml 1× DPBS.
7. Add 1 µl of the Vybrant cell labeling DiO dye to the cell suspension and mix well by pipetting.

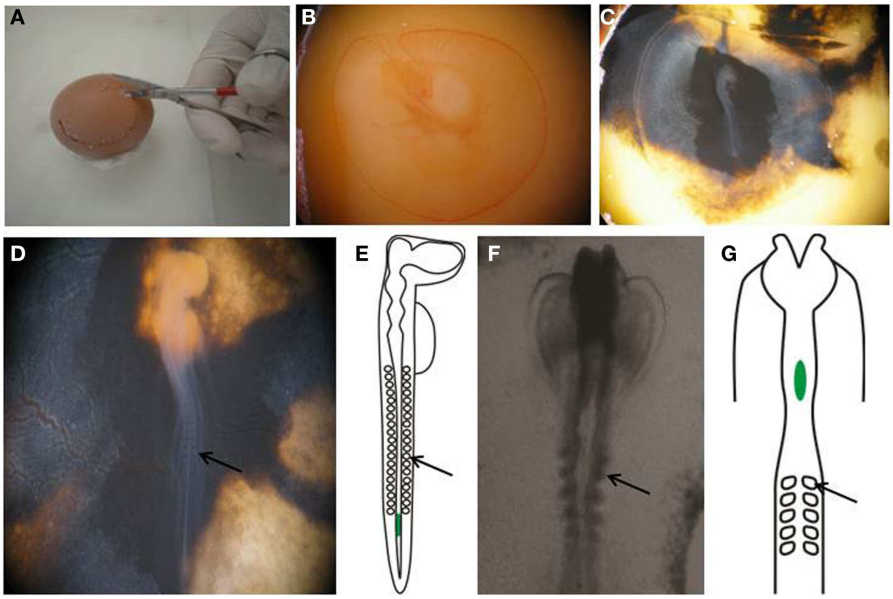

FIGURE 1 | Preparing egg for transplantation. **(A,B)** Create a window in the eggshell and **(C)** inject with India ink to better visualize the embryo. **(D)** Perform staging of the embryo by counting the number of somites. **(E)** An E2.5, or HH stage 14 embryo, showing the location of the transplanted melanoma cells in green. **(F)** Wholemount view of the cranial region of an E1.5, or HH stage 8+ embryo. **(G)** Schematic of an E1.5/HH8+ embryo, showing the location of transplanted melanoma cells in green. Arrows point to somites.

8. Incubate cells with labeling solution at 37°C for 20 minutes. The DiO label is a lipophilic tracer dye. After 20 min, add 10 ml of RF-10 media.
9. Centrifuge the tubes at 1800 rpm for 5 min. Aspirate the supernatant and resuspend cells in media. Repeat the wash procedure two more times.
10. Resuspend cells in media at a density of 5×10^4 cells per 25 μl media. Pipette four 25 μl drops of resuspended cells on the lid of a 35 mm culture petridish. Place the lid on the culture dish filled with 2 ml media. Incubate cells at 37°C for 24 h under standard cell culture conditions.

Preparation of eggs:

11. Rinse eggs with 70% ethanol and wipe clean. Do not turn eggs over at this time, the embryo will be on the uppermost side.
12. Mount the egg on the stage of a dissection microscope. Using a 5 cc syringe fitted with an 18 gauge needle, pierce a small hole in the eggshell and withdraw 3 ml of albumin from the caudal region of the egg.
13. Cut a circular hole in the eggshell with scissors to create a window to enable visualization of the yolk (**Figures 1A,B**).
14. Using a 1 cc syringe fitted with a 27 gage needle, inject 10% India ink in PBS-Pen/Strep below the blastodisc to enable better visualization of the embryo (**Figure 1C**).
15. Count the somite pairs in the embryo to determine the embryonic stage (28). Stage HH 12-14 embryos are used for this procedure (**Figure 1D**).
16. Make a small nick with a needle in the vitelline membrane overlying the most caudal end of the neural tube. Other procedures use similar processes to prepare chick embryos for grafting experiments (29–31).

Transplantation of melanoma cells into the neural tube:

17. Melanoma cells cultured as hanging drops form clusters (**Figure 2A**). Disaggregate the large cell clusters with needles into similar sized smaller clumps of approximately 500 to 1200 cells (**Figure 2B**). We use this method as single cells injected into the neural tube often float out. The clumps can be variable in size but they are malleable. The size of clump that can be inserted into the neural tube is limited by the width of the neural tube; therefore, the width of the clump is typically reproducible following placement in the neural tube, with greater variability seen in the length of the clump along the neural tube.
18. Aspirate a small clump of melanoma cells into the pulled glass pipette (**Figure 2C**).
19. Transplant the melanoma cells into the lumen of the neural tube in the trunk region, just caudal to the most caudal somites (**Figures 1E** and **2E**). The glass pipette can be guided into the neural tube through the open neuropore, and cells injected into the neural tube caudal to the last somite (**Figure 2E**). Alternatively, a clump of cells can be maneuvered into the neural tube using tungsten wire needles through the open neuropore or a slit cut carefully into the dorsal neural tube closer to the most caudal somite. This is a delicate process with variable success rate. It is useful to incubate approximately 2 dozen eggs to generate roughly 6–12 injected embryos.
20. For transplantation into the cranial region, eggs are incubated for 1.5 days prior to injection, and embryos used between stages HH 8–10. Melanoma cells are placed into the neural tube at the

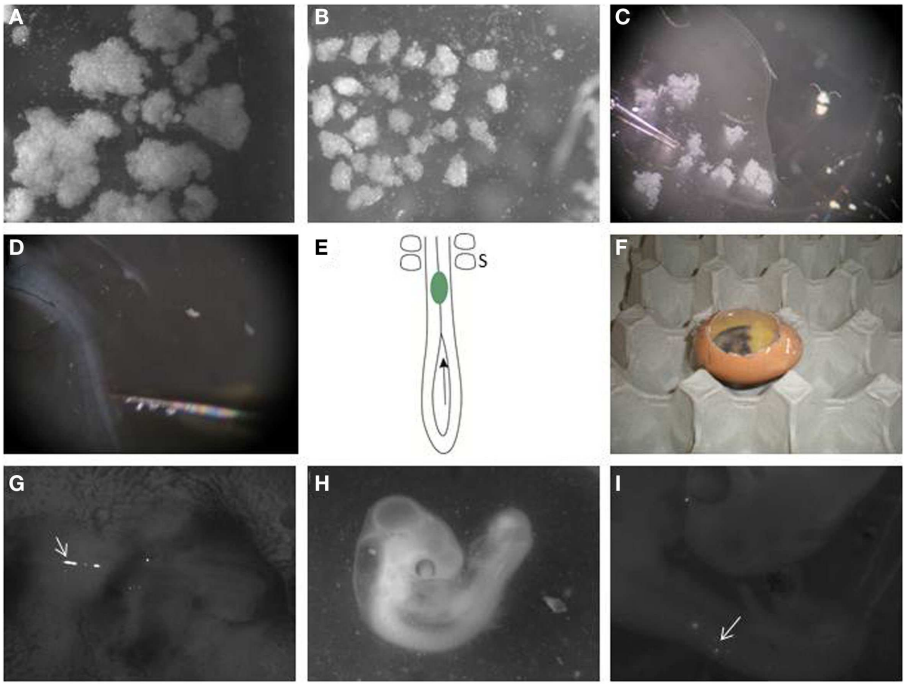

FIGURE 2 | Transplanting melanoma cells into the embryonic chick neural tube. **(A)** Melanoma cells are labeled with DiO and allowed to grow as hanging drop culture. They form variable sized aggregates after 24 h. **(B)** Large clumps are dissected into similar sized small clusters using dissection tools. **(C)** A pulled glass pipette is used to aspirate a small cluster. **(D)** Melanoma cells are transplanted to the neural tube in the trunk region of the embryo. **(E)** Schematic of the caudal trunk region of an E2.5 embryo. The arrow is located in the posterior neuropore, showing the entry for a cluster of melanoma cells (green), which can then be pushed or injected into the neural tube so it lies just caudal to the most caudal somites (S). **(F)** Eggs are then re-sealed with transparent tape and allowed to incubate for 48 h. **(G)** Clusters of DiO-positive melanoma cells can be seen in the trunk neural tube immediately after injection when imaged using a fluorescence stereomicroscope (arrow). The head of the embryo is to the right. **(H)** Embryo is processed 2 days following injection. **(I)** DiO-positive melanoma cells can be seen under a fluorescence stereomicroscope in the trunk neural tube and in the surrounding tissue (arrow).

level of rhombomere 1–2 (HH 8) to rhombomere 4 (HH 10) (**Figures 1F,G**).

21. Seal the window in the eggshell with transparent tape and re-incubate eggs in the incubator at 38°C for 48 h to allow melanoma cells to invade the host tissue (**Figure 2F**). Following injection, fluorescently labeled cells can be seen in the lumen of the trunk neural tube (**Figure 2G**).

Harvesting chick embryo for image processing:

22. After 2 days, select the surviving embryos and harvest the embryos individually and place it in 1× DPBS in a petridish (**Figure 2H**).
23. Under the fluorescence stereomicroscope, dissect out the membranes covering the embryo using microscissors and microforceps.
24. Place embryos dorsal side up with needles on a Sylgard petridish. Locate the fluorescent labeled melanoma cells within the embryo using the upright stereomicroscope (**Figure 2I**).
25. Whole mount pictures are captured of the embryo containing fluorescently labeled melanoma cells.
26. Using microscissors dissect the region of interest and fix it in 4% paraformaldehyde for 1 h on a shaker.
27. Wash the tissue in 1× DPBS and embed the embryos in 30% sucrose in DPBS overnight at 4°C.
28. Transfer the tissue into a cryomold filled with Tissue-Tek OCT Compound. Using forceps, position the tissue so cross-sections can be cut and remove air bubbles.
29. Freeze in liquid nitrogen. Section tissues at 20 μm using a cryostat. Sections can be frozen and stored prior to mounting.
30. Mount slides in fluorescent mounting media or counterstain with antibodies to visualize proteins of interest. DiO does fade over time; therefore, it is advisable to examine and image sections shortly after processing and mounting. Melanoma cells can also be detected using anti-human antibodies, such as Abcam ab7856 mouse anti-HLA DR + DP + DQ (CR3/43), used at 1:200 dilution with a fluorescent secondary anti-mouse antibody.

RESULTS AND DISCUSSION

Typical results of motility of melanoma cells within the embryonic chicken are shown in **Figure 3**. DiO was used to mark the melanoma cells; however, anti-human HLA antibody also identifies melanoma cells (Figure S1 in Supplementary Material). We initially transplanted melanoma cells into both head and trunk regions of the neural tube. Analysis of two cell lines (LM-MEL-3, LM-MEL-8) showed that cells transplanted into the head region migrated far less readily than cells transplanted into the trunk

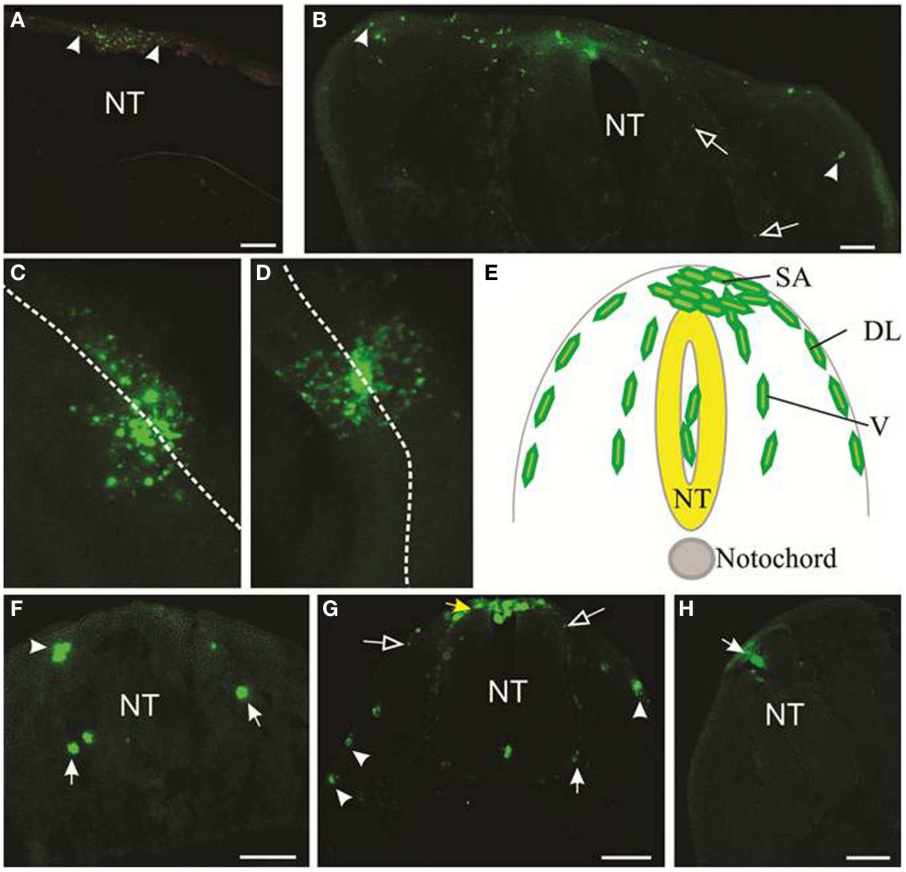

FIGURE 3 | Migration of melanoma cells from the neural tube. Melanoma cells labeled with DiO are green. **(A,B)** Cross-sections of cranial **(A)** and trunk **(B)** levels showing the extent of migration of LM-MEL-3 cells at 4 days post-injection (DPI). Arrowheads indicate the cells that migrated the greatest distance from the middle of the dorsal neural tube. **(A)** LM-MEL-3 cells are located dorsal to the hindbrain. **(B)** LM-MEL-3 cells migrate further in trunk regions at 4 DPI. **(C,D)** Dorsal view of melanoma cells migrating from the neural tube at 2 DPI. White dotted line indicates the midline of the neural tube. **(C)** LM-MEL-3. **(D)** LM-MEL-8. **(E)** Schematic of a cross-section through the trunk region of the neural tube during neural crest or melanoma cell migration. Melanoma cells are injected into the lumen of the neural tube (NT) and some remain in this position without migrating. Cells emerge from the neural tube at the dorsal surface (top) into the staging area (SA). They then migrate along one of two major pathways: the dorsolateral pathway under the ectoderm (DL) or the ventral pathway (V). **(F,G)** Cross-sections of trunk at 2 DPI showing the location of **(F)** LM-MEL-3 and **(G)** LM-MEL-8 cells. Cells can be seen along dorsolateral (arrowheads) and ventral (arrows) pathways. The SA is indicated with a yellow arrow in **(G)**. **(H)** Melanocyte cells are found in the roof of the neural tube (arrow) but very few migrate outside the neural tube. NT, neural tube. Scale bar is 100 μm. Open arrows indicate speckles of DiO that have transferred to non-melanoma cells.

region. A comparison of the migration of LM-MEL-3 at 4 days post-injection is shown for the cranial region (**Figure 3A**, $n = 4$) and the trunk (**Figure 3B**, $n = 3$). A similar difference in migration between head and trunk regions is also seen using LM-MEL-8 ($n = 5$ for each of head and trunk), which is a cell line with an epithelial-like phenotype and deemed to be non-invasive on the basis of *in vitro* characterization.

Previous work using the chicken embryo to assess melanoma migration has used cranial regions (11) and trunk (9). Kulesa et al. found that at cranial levels, a highly aggressive melanoma cell line C8161 migrated well, while a poorly aggressive cell line C8161 was less responsive (11). In our hands, both LM-MEL-3 (invasive mesenchymal-like) and LM-MEL-8 (non-invasive epithelial-like) cell lines showed poor migration at cranial levels and much greater migration at trunk levels, indicating that there were significant differences in the environmental signals between these regions. During normal development, the majority of melanocytes arises from trunk levels (18, 19), see **Figure 3E**. We concluded that the trunk was a preferable region for analyzing the migration of melanoma cells, but comparison of migratory ability between cells transplanted at cranial compared to trunk levels could be very informative.

During development, neural crest cells in the trunk migrate along two main pathways, the ventral pathway, which gives rise to neurons and glia in dorsal root and sympathetic ganglia, Schwann cells along nerves, melanocytes and adrenal chromaffin cells, and the dorsolateral pathway, which gives rise solely to melanocytes (19, 32–34). We analyzed cross-sections of embryos containing LM-MEL-3 and LM-MEL-8 cells to see if the cell lines followed these pathways. Cells from both LM-MEL-3 and LM-MEL-8

migrated from the trunk neural tube and could be seen outside the neural tube at 2 days post-injection by a dorsal view of whole mount embryos (**Figures 3C,D**). These migrated along the dorsolateral and ventral pathways (**Figures 3F,G**). We observed numerous cells located dorsal to the neural tube, a region termed the staging area, where neural crest cells are found prior to migration along a pathway (**Figure 3E**, yellow arrow, **Figure 3G**). Cells were also observed along the dorsolateral pathway adjacent to the ectoderm, along the ventral pathway in association with the dorsal root ganglia, and a small proportion of cells were observed in between the ventral and dorsolateral pathways. Neural crest cells are found at each of these locations (35). This finding is in line with earlier studies that have demonstrated that melanoma cells migrated along both dorsolateral and ventral routes (11, 13). Melanoma cells from another epithelial-like melanoma cell line LM-MEL-71 (Figure S2 in Supplementary Material) also showed migration outside the neural tube, along the dorsolateral and ventral pathways. EMT in melanoma often occurs with acquisition of motility and concomitant decrease in the expression of E-cadherin and gain in the expression of N-cadherin (12, 36). Culturing both epithelial- and mesenchymal-like melanoma cell lines as hanging drops do not induce changes in the expression of *E-cadherin* (epithelial marker) and *N-cadherin* (mesenchymal marker) (Figure S3 in Supplementary Material).

We also transplanted normal human melanocytes into the trunk region. A very small number of melanocytes migrated away from the neural tube, but this proportion was far less than the migration observed from melanoma transplantations. Some cells were observed in the roof plate of the neural tube, while others remained in the lumen of the neural tube (**Figure 3H**). Previous studies have reported that melanocyte aggregates integrated into the roof plate but did not migrate outside the neural tube (13).

The microenvironment of the neural tube of chick embryos affects the behavior of melanoma cells and promotes their migration along neural crest migratory pathways. In addition to chick embryos, zebrafish and mouse embryos have been used previously to provide either a receptive or potentially normalizing microenvironment that alters the behavior of human melanoma cells. Some factors active during gastrulation and early organogenesis within these embryonic microenvironments change the gene expression pattern of melanoma cells, limiting tumor formation, and enabling migratory potential (11, 26, 37). Bailey et al. identified a number of genes associated with EMT and migration upregulated in melanoma cells post exposure to the chick microenvironment (22). These gene products are excellent candidates to send and receive signals instructing and maintaining migratory potential in melanoma cells. Furthermore, epithelial-to-mesenchymal transitions are generally described as a process requiring external stimuli for their initiation (38). The process of neural crest development and the molecular mechanisms involved show similarity across species. For example, members of the BMP and Wnt families are involved in regulating neural crest induction and emigration in multiple species (39).

In conclusion, this model represents an accessible and potentially powerful system for examining the invasive behavior and remarkable plasticity of metastatic melanoma cells *in vivo*. It is proving helpful for identifying intrinsic and microenvironmental regulators and drivers of cellular migration. This should prove valuable for the identification and validation of molecules involved in metastatic behavior as well as for the development of therapies that target related pathways.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2015.00036/abstract

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