Epithelial to mesenchymal plasticity: role in cancer progression

Remya Raja\textsuperscript{1,3}, Akhilesh Pandey\textsuperscript{1-5}, Prashant Kumar\textsuperscript{1-3}

\textsuperscript{1}Institute of Bioinformatics, International Technology Park, Bangalore, 560066, India, \textsuperscript{2}Centre for Molecular Medicine, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, 560029, India, \textsuperscript{3}Manipal Academy of Higher Education (MAHE), Manipal, 576104, India, \textsuperscript{4}Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN55905, US, \textsuperscript{5}Center for Individualized Medicine, Mayo Clinic, Rochester, MN55905, US

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1. ABSTRACT

Epithelial-mesenchymal transition (EMT) is a dynamic process by which the cells transdifferentiate into two or more somatic states. The metastatic spread begins with tumor cells disseminated from the primary tumor via intravasation, hematogenous transit and extravasation to reach the distant organs to form micro- or macrometastasis. Dissemination of tumor cells or metastasis is a crucial stage in cancer progression and accounts for majority of cancer associated morbidity and mortality. Advances in technology has now enabled detection and capture of tumor cells that escape from primary site into the bloodstream. Such tumor cells which are found in transit in the blood are referred to as circulating tumor cells (CTCs) and they represent the early step in metastatic cascade. The dynamic changes in EMT phenotype in CTCs plays a key role in cancer metastasis. This review will focus on the role of EMT in cancer progression, circulating tumor cells and its clinical relevance.

2. INTRODUCTION

The epithelial-to-mesenchymal transition (EMT) is a well-coordinated cellular program
during embryonic development wherein epithelial cells transition towards a mesenchymal phenotype. Epithelial cell layer is an organized structure which is separated from the adjacent tissue by a basal lamina. The epithelial cell maintains apical-basal polarity and contact with adjacent cells through adheren junctions, tight junctions and desmosomes. In contrast, mesenchymal cells are loosely organized in an extracellular matrix and do not have a basal lamina separating them from adjacent tissue. Mesenchymal cells also do not have the distinctive apical-basolateral polarity as epithelial cells. The shift from epithelial to mesenchymal state is marked by several phenotypic changes including the loss of cell polarity, cell-cell adhesion, cytoskeletal reorganization and gain of migratory and invasive properties. The reverse of this process-mesenchymal to epithelial transition (MET) is associated with loss of the migratory potential and a gain of cell polarity through expression of junctional proteins.

Originally EMT and MET were studied in the context of cellular differentiation during development. The EMT can be triggered by several extracellular cues and studies have shown extensive crosstalk between signaling pathways that can either activate or repress the transition process (1, 2). Most frequently observed change during EMT is the loss of E-cadherin expression along with induced expression of specific transcription factors (EMT-TFs) that includes Snail, Zeb and Twist among others (3). EMT is orchestrated through complex molecular pathways that involves microRNAs, epigenetic and post-translational regulators along with alternative splicing events (4, 5). Researchers have drawn strong parallels between cell plasticity observed during embryonic development and cancer which underlaid the hypothesis for EMT as a driver of epithelial cancers (6-8). EMT has since been shown to impart cells with migratory and invasive properties, stemness, resistance to apoptosis and senescence, as well as aid in immunosuppression (9). However, much of the research into mechanisms underlying EMT transdifferentiation program has been restricted to cultured cells and hence its relevance under in vivo settings has been debated for long (10). Accumulating evidences points towards a more fundamental role for EMT in cancer progression.

Unlike the classic description of EMT which refers to a shift between two alternative cellular states, EMT in cancer is rarely complete. Recent studies indicate to the presence of multiple transition states in the tumor suggesting that the cells could frequently express a mix of both epithelial and mesenchymal genes (11, 12). This partial EMT program can acce the cells with greater plasticity as they change through a spectrum of intermediary phases. Such hybrid cells can move as clusters and have been found to be more aggressive than the cells that undergo complete EMT (13). The intermediary phenotypic states which were previously observed under in vitro settings have also been identified during organ fibrosis and in CTCs (14-17).

Circulating tumor cells (CTCs) are precursors of metastasis and their presence in peripheral blood points to invasion and aggressive nature of the malignancy. Detection and molecular characterization of CTCs from the patients with solid tumors can aid in disease categorization. Considering the low invasive nature of testing, CTCs as “liquid biopsies” can be used as an index to serially track the disease progression and monitor therapeutic response (18). Prognostic value of enumerating CTCs have been shown in multiple cancers. Mesenchymal CTCs have been shown to correlate with poor outcome and shorter survival rate in breast cancer patients (19). Here, we will review our current understanding of dynamic changes in EMT observed in CTC and its relevance in the design of appropriate therapies.

3. EPITHELIAL TO MESENCHYMAL TRANSITION

Distinct differences in morphologies of epithelial and mesenchymal cells were recorded as early as 1897, however EMT as a process came to limelight following pioneering work from Elizabeth Hay at Harvard Medical School. Greenburg and Hay were the first to show the loss of polarity and gain of mesenchymal properties when epithelial cells from embryonic and adult anterior lens were suspended in
three dimensional collagen gels. The phenomenon was termed “epithelial to mesenchymal transformation” (20). The finding prompted a flurry of research in the area, soon EMT was recognized to be an evolutionarily conserved process fundamental to embryogenesis (21). Mesenchymal to epithelial transition observed during kidney development was one of the early indicators for the reversible nature of EMT (22). However the first link to cancer did not emerge until Jean-Paul Thiery’s group demonstrated the effect of ‘scatter factor’ or hepatocyte growth factor on the invasiveness of rat bladder cancer cell line (23). Subsequently, EMT was shown in organ fibrosis (24). The decision to replace the term “transformation” with “transition,” was taken at the first meeting of The EMT International Association (TEMTIA, 2003), in Port Douglas, Australia. The renaming consolidated a decade old research in EMT that emphasized the reversibility of the process and reinstated the fact that it is distinct from neoplastic transformation (25).

3.1. Overview of EMT

Epithelial cells forms a layer and act as a barrier which is separated from adjacent tissue via a basement membrane. The structural integrity of epithelial cells is often maintained by desmosomes, adherens junctions and tight junctions which are sites for intercellular anchoring and signaling. EMT involves extensive cellular reprogramming that confers epithelial cells with properties like increased motility, invasiveness and the ability to degrade extracellular matrix (ECM) (8, 12). One of the early steps in EMT is loss of apico-basal polarity and cell-cell cohesion due to disintegration of tight junctions and dysregulation of cell adhesion molecules (26). Concomitant activation of proteases facilitates breakdown of basement membrane and ingestion of cells (27). A switch in expression from E-cadherin to N-Cadherin, referred to as ‘cadherin switching’ is one of the prominent features associated with EMT (28, 29). The expression of tight junction proteins such as occludins, claudins and desmosomal proteins have been shown to be altered during EMT (30, 31). Cadherins are linked to cytoskeletal actin via catenins and loss of cadherins triggers cytoskeletal reorganization during which peripheral actin cytoskeleton is replaced by stress fibers. Moreover, cytokerin intermediate filaments in the epithelial cells are replaced by vimentin. Towards the later stages of EMT, the expression levels of adhesion proteins are maintained low through active transcriptional repression mechanism. Collective downregulation of adherens proteins have been reported in prostate cancer that clearly indicates to a coordinated regulation of transcription for junctional genes during EMT (32).

At cellular level, EMT is associated is three major changes (a) a phenotypic shift from cobblestone morphology of epithelial cells to spindle like appearance of mesenchymal cells (b) altered expression of cell surface markers, cytoskeletal and ECM proteins, notable is the aforementioned cadherin switch (c) and the most important one is acquisition of motility and invasiveness by single cells (33, 34). In many instances, ability to invade ECM is considered as the foremost and important hallmark associated with EMT (35). Figure 1 represents the classical description of EMT process.

4. EMT IN CANCER

The initial threads linking EMT to cancer was reported as early as the 1980’s. Later on, much of the information on EMT and cancer came from culture based studies, this raised skepticism regarding its clinical relevance and was a matter of intense debate (36). Lack of substantial evidences for EMT phenotype in vivo has been attributed to the transient nature of process as well as to intrinsic tumor heterogeneity. It is well documented that most solid tumors have partial EMT features with cells expressing both epithelial and mesenchymal markers. Though paradoxical, EMT related processes have also been evidenced in carcinosarcomas, a specific subtype of sarcomas which are tumors of mesenchymal origin (37). Another example for EMT in cancer can be observed with basal B or claudin low subtype breast cancer, where EMT signature is correlated with poor clinical outcome (38, 39). Recent findings suggest an active role for EMT in metastasis, as gain of mesenchymal markers in breast CTCs were predictive of worse prognosis (40). Additionally the ability to specifically tag and track cancer cells in transgenic mouse models have now enabled researchers to address
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Figure 1. Overview of EMT. The classical depiction of transition from epithelial cells to mesenchymal phenotype, reverse of the same process, MET is shown. The transition process begins with dissolution of tight junctions, followed by disassembly of adherens junction and desmosomes. The currently used markers for epithelial and mesenchymal phenotypes are also listed.

the role of EMT in in vivo tumor progression and its potential involvement in the seeding of distant organs (41, 42). These clinical findings combined with experimental models are throwing more light into the versatile role of EMT in cancer progression.

EMT in cancer is more or less defined using the presence or absence of mesenchymal or epithelial cell surface markers respectively, especially loss of E-cadherin. Based on the expression profiles of these markers, it is quite apparent that unlike homogenous cell line models, execution of EMT is not uniform in primary tumors. Brabletz et al. had reported that in colorectal carcinomas, invasive front has marked expression of nuclear β-catenin along with decreased E-cadherin and proliferative activity which was distinct from bulk of the tumor. The report points to the heterogeneous nature of the EMT program within the tumor, contributed in part due to the molecular heterogeneity arising from somatic driver mutations and the microenvironment (43). Loss of E-cadherin expression has been a central theme linked to EMT in cancer. However, studies show that loss of E-cadherin alone might not be sufficient to trigger EMT associated changes (44). In fact, in lobular cancer in situ which is characterized by loss of E-cadherin do not show aggressive phenotype whereas invasive lobular breast carcinoma with low E cadherin has a favorable outcome compared to invasive ductal breast carcinoma which retains E cadherin expression (45).

Importantly, EMT drivers such as Snail 1/2 and Twist1 have been shown to correlate significantly with recurrence and survival in patients with
colorectal, breast and ovarian carcinoma (46-53). In a conditional transgenic mouse model for recurrence of HER2/neu-induced mammary tumors, Moody et al. revealed the presence of Snail in recurrent tumors that was accompanied by EMT (54). A recent study reported that Zeb1 overexpression with concomitant reduction in miR200c levels in invasive fronts was predictive of clinical outcome in colon cancer that correlated with PD-L1 overexpression (55). Accumulating lines of evidences from clinical tissues have pointed towards a key role for EMT-TFs in cancer, next we will address the pleiotropic function of these EMT-TFs in regulating multiple facets of neoplastic progression.

4.1. EMT in cell survival, resistance to anoikis and acquisition of stem cell-like characteristics

Acquisition of resistance to cell death is one of the features of cellular transformation and hallmark of cancer. Role of EMT in evasion of apoptosis has been increasingly documented (56). Slug was one of the first transcription factors reported to confer radioprotection to early hematopoietic cells in vivo (57). Slug functions as a downstream target to p53, which gets activated upon genotoxic stress (58). Further, increased Slug expression promotes cell survival by preventing p53 mediated transactivation of PUMA, a key antagonist to Bcl2 protein in hematopoietic cells (59). Snail1 was also shown to block cell cycle by suppressing Cyclin D2 transcription resulting in concomitant increase in p21/Cip1. Snail can protect the cells from lethal effects of serum depletion or TNF-α administration by triggering the MAPK and PI3K-mediated survival pathways (60, 61). Both Slug and Snail can mediate chemo- and radioresistance by repressing targets of p53 which includes apoptotic molecules such as Bid, PIG8, Caspase 6 and PTEN among others (62, 63). Zeb2/SIP1 can cause cell cycle arrest in A431 cells by targeting Cyclin D1 expression (64). In addition, Zeb1 through direct interaction with E-box sites present in the intron 1 region of p73, downregulates the pro-apoptotic tumor protein p73 (Tp73) (65). In N-myc amplified neuroblastomas, Twist is frequently overexpressed which in turn directly inhibits the expression of p14ARF gene leading to inhibition of apoptosis (66). These molecular changes provides survival advantage to the invasive and migratory cells during tumor dissemination.

Anoikis, a programmed cell death induced upon detachment of cells from the extracellular matrix, serves as a cellular checkpoint that prevents anchorage-independent survival and growth. For a cell to metastasize, it needs to escape from the primary tumor and enter the vasculature and its survival during the journey impinges upon acquisition of resistance to apoptosis. Studies have revealed multiple mechanisms through which cancer cells evade anoikis, which includes sustained activation of signaling pathways leading to upregulation of anti-apoptotic molecules thereby promoting cell survival. In particular, altered integrin repertoire as well as integrin mediated signaling contribute to pro-survival signals (67). The E-box-binding factors, Snail, Slug, Twist and Zeb1/2 are known to transcriptionally repress cell adhesion genes that are part of the adherens junctions, desmosomes and tight junctions. Alterations in cell surface markers including junctional proteins can potentially aid in suppressing anoikis (10, 56).

Snail can directly repress genes involved in anoikis, like PTEN, which leads to activation of PI3K/Akt pathway. Further inactivation of the pro-apoptotic protein Bad can contribute to anoikis resistance (68). Sustained presence of β-catenin in the cytosol results in anoikis resistance through activation of c-Myc, cyclin D1, and MAPK-mediated survival pathways (69). Knockdown of Zeb1 was found to suppress anchorage independent growth in lung cancer cells (70). Twist1 and 2 by inhibiting p16/Ink4a and p21/Cip1 prevent cells from undergoing senescence, these findings established a link between EMT program and acquisition of resistance to premature senescence (71). Similarly, Zeb1 was also found to protect mouse embryonic fibroblasts from senescence (72). Taken together, EMT can aid the survival of metastatic tumor cells by overcoming the inherent safeguard measures put up by the cell against turning cancerous.

Mani et al. first reported that treating human mammary epithelial cells with TGF-β or induced expression of Snail1/Twist1 resulted in morphological transformation (EMT) of a sub-population of cells with
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stem cell characteristics that has the capability to form mammospheres, (73). Using genomic approaches, it was demonstrated that ablation of p53 in mammary epithelial cells led to reduced miR-200c levels that activated the EMT program and increased the stem cell population (74). In pancreatic cancer, Zeb1 acted as a strong promoter of EMT by targeting miR-200 family members, which are known to negatively regulate stem cell factors including Sox2 and Klf4 (75). Along the same lines, Kong et al. reported that mesenchymal prostate cancer cells had elevated levels of pluripotency genes such as Sox2, Nanog, and Oct4. Consistent with the findings the mesenchymal cells displayed enhanced clonogenic, sphere forming ability and tumorigenicity in vivo along with decreased expression of miR200 and let-7 family members (76). Bmi1 is a polycomb-group protein that helps maintain self-renewal capacity and is reported to be frequently overexpressed in cancers. Yang et al. reported that Twist1 and Bmi1 cooperated to promote EMT and tumor-initiating capability via regulation of E-cadherin and p16INK4a. The augmented expression of both Twist1 and Bmi1 correlated with worst prognosis in head and neck cancer patients (77). Furthermore, mutual action of Twist1 and Bmi1 suppressed the expression of let-7 miRNA which resulted in upregulation of NEDD9 and DOCK3 that further led to RAC1 activation and acquisition of stem like properties (78). Recently, downregulation of let-7 was shown to upregulate the chromatin modifier ARID3A and B, leading to its nuclear transport. Recruitment of histone demethylase 4C by ARID3B complex leads to reduced H3K9 trimethylation which enhances the transcription of stemness factors (79). In breast epithelial cells, cooperation between Slug and Sox9 transcription factors help maintain the stem cell state (80). Interestingly in colorectal cancer stem cells, signaling pathway involving Snail, microRNA-146a and Numb is now shown to influence the switch between symmetric and asymmetric cell division (81).

On the contrary, recent studies suggests that it is often not necessary for EMT and stemness to go hand in hand (82). In BT549 breast cancer cells, silencing paired-related homeobox transcription factor 1 (Prrx1), a known inducer of EMT led to increased self-renewal and mammosphere forming capacity (83). A separate study reported that transient activation of Twist1 leads to acquisition of stem cell properties by mammary epithelial cells and the cells gained mesenchymal phenotype only after Twist1 deactivation (84). Along the same lines, Celia Terrassa et al. have reported that in prostate and bladder cancer cells, constitutive overexpression of Snail led to mesenchymal phenotype while suppressing their self-renewal capacity (85).

4.2. EMT and chemoresistance

A plethora of studies have demonstrated that the cells or tumors undergoing EMT acquired resistance to chemotherapy (86, 87). Moreover, the studies show that overexpression EMT specific transcription factors such as Twist and Snail were enough to confer drug or radioresistance in multiple cancers (58, 63, 88, 89). Chemoresistance effected through drug efflux transporters are well documented, Saxena et al. have reported that Snail and Twist can increase transcription of ABC genes by direct binding to its promoter (90). Studies have shown that EMT can be activated in response to reactive oxygen species (ROS) (91, 92). Additional factors such as hypoxia also contributes to generation of ROS through HIF1α/VEGF pathway which can adversely influence therapeutic response (93). Factors that promote EMT has been linked to genomic instability which can have potential consequences on therapeutic resistance. TGFβ has been shown to trigger DNA damage response pathway via activation of ataxia telangiectasia mutated ATM (94). Recently TGFβ was shown to cause mitotic abnormalities as a result of failure in cytokinesis which led to aneuploidy (95). The mitotic defects was mediated through suppression of Lamin B1 and occur especially in epithelial cells that did not go into cell cycle arrest upon induction of TGFβ induced EMT. Along the same lines, Krishnan et al. demonstrated that stromal TGFβ can result in double stranded breaks in cancer cells that lack RUNX3 expression (96).

4.3. Role of stromal compartment in regulating EMT in primary tumor

The complex cellular interactions in the tumor milieu forms an important determinant of cancer progression. There are substantial evidences that highlights the role of stroma-tumor interactions in
metastasis (97). Tumor microenvironment comprises of multitude of cell types including fibroblasts, immune cells, endothelial cells, adipocytes, pericytes and ECM components. One of the mechanisms by which tumor associated stromal cells promote metastasis is by generating a pro-inflammatory setting that modulates EMT pathways (98). In a seminal study, Orimo et al. have demonstrated that cancer-associated fibroblasts (CAF) isolated from primary breast tumors can promote growth of breast tumor cells when co-injected in mouse. Stromal derived factor 1 (SDF-1/CXCL12) secreted by CAFs played a role to recruit endothelial precursor cells and promote tumor angiogenesis (99). In prostate cancer, CAFs promote EMT via activation of HIF1α/ NFκB/ COX-2 pathway (100). Ao et al. detected CAFs co-expressing FAP and α-smooth muscle actin in peripheral blood of patients with metastatic breast cancer, hinting at the clinical utility of CAFs as a potential biomarker along with CTCs (101). Recently, CAF derived IL6 was shown to induce a mesenchymal and resistant tumor cell state in esophageal adenocarcinoma while inhibition of IL-6 led to reversion of mesenchymal state and resensitized tumor cells to therapy (102).

Toh et al. demonstrated preferential infiltration of myeloid-derived suppressor cells (MDSC) in melanoma that led to increased metastasis. Furthermore, accumulation of MSDC’s in the primary tumor was mediated by increased secretion of chemokine, CXCL5 by tumor cells and the MSDC’s were shown to induce EMT (103). Su et al. reported that co-culture of mesenchymal breast cancer cells with macrophages resulted in a positive feedback loop involving CCL18. Tumor derived CCL18 induced a TAM like phenotype in macrophages whereas macrophage derived CCL18 potentiated EMT and invasiveness in breast cancer cells (104). On the other hand, depletion of pericytes resulted in enhanced hypoxia, EMT and activation of Met receptor in breast cancer (105).

4.4. EMT and immune evasion

Expression of Snail in melanoma cells induced regulatory T cells and impaired dendritic cells both in vitro and in vivo, which was partly mediated through TSP1 production, suggests a potential role for EMT in immune resistance (106). Ye et al. demonstrated that hypoxia treated hepatoma cells induced the expression of indoleamine 2, 3-dioxygenase (IDO) in monocyte-derived macrophages in a CCL20-dependent manner. Consequently, IDO+ macrophages abrogated T-cell proliferation and promoted the proliferation of immunosuppressive regulatory T cells. The HIF1α/CCL20/IDO axis in hepatocellular carcinoma presents a novel mechanism for EMT in promoting an immunosuppressive behavior (107). Moreover, HIF1α was shown to directly regulate PD-L1 expression in tumor infiltrating myeloid cells such as myeloid derived suppressor cells (MDSC), which represents additional mechanisms through which cancer cells evade immune response (108).

Recently, the role of EMT in immune evasion was addressed using MCF7 breast cancer cell line. Wnt1-inducible signaling pathway protein 2 (WISP2/CCN5) is reported to be a key regulator of plasticity associated with estrogen receptor positive cells. Knockdown of WISP2 in MCF-7 decreased tumor susceptibility to cytotoxic T-lymphocyte (CTL)-mediated lysis. This observed immune evasive function was mediated through induction of Kruppel-like factor-4 (Klf4) and subsequent microRNA-7 (miR-7) downregulation. (109). Recently, EMT process was also linked to PD-L1 mediated immune evasion (110). Furthermore, in a panel of EMT activated breast cancer cell lines, immune checkpoint ligand PD-L1 was found to be upregulated which was mediated through Zeb/miR200 signaling axis (111). Interestingly, Dongre et al. demonstrated that mammary tumors from epithelial carcinoma cell lines expressed low levels of PD-L1 and had higher number of stromal CD8+ T cells and M1 (antitumor) macrophages. In contrast, tumors arising from more mesenchymal carcinoma cell lines expressed higher levels of PD-L1 had regulatory T cells, M2 (pro-tumor) macrophages and had minimal CD8+ T cells in their stroma (112). These observations were independently confirmed by Lou et al. in NSCLC adenocarcinomas. Elevated expression of multiple immune checkpoint molecules including PD-1, PD-L1, PD-L2, B7-H3, TIM-3, CTLA-4 and BTLA were found in lung adenocarcinomas with EMT phenotype. Moreover the lung adenocarcinomas also showed an increased infiltration of CD4+Foxp3+ regulatory T
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cells (113). These findings support the rationale for EMT blockers as an additive to boost immunotherapeutic responses.

4.5. EMT and metastasis

The systemic dissemination of cancer cells from primary tumors and their subsequent colonization in distant sites have been considered as a complex and multi-step process, referred to as the invasion-metastasis cascade (114-116). The process begins with the local invasion of primary tumor cells into adjacent tissues followed by intravasation of these cells into the blood stream. The tumor cells that survive hematogenous transit will then extravasate through blood vessels into the parenchyma of distant tissues and with time proceed from micrometastatic colonies to, clinically detectable metastatic lesions, this last step is termed colonization (117, 118). In 1889, Stephen Paget’s “seed and soil” theory proposed that cancer cells can metastasize only to those tissues that has permissive microenvironment supporting the growth of cancer cells. The hypothesis still holds forth to date as additional discoveries including CTC, the pre-metastatic niche and mesenchymal to epithelial transition of CTCs have provided better insight into this complex process of metastatic dissemination (114).

Invasion of cells into the extracellular matrix is considered as one of the initial step in the metastatic cascade. The cells acquiring the ability to migrate and invade matrix has long been considered a hallmark of EMT and has been used as a surrogate to describe the role of EMT in metastasis (119). Multiple studies have documented distinct mechanism(s) by which EMT promote cell invasion which includes cytoskeletal reorganization, altered expression of cell adhesion molecules, degradation of basement membrane through activation of matrix metalloproteinases (MMPs), especially, MMP-2 and -9, (120, 121) as well as sustained autocrine growth factor signaling to evade apoptosis and/or anoikis (8, 12, 25). EMT-inducing transcription factors, notably Snail, Slug, Twist and Zeb1 organize these complex programs in a coordinated fashion (3, 122). Metastasis has always been considered to occur at advanced stages of tumor progression, however some studies are pointing towards early acquisition of EMT-associated traits which initiates dissemination process to occur relatively early as evidenced in certain pre-neoplastic lesions (41).

EMT program when activated in cancer cells can exert pleiotropic effects favoring the acquisition of metastatic and malignant properties leading to tumor progression (12, 118). However it is also evident that most distant metastases present the same epithelial phenotype as that of the primary tumor indicating possibility of MET occurring at distant sites (123, 124). Though, the requirement of EMT/MET pathways for metastasis was proposed (8), there were relatively no supporting evidence to the MET claim. Recent studies have however provided evidences that underscore the role of MET pathway in regulating tumor metastasis (83, 125-127).

Understanding the role of EMT in metastasis in vivo has been complicated as it is difficult to distinguish epithelial cells with mesenchymal features from the stromal counterpart that expresses high level of mesenchymal markers. Though not ideal, mouse xenograft models generated with genetically modified cell lines were used as experimental systems to demonstrate the role of EMT inducing transcription factors in metastasis. These studies provided evidences that underscored the role of transcription factors such as Snail, Slug, Twist and Zeb in acquisition of metastatic capabilities in cancer (119, 128-130). Tsai et al. employed a spontaneous squamous cell carcinoma mouse model to demonstrate the role of Twist1 in metastasis. The study further concluded that inactivation of Twist1 was essential for generation of metastases at distant sites (127). In a separate study, using a mouse model with an inducible SNAI1 transgene, Tran et al. showed that Snail1 expression was essential for breast cancer metastasis (131). However recent studies using lineage tracing mouse models put forth questions relating to the role of EMT in metastasis. Zheng et al. showed that genetic ablation of either Twist or Snail in a spontaneous pancreatic ductal adenocarcinoma (PDAC) model did not impede metastasis (89). By using a mesenchymal specific Cre-mediated fluorescent marker switch with either Fsp1 or Vimentin in a spontaneous breast to lung cancer metastasis
mouse model, Fischer et al. reported that a small proportion of primary tumor cells underwent EMT, however those cells did not contribute to metastatic colonization (88). There are several arguments against these disparate observations which include (i) lack of reliability of Fsp1 and Vimentin as universal mesenchymal markers (ii) compensatory mechanisms against loss of Twist or Snail (iii) presence of EMT markers in circulating tumor cells. In support of the compensatory mechanisms, Kerbs et al. have shown that knockout of Zeb1 in a mouse model of pancreatic cancer decreases the metastatic burden to about 30% without any effect on the expression of Snail1 or other EMT-TFs (132). Taken together, there is still a need to define role of EMT in cancer dissemination by engaging better experimental models and contribution of EMT to metastatic process cannot yet be discounted. Importantly, circulating tumor cells (CTCs) are considered as precursors of metastasis and learning the molecular attributes of these cells will provide a unique window into the mechanisms underlying malignant spread.

5. CIRCULATING TUMOR CELLS IN CANCER

A multitude of factors govern the malignant nature of solid tumors. Foremost among them is the acquired capacity of tumor cells to break away from the primary tumor or metastatic lesion to enter blood circulation leading to colonization at a distant organ. The first report of CTC in breast cancer patient was made by John Ashworth in 1869, however the research did not gain much traction owing to the technical challenges in identifying these rare cell population. Most metastatic cancers have CTC counts as low as 1-10 cells per 10 ml blood (133) however their accessibility in blood makes them an attractive choice for disease monitoring or for use as "liquid biopsy". Due to considerable advances in technology, molecular analysis of CTC is one of the most dynamic field in the area of cancer diagnostics.

Enumeration and characterization of CTCs have been reported across multiple cancers including breast, lung, prostate and colorectal carcinomas where the baseline CTC count could act as a reliable prognostic indicator for disease outcome (134-137). Recently, Adams et al. showed an increased number of mitotic CTCs in advanced metastatic breast cancer patients and the mitotic state of CTCs correlated with shorter survival in breast cancer patients (138). Given their clinical utility, multiple methods have been employed for isolation and detection of CTCs. Broadly, CTC’s are separated based on their physical features including size, density and deformability as well as antibody-based capture approaches that target either cell surface proteins or phenotypic markers such as HER2 or Vimentin (139). The ability to enrich CTCs vary depending on individual approaches and hence should be evaluated based on the respective limitations associated with each method. For instance, CTC size ranges from 9 to 30μm and two separate studies that employed size based CTC selection showed enrichment of CTC's with mesenchymal features (140, 141). Shaw Bagnall et al. reported that selection based on deformability represents an unbiased approach to capture CTC’s with both epithelial and mesenchymal phenotype (142). However studies have shown that isolation strategies combining both physical and biological properties of CTCs to be superior over singular approaches (143, 144).

CellSearch, the FDA approved device for CTC isolation relies on the most widely used approach employing antibodies against EpCAM, a cell surface receptor for positive selection of CTC’s. However EpCAM based approaches fail to detect cells that express very low to negligible levels of EpCAM which leads to exclusion of mesenchymal CTCs. Schneck et al. demonstrated that CTCs which are not detected by EpCAM based approaches could be detected using antibodies against mesenchymal markers (145). However one of the biggest challenge in isolation of CTCs is the plasticity of these cells which exhibits dynamic changes in epithelial and mesenchymal phenotype. The recent findings suggest that CTCs exhibits a spectrum of EMT phenotypes. This also reinforces the need to go deeper to understand the biology of these cells. Most of the methods used for the isolation of CTCs are based on cell-surface markers or their physical properties. The current studies indicate that use of antibody cocktails against mesenchymal and epithelial can compensate the limitations associated with EpCAM-based approaches.
5.1. EMT in CTC

Tumor cells are known to hijack the conserved EMT program to effect a plethora of functional changes including enhanced motility, degradation of basement membrane, invasiveness, stemness and resistance to anoikis. Acquisition of these cellular properties enable individual cells from primary tumor to intravasate the vasculature either from adjacent tissue or within the tumor. CTC’s are mostly single cells, however it can travel in small clusters as well. The relative contribution of single cells vs clusters towards generation of metastasis is not clearly understood, however, clusters were found to be more efficient than single cells in seeding metastatic colonies when introduced into circulation in mouse models (146). Importantly, CTCs transiting alone or in group are known to exhibit varying degree of epithelial and mesenchymal characteristics, the findings coincides at a time when definition of EMT is getting reshaped (17, 118).

Currently, EMT is no longer considered as binary switch that enables the cells to shift between two states instead EMT is seen as a process that can generate cells with a spectrum of intermediary stages (28, 147). In fact several lines of evidences have reported the existence of ‘partial EMT’ in both adult tissues and tumors (11). The cells in this phenotype have mixed epithelial and mesenchymal properties. Gross-wilde et al found that these hybrid cells (E/M) with mixed epithelial (E) and mesenchymal (M) signatures had increased stemness as evidenced by their increased potential in vitro for self-renewal, plasticity and mammosphere formation when compared to the differentiated mesenchymal and epithelial cells. Moreover co-expression of epithelial and mesenchymal signatures were found to be associated with poor outcome in luminal and basal breast cancer patients (148). The intermediary phenotypes are also referred to as ‘metastable’ to reinstating the flexible state of the cells where it can either move forward or backwards depending on environmental cues (149). Schleikelman et al. took an integrated approach combining information from mRNA, miRNA, DNA methylation and proteomic profiles from a panel of 38 NSCLC cell lines. The analysis revealed that a subset of cells of hybrid phenotype (E/M) had increased expression of cytoskeletal proteins (150).

Importantly, Armstrong et al. demonstrated that majority of CTCs isolated from metastatic castration resistant prostate cancer and breast cancer patients co-expressed epithelial and mesenchymal markers including E-cadherin, EpCAM, cytokeratins, vimentin, N-cadherin, O-cadherin as well as CD133 which is a stem cell marker (151). Fustaino et al. have reported that hybrid phenotypes in non-small cell lung carcinoma cell lines result in collective migration, stem like properties and resistance to erlotinib, an EGFR inhibitor (14). In fact, the biphenotypic tumor cells that express both epithelial and mesenchymal markers may have better plasticity and could potentially be the cells that are most likely to contribute to metastatic outgrowth (16). Triple negative breast cancer and basal-like breast cancers which have poor clinical outcome showed an increased prevalence of biphenotypic cells, underscoring the association between E/M phenotype and aggressiveness (17). Using RNA in situ hybridization method, E/M phenotypes have now been detected in CTCs from multiple cancers including liver, nasopharyngeal, colon and gastric cancer. Wu et al. further reported that the frequency of mesenchymal CTCs increased depending on the TNM stage of these cancers (141). Taken together, hybrid E/M phenotypes result in enhanced metastatic and tumor initiation potential as well as resistance in multiple tumor types (152).

Interestingly, Aiello et al. using a lineage tracing mouse model system, showed that pancreatic tumors adopt two distinct programs to undergo EMT. Minority of the tumors followed the conventional system of transcriptional repression of epithelial genes whereas majority of the tumors relied on re-localization of epithelial proteins resulting in a partial EMT state. The observed partial EMT resulted from internalization and re-localization of E-cadherin to late recycling vesicles in pancreatic cancer cells. Importantly, cells exhibiting partial EMT underwent collective migration over the cells that had complete EMT phenotype (153). Of note, cells that have both epithelial and mesenchymal (E/M hybrid cells) traits can aid in collective cell migration. Instances of
migration of multicellular aggregates or CTC clusters have been observed in breast, lung, and prostate cancer patients (16, 151, 154). These clusters, referred to as “microemboli” were found to have increased metastatic propensity over individual CTCs. Further CTC clusters were found to be more resistant to apoptosis as well as presence of CTC clusters indicated to poor prognosis in breast and prostate cancer patients (146). In a separate study, in vivo xenotransplantation of CTC clusters in zebrafish embryos demonstrated that these clusters can traverse through blood vessels which are 5-10 µm in diameter and can easily exit blood vessels than previously imagined (155).

Multiple studies have looked into the underlying mechanisms guiding the transitions and maintenance of hybrid phenotype. A study with MCF-10A cell line model has revealed that the Snail1/miR-34 double-negative feedback loop regulates the initiation of EMT, whereas the Zeb/miR-200 feedback loop is accountable for establishment of the mesenchymal state. In addition, an autocrine TGF-β/miR-200 feedback loop makes the switch irreversible, thereby contributing to the maintenance of EMT (156, 157). Using a systems biology approach, OVOL2 transcription factor was found to modulate transition states and form an inhibitory loop involving Zeb1 (158). Recently Jolly et al. showed that certain phenotypic stability factors (PSF) can enhance the stability of the hybrid phenotype and facilitate collective migration. In H1975 lung cancer cells, knockdown of OVOL or GRHL2 impeded collective migration (159). The same study also reported a miR-200/Zeb/miR-145/OCT4 circuit in addition to the miR-200/Zeb/OVOL and miR-200/Zeb/GRHL2 loops that helps to stabilize the E/M state. A schematic representation of the core EMT regulatory loop and its interaction with phenotypic stability factors is shown in Figure 2.

These CTC clusters intravasate by collective migration of cells, therefore retaining epithelial characteristics could endow selective advantage to these cells to rapidly grow to form macrometastases at target organs (160). However, it is important to understand that CTC clusters are not only hybrid E/M cells, but also admixtures of epithelial and mesenchymal cells along with stromal cells which includes leukocytes, platelets and megakaryocytes (16). Hence, it is imperative to better comprehend the cellular heterogeneity to fully exploit the clinical potential of CTCs.

5.2. Role of stroma in regulating EMT in CTC

Tumor microenvironment is known to play a critical role in tumor progression which is discussed in detail in the earlier section. Tumor cells recruit stromal cells, which in turn, provides an environment conducive to tumor progression as well as metastasis. Similar to what we observed in primary tumor, current evidences indicates that circulating tumor cells also interact with stromal cells which then contributes to the increased persistence and metastatic capability of these cells (161).

It is estimated that only 0.01% of the cancer cells that enter hematogenous circulation ultimately develops into macroscopic metastases which suggests that the process of metastasis and subsequent colonization to be an inefficient process (162). Based on the current studies, the average half-life of CTCs in circulation is around 1-2.4 hours (163). In the bloodstream, CTCs are subject to various stress factors that includes matrix detachment, oxidative stress, physical stress arising from collisions with endothelial cells lining the vessel walls as well as attack from the immune system (164). CTCs survive these insults through its interactions with platelets. Platelets protect CTCs from natural killer cells and helps to recruit neutrophils that enable CTCs to adhere on to the walls of the blood vessels. Labelle et al. have shown that platelet derived TGF β induces TGF β/Smad and NF-κB signaling in cancer cells leading to EMT and increased metastatic potential in vivo (165). Interactions between platelet derived autotaxin and αvβ3 integrins in tumor cells have been reported to influence metastasis of breast cancer cells to bone (166). Yu et al. demonstrated that loss of TLR4 specifically inhibited lung cancer metastasis. The study further reported that tumor derived HMGB1 to be a TLR4 interactor and a critical mediator of platelet-tumor cell interactions that drives metastasis (167). Takagi et al. reported that antibody mediated disruption of podoplanin and platelet receptor CLEC2 resulted in strong anti-tumor activity.
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Figure 3 illustrates the role of complex tumor-stroma interactions, cellular plasticity and CTCs in the metastatic cascade.

5.3. EMT signatures associated with CTC

The flexibility in EMT program combined with genetic heterogeneity precludes the possibility of universal molecular signature which can be used for unbiased capture of CTCs. Some of the most
commonly used markers to identify CTCs of epithelial origin include EpCAM, cytokeratin-8, 18 and 19 and E-cadherin (16). The strategy however fails to detect mesenchymal CTCs as epithelial markers are often downregulated during EMT. In addition, cytokeratins may be partially or fully replaced by vimentin in mesenchymal cells. Hence, inclusion of vimentin as CTC marker comes with its own downside owing to its ubiquitous expression (40, 169). Recently, a quantitative EMT scoring system was established based on gene expression profiles, ranking the EMT state from -1.0 to +1.0, Tan et al. demonstrated that breast cancer cell lines with high EMT score had increased vimentin and N-cadherin levels compared to cell lines with epithelial characteristics. Consonant to the findings, cell lines with low EMT score had high CK19 and E-cadherin levels while cell lines with intermediate EMT status expressed both CK19 and E-cadherin. To sum up, the studies confirmed the suitability of these marker combinations to reliably identify EMT states (170).

Additional mesenchymal markers such as N-cadherin (CDH2), fibronectin1 (FN1), SERPINE1/PAI1 (serpin peptidase inhibitor, clade E) and foxc1 (FN1 forkhead box protein C1) are expressed in CTCs and are used in tandem with other cell surface markers for CTC capture (171). In colorectal cancer, plastin 3 (PLS3) was identified as a novel marker for mesenchymal CTCs which was not expressed in blood cells (172). Ueo et al. reported that breast cancer patients with PLS3 + CTCs had shorter overall survival over patients with PLS3- CTCs, suggesting the prognostic potential of plastin 3 marker (173). Another novel mesenchymal CTC marker identified was plakoglobin (JUP), a cell
adhesion molecule which was found to be a prognostic indicator for clinical outcome in breast cancer patients (174). Jolly et al. have proposed that potential signature of CD24+/CD44+ could be employed to detect CTCs with partial EMT phenotype (16). Interestingly, CD24/CD44+ cells were found to highly express P-cadherin which is a breast cancer stem cell marker. A recent study has shown P-cadherin to be a potential marker to identify metastable phenotype (175). In silico analysis of gene expression studies in CTCs by Yadavalli et al. revealed an enrichment of leukocyte extravasation pathway along with epithelial and mesenchymal markers (176). Consequently, considerable efforts are being made by research groups to identify newer markers of EMT for reliable detection and capture of CTCs (177). However, it is important to note that most approaches that employ markers for epithelial and mesenchymal phenotype are not tumor specific but can also be expressed in blood cells and CD45-endothelial cells. Table 1 summarizes the CTC markers used across multiple cancers and its clinical relevance. The inclusion of tumor-specific genetic signatures could further complement existing capture technologies (178). For instance, copy number gain of ERBB2 in breast cancer is exploited in conjunction with EpCAM for CTC capture and enumeration. Recently through targeted sequencing, several driver mutations/amplifications including APC, KRAS, TP53, ERBB3, FBXW7 and ERBB2 were identified in CTCs from colorectal patients which matched with the signatures from primary tumor (179). Collectively, these studies underscore the need to identify druggable mutations from CTCs to facilitate timely execution of individual centric therapeutic interventions for effective management of cancer.

5.4. Clinical perspectives of CTC

CTC enumeration and their molecular characterization would improve our understanding of the biology of refractory cancer. It has a huge potential in monitoring early relapse and treatment resistance of the disease. The CTC culture has enabled researchers to utilize primary cell populations for drug susceptibility assays which could guide clinicians for treatment selection. Multiple studies have evaluated the prognostic value of monitoring and phenotyping CTCs during the course of adjuvant or neoadjuvant therapies (166, 180). Yu et al. have reported alterations in number of CTCs and phenotype in response to therapy. The study demonstrated that breast cancer patients who are responders had significantly lower CTCs with more of an epithelial like phenotype whereas treatment refractory patients had more mesenchymal like CTCs (17). Short term expansion of CTCs got affected in case of responders while clusters persisted for treatment refractory cases and could predict therapeutic response in breast cancer patients (180). A large scale study in colorectal cancer found that biphenotypic and mesenchymal CTCs positively correlated with clinical stage, lymph and distant metastasis (181). Sateli et al. reported that prostate and colorectal cancer patients with CTCs which are vimentin and nuclear PD-L1 positive were associated with shorter survival (182). Yu et al. in a proof of concept study established cultures of CTCs isolated from estrogen receptor positive breast cancer patients and reported that majority of the cultures had tumorigenic potential in mice. The CTC cell lines were also used for drug sensitivity testing, thus providing a novel approach to develop targeted therapies (183). CTC-derived patient explants in immunocompromised mice exhibit correlation with those of patient’s CTCs. However the success rate is very low. Currently, an extensive panel of 60 SCLC CDX model is in existence demonstrating the utility of CDX to investigate therapies. Several studies have provided substantial evidences towards the enhanced metastatic potential of CTC clusters over individual CTCs (146, 184). Detection of clusters was also associated with worse outcome in breast cancer and colorectal cancers (185, 186). Despite the mounting evidence that supports the potential of CTCs in prognostication, differences in baseline number of CTCs across different tumor types pose considerable challenge to its successful implementation in clinical setting.

6. FUTURE DIRECTION

A decade of intensive research in the field of EMT has improved our understanding of this complex process. Moving away from the original concept, EMT is now seen as a spectrum of transition states that attributes significant plasticity to the cells, which in many ways enable the tumor cells to adapt
Table 1. Currently used markers for CTC detection and isolation

<table>
<thead>
<tr>
<th>Marker</th>
<th>Phenotype</th>
<th>Clinical relevance of marker positivity in cancers</th>
<th>References</th>
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<tbody>
<tr>
<td>EpCAM</td>
<td>Epithelial</td>
<td>EpCAM, CK 8/18/19, VIM, Twist1, AKT2 and SNAI1 were used to detect epithelial, biphenotypic and mesenchymal CTCs in colorectal cancer.</td>
<td>(181)</td>
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<tr>
<td>EGFR</td>
<td>Mesenchymal</td>
<td>Prognostic marker in castration resistant prostate cancer.</td>
<td>(187)</td>
</tr>
<tr>
<td>HER2</td>
<td>Epithelial</td>
<td>No significant relationship was found between HER2+ CTCs and survival in metastatic breast cancer patients.</td>
<td>(188)</td>
</tr>
<tr>
<td>CSV</td>
<td>Mesenchymal</td>
<td>CSV+ CTC correlated with therapeutic outcome in metastatic colon cancer patients.</td>
<td>(189)</td>
</tr>
<tr>
<td>MUC-1</td>
<td>Epithelial</td>
<td>CTCs co-expressing MUC-1, HER2 and EpCAM detected in breast cancer patients refractory to treatment.</td>
<td>(190)</td>
</tr>
<tr>
<td>CK5,7,8,18 and 19</td>
<td>Epithelial</td>
<td>Loss of cytokeratins combined with increased levels of ALDH1, CD133 and CD44 in CTCs can be predictors of recurrence in pancreatic ductal adenoma carcinoma patients</td>
<td>(191)</td>
</tr>
<tr>
<td>Twist1</td>
<td>Mesenchymal</td>
<td>Increased nuclear Twist1 with high ALDH1 expression observed in CTCs with metastatic breast cancer.</td>
<td>(192)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial</td>
<td>CTC’s with high c-Met and low E-cad expression indicative of better prognosis in small cell lung carcinoma patients.</td>
<td>(193)</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Mesenchymal</td>
<td>N-cadherin positive CTC in HNSCC patients is associated with shorter survival.</td>
<td>(194)</td>
</tr>
<tr>
<td>CD44/CD24</td>
<td>Stem cell marker</td>
<td>CTC subsets with CD24/CD44, EpCAM and N-cadherin were found to be altered during the course of neoadjuvant therapy.</td>
<td>(195)</td>
</tr>
<tr>
<td>Nuclear PD-L1</td>
<td>Mesenchymal</td>
<td>CTCs positive for nuclear PD-L1 correlated with shorter overall survival in prostate and colorectal cancer.</td>
<td>(196)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchymal</td>
<td>Increased vimentin+ CTCs were identified in NSCLC patients however only baseline CTC numbers correlated with poor prognosis.</td>
<td>(197)</td>
</tr>
<tr>
<td>Plastin-3</td>
<td>Mesenchymal</td>
<td>Further, Plastin3+ CTC were found to independently correlate with poor prognosis in colorectal cancer.</td>
<td>(172)</td>
</tr>
<tr>
<td>Snail</td>
<td>Mesenchymal</td>
<td>Mesenchymal CTCs characterized by co-expression of Snail1, Twist, Vimentin and Akt2 were found to correlate with poor progression free survival.</td>
<td>(197)</td>
</tr>
<tr>
<td>Slug</td>
<td>Mesenchymal</td>
<td>Increased number of CTC’s with marked expression of SLUG and other mesenchymal markers correlated with lymph node metastasis in breast cancer patients.</td>
<td>(198)</td>
</tr>
<tr>
<td>Zeb1</td>
<td>Mesenchymal</td>
<td>Zeb1+ CTCs were almost exclusively identified in patients with metastatic disease however the CTC number or Zeb1 positivity did not correlate with overall survival.</td>
<td>(199)</td>
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<tr>
<td>AKT2</td>
<td>Mesenchymal</td>
<td>Molecular marker panel of AKT2, CK8/18/19, VIM, Twist1 and SNAI1, could successfully identified metastatic CTCs which corresponded with recurrence in hepatocellular carcinoma.</td>
<td>(200)</td>
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<tr>
<td>PIK3q</td>
<td>Mesenchymal</td>
<td>EMT-like CTCs with PI3Kq and Twist expression CTCs which could contribute to therapeutic resistance in ovarian cancer patients.</td>
<td>(201)</td>
</tr>
<tr>
<td>SERPINE1/PAI1</td>
<td>Mesenchymal</td>
<td>EMT marker panel including KRT 5, 7, 18, 19, EpCAM, CDH1, FN1, CDH2 and SERPINE1/PAI1 has been successfully employed to categorize different CTC phenotype.</td>
<td>(17)</td>
</tr>
<tr>
<td>OB-cadherin</td>
<td>Mesenchymal</td>
<td>OB-cadherin positive CTCs were found in castration resistant metastatic prostate cancer and the increased number CTC’s co-expressing O-cadherin correlated with bone metastasis.</td>
<td>(151)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Mesenchymal</td>
<td>Fibronectin A multi-marker panel comprising of FN1, CK7 and CK19 were used to successfully differentiate advanced NSCLC patients from healthy controls.</td>
<td>(202)</td>
</tr>
<tr>
<td>Plakoglobin</td>
<td>Epithelial</td>
<td>Elevated plakoglobin expression had significant correlation with poor distant-metastasis-free survival (DMFS) in breast cancer patients.</td>
<td>(174)</td>
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and evolve in response to extraneous cues. As we continue to explore the complex mechanisms that drive metastatic dissemination, improved experimental models are required to adequately
address the essentiality of EMT in metastasis. EMT in cancer diagnosis and treatment is limited, in part due to tumor heterogeneity. Recent studies described an EMT gradient in different tumor types reflecting intrinsic tumor heterogeneity in EMT execution. Importantly, EMT status correlates with overall (OS) and disease-free survival (DFS). The new era of personalized medicine would facilitate a tailored cancer treatment at different stages of cancer progression. The advancement of technologies has provided a wider avenue to understand EMT pathway and further target them based on intrinsic EMT status of a cancer. In the era of deep sequencing technologies, single cell sequencing approaches holds the potential to shed more information on the spectrum of EMT transition states and reveal its contribution to intratumoral heterogeneity. Further, isolation and characterization of CTCs have enabled us to advance our knowledge as to how EMT contributes to cancer spread. EMT markers are now widely employed for enumeration of CTCs and it is proven that mesenchymal CTCs are associated with worse prognosis in cancer patients. CTCs as liquid biopsy has immense potential to become one of the promising tools for disease monitoring and therapeutic response. The data from existing studies are very encouraging, however clinical utility of CTCs still remains to be evaluated owing to lack of standardized isolation strategies as well as the inability to capture all of the cellular repertoire. Future analyses should focus on consistent isolation strategies that will enable accurate tracking of CTCs for monitoring residual disease and therapeutic response. Standardized procedures combined with detailed molecular characterization of CTCs will be essential to conduct studies on larger cohorts of patients. Future investigation can lead to a nuanced understanding of role of EMT in CTC generation, survival and colonization. It will pave the way forward for developing new treatment modalities that can complement current therapies more effectively.

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Send correspondence to: Remya Raja, Institute of Bioinformatics, Discover Building, International Tech Park, Bangalore- 560066, India, Tel: 91-80-28416140, Fax: 91-80-28416132. E-mail: remya@ibioinformatics.org