Alessandro Fatatis *Editor*

Signaling Pathways and Molecular Mediators in Metastasis

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To Mara, Enrico and Fabrizio,
for introducing me to life.

To Olimpia and our son Andrea,
for making my life worthwhile.

Preface

Since the dawn of modern medicine, the spreading of a tumor has always been regarded as an unfavorable event. However, until recently patients would refer to this condition as a tumor that had 'returned' and simply prepare for additional rounds of treatment, as if the recurrence would be a replica of the original disease, just in a different site.

Today, patients and physicians alike are fully aware that the detection of metastases is a negative prognostic factor, in most cases leading to serious repercussions on the quality of life and overall survival. Indeed, the treatment of metastatic patients with curative intent is frequently a daunting task, albeit not always bond to fail. The first chapter of this book provides an excellent overview of the problem.

The gravity of metastatic disease is often due to the multiplicity of lesions to be treated, combined to locations not easily amenable of surgical excision or irradiation. Although cytotoxic, targeted and in selected cases hormone-deprivation systemic therapies can initially bypass some of these limitations, the onset of resistance mechanisms will eventually obligate physicians to change chemotherapy protocol, switch to different targeted drugs and eventually resolve to palliative measures.

Furthermore, we now recognize that metastatic lesions might share very little with their tumors of origin as a myriad of events occurring either at primary or secondary sites can dramatically alter genotypic and phenotypic features of cancer cells during the progression of the disease.

Thus, aiming to the successful treatment of metastatic lesions based on information gained from primary tumors should be considered a dangerous overlook. Several chapters of this book provide a compelling review of the current knowledge on the changes occurring in different organ microenvironments that permit malignant colonization and subsequent progression of clinically overt metastases. These changes affect not only epithelial cancer cells, but also the resident cells of the surrounding stroma, immune cells and bone marrow-derived cells that can be locally recruited to create a pre-metastatic niche.

A significant percentage of solid tumors are currently diagnosed at their initial stages. However, the progress in screening and diagnostic procedures only marginally translates into major survival benefits for patients, as too many still succumb to

metastatic disease often years after the initial diagnosis. A better understanding of the mechanisms and molecular mediators promoting local tumor progression and invasion into the circulatory and lymphatic systems will lead to appropriate therapeutic strategies aiming to limit the extent and duration of cancer spreading. A number of chapters in this book very effectively address these particular issues.

This volume presents the work of scientists at the forefront of the metastasis research field and it is a testimony of the power of their intellect, dedication and efforts to improve the range of treatment options for cancer patients and effectively counteract the most lethal complication of their disease. I knew some of them personally prior to undertaking this project and learned more about the others because of it. It has been a true privilege for me to work on this text with such a group of brilliant co-authors. They all have been enthusiastic about this book from the very beginning and demonstrated a remarkable willingness to participate, despite the variety of academic and clinical commitments they had to attend and the demands of their research groups and medical teams. For this, and for their contribution of competent writing and effective illustrations, I am extremely grateful.

I am also indebted to all the staff at Springer, especially Melania Ruiz (Publishing Editor), Ilse Hensen (Publishing Assistant) and Sunil Padman (Project Manager). Their enthusiasm and knowledgeable assistance during the different phases of this project have been very much appreciated.

The ensuing of metastatic disease is too often responsible for the demise of patients that would be otherwise successfully treated for their primary tumors. The scientists involved in this project, along with many others in the field, intend to change this grim scenario forever.

Philadelphia, PA Alessandro Fatatis

Contents

Chapter 1
Introduction

Robert B. Den, MD and Adam P. Dicker, MD, PhD

Abstract Local therapies have continued to evolve with advances in surgical and radiation therapy techniques. This has contributed to improvements in survivorships in early stage disease. However, survival rates for metastatic disease remain poor. There has been increasing evidence that local therapy directly influences metastatic growth and overall survival. Both clinical and laboratory evidence support this hypothesis and an increasing understanding of the role of circulating tumor cells has further galvanized the idea of crosstalk between distant sites and the local disease. The current clinical arena is ripe for new innovations and the scientific advancements described in the remainder of this text set the stage upon which such progress will occur.

Cancer is the second leading cause of death in the United States for both men and women [1]. Currently, there are 1.5 million new cases diagnosed per year with a declining cancer-specific mortality rate of 600,000 per year due in large part to early detection and improved therapeutics. Five-year survivorship is markedly decreased between localized and metastatic disease. Regardless of the initial tumor type, 5-year overall survival with stage IV malignancy ranges from 2% to 30% in comparison to

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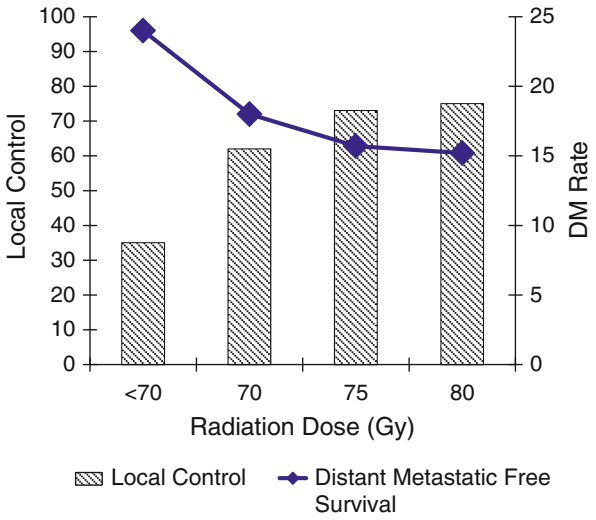

Fig. 1.1 Increasing radiation dose results in both an increase in local control as well as a decrease in distant metastatic free survival (Data based on Zelefsky et al. [22])

localized disease where 5-year survival is between 20% and 100% [1]. Thus, it is clear that the development of distant disease portends to patient death.

For the vast majority of malignancies, patients are diagnosed when the disease is localized. Local control rates continue to improve and have shifted the management of cancer from pure palliation into a chronic disease. Local control has greatly improved due to advances in surgical technique [2], improved understanding of pathologic features that are prognostic for further adjuvant [3–5], and more sophisticated planning and delivery of radiation therapy [6, 7].

In the field of prostate cancer, multiple phase III trials have demonstrated the benefit to radiation dose escalation [8–15]. These trials have shown statistically significant improvements in biochemical free survival without compromise of higher toxicities in either genitourinary, gastrointestinal, or sexual metrics as reported by both patients and physicians [16–21]. Retrospective series have demonstrated that increased radiation doses reduced the rate of persistent positive disease on post treatment prostate biopsy [22] (Fig. 1.1). A meta-analysis of these trials demonstrated a linear correlation between total dose and biochemical progression free survival in all risk groups [23]. Biochemical progression has been shown to be a surrogate endpoint for prostate cancer specific mortality [24].

At the same time, the combination of androgen suppression therapy with radiation therapy has consistently demonstrated improvements in not only local control [22] and biochemical control, but in overall survival benefit as well. Multiple studies have shown that even short term (4–6 months) of androgen ablation with radiation therapy translates into improved cause-specific survival [25–32]. Further, in patients with high risk or locally advanced disease long-term hormonal suppression (2–3 years) in combination with radiotherapy results in improved local control and overall survival [33–38].

Another primarily hormonally driven tumor, breast cancer, has seen decreased rate of in field breast recurrences with increased doses of adjuvant radiation therapy [39–41] and the addition of systemic agents such as tamoxifen [42]. While hormonal manipulation improved local control, it has been demonstrated in multiple large randomized studies to be insufficient to prevent local regional recurrences as monotherapy [43–45]. Further, local control has been demonstrated to lead to improvement in overall survival as demonstrated in the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) most recent meta-analysis [46]. A 20% absolute improvement in local control with the addition of radiation therapy at 5 years translated into a 5% improvement in overall survival at 15 years. This was seen both for women undergoing breast conserving surgery as well as those receiving post mastectomy radiation therapy.

A different approach to improve local therapy was adopted in the field of head and neck cancer with regard to radiation therapy. Instead of increasing the dose of radiation, alternative fractionation schemes were used to provide greater biologically equivalent doses. There are numerous phase III trials supporting non-standard fractionation for head and neck cancer resulting in a 10% absolute improvement in local control [47–49]. In addition, the addition of chemotherapy, specifically a platinum-based regimen, has consistently increased both local control and overall survival [50–53]. Further, given the high levels of EGFR seen in head and neck cancers, the targeting of this receptor using the monoclonal antibody Cetuximab combined with radiation therapy results in improved local control and survival as well [54].

While the addition of systemic agents to local therapy (surgery or radiation) has clearly shown benefit in improvement in overall survival, more recently there has been increasing evidence that the corollary is true; the addition of local therapy to systemic therapy translates into increased overall survival. Two phase III trials within prostate cancer have demonstrated the survival benefit with the addition of radiation therapy to androgen suppression therapy [55, 56]. The SPCG-7/SFUO-3 trial [55] revealed a 12% absolute improvement in cause specific survival and a 9% improvement in overall survival with the addition of radiation therapy. The Intergroup trial [56] showed a similar 8% improvement in cause specific survival and a 5% absolute improvement in overall survival. These patients with locally advanced and high-risk features had previously been thought to have subclinical microscopic metastatic disease and it was presumed that there was no role for localized therapy. Further, the addition of radiation did not adversely impact the quality of life of these patients [57]. A post hoc analysis of SWOG 8894 revealed that amongst patients with metastatic cancer those who underwent prior radical prostatectomy had a better response to androgen ablation and better survival than those with an untreated prostate [58]. In a study of hormone refractory metastatic prostate cancer the patients who underwent prior prostatectomy/radiation had better survival than those who had no prior local treatment [59]. These clinical studies suggest that removing the prostate in metastatic prostate cancer might result in a more durable response to systemic treatment.

The benefit of local control to improve overall survival even once the disease has metastasized is clearly demonstrated in kidney cancer. Cytoreductive nephrectomy is well established as a critical component of the management of metastatic renal cell carcinoma. This practice is based on results of two large randomized trials [60, 61] that

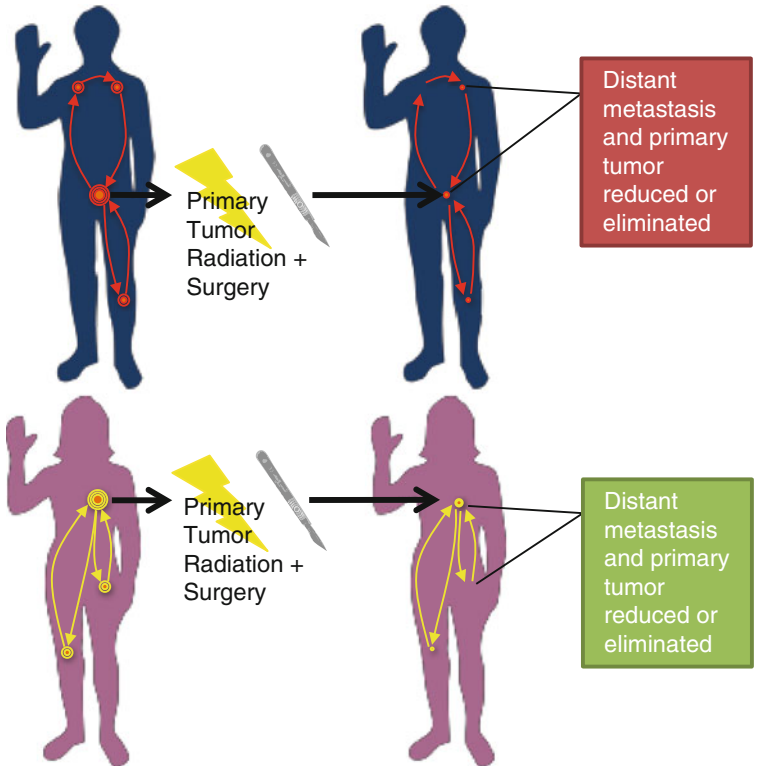

Fig. 1.2 Treatment of the primary tumor with a localized modality directly impacts sites of metastatic disease resulting in improvements in cancer specific and overall survival

compared surgery with interferon to interferon monotherapy. Both trials [62] showed significant survival advantages with the addition of surgery (median survival 13.6 vs. 7.8 months) and recently, retrospective data [63] suggested the benefit of nephrectomy in the setting of vascular endothelial growth factor targeted therapy, which has become the new standard of care for patients with metastatic renal cell carcinoma.

Recently, there has been increasing interest in understanding the interplay between local disease and metastatic deposits. Historically, cancer cells have been thought to possess the ability to leave the primary tumor and seed metastatic deposits and a clear temporal relation between local failure and metastasis has been documented. William Halstead, who posited that breast cancer spread contiguously from the primary site through local and regional nodes before reaching metastatic sites, pioneered this relationship in the breast cancer literature.

Currently, it is unclear whether locally persistent disease results in metastatic formation or is a prognostic factor for more virulent disease. If the former is true, then improvement in local therapy should translated into improved distant control (Fig. 1.2). In a trial randomizing men with advanced prostate cancer following prostatectomy to adjuvant radiation therapy or observation not only was there an increase in overall

survival and but in distant metastatic free survival as well [64]. As well, Kuban et al. [14] demonstrated that the addition of 4 days of localized treatment corresponded to a reduction in the distant metastatic rate of 96% vs. 83% at 8 years. Zelefsky et al. [22] also demonstrated that post treatment biopsy positivity rate was significantly associated with worse distant metastases free survival (DMFS) and increased prostate cancer specific death. Coen et al. [65] showed a statistically significant difference in DMFS between those patients with local control vs. locally persistent disease (87% v 80%, 77% v 61%, 72% v 37%) at 5, 10, and 15 years respectively. Further, it was demonstrated that there was an increasing risk of distant metastasis over time in patients who ultimately develop local failure. Thus, through improved treatment of the primary site, there is decreased widespread metastatic burden.

The identification of circulating tumor cells and the observation that these cells can colonize tumors of origin [66] has led to the "tumor self seeding" hypothesis [67]. This theory highlights the increasing importance of not only localized therapies, but also improvements in chemotherapy and targeted biologics.

There has also been increasing excitement in the use of "local" approaches to treat oligometastatic disease. Stereotactic body radiation therapy or surgery has been used in the setting of patients with limited distant disease burden with success [68]. Currently, there are phase I trials examining the incorporation of targeted agents with radiation therapy in this patient population [69].

Historical response to single agent chemotherapy and combination therapy has poor results [70]. With increasing understanding of the underlying molecular pathways and drivers of cancer, improvements in survival have been demonstrated [71]. In fact, the BATTLE (Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination) trial presented at the 2009 ASCO meeting [72] and updated at the 2010 AACR Meeting [73] demonstrated the ability to select patients based on mutational status to different biologic agents. Further, these findings suggested that this approach improves the response rate in patients when compared to historical controls treated with traditional chemotherapy. The true benefit of such an approach is that it allows the optimal therapy to be delivered to a patient population which generally has overall poor functional status and impaired reserve to tolerate aggressive therapy.

Clinically there is great interest in improvements to current treatment options and a need for more robust understandings of the molecular drivers of both formation and spread of metastases. While improvements in local control have increased greatly over the past decade, large breakthroughs in the management of metastatic disease have yet to be fully realized. Thus, the scientific advancements described in the remainder of this work set the stage upon which such progress will occur.

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Part I
Local Invasion

Chapter 2
Reactivation of Epithelial-Mesenchymal Transition in Invasive and Metastatic Cancer

Michael D. Amatangelo and Mark E. Stearns

Abstract During development cells of epithelial and mesenchymal origin convert between the two phenotypes in what has been described as Epithelial-Mesenchymal Transition (EMT) and Mesenchymal-Epithelial Transition (MET). The common characteristics exhibited during EMT are a loss of epithelial cell contacts, a reorganization of cytoskeletal components to promote a motile phenotype, and a remodeling of the surrounding extracellular matrix to allow for invasion. These events are tightly regulated and required for proper cellular organization and organogenesis during development. Studies in cancer models have identified an analogous plasticity of some epithelial cancer cells which acquire mesenchymal features as a means to escape the primary tumor mass. During the initial stages of tumor metastasis a complex series of events occur in which cancer cells leave the original tumor site and migrate to other parts of the body via the bloodstream and/or the lymphatic system. All metastatic cells must first acquire the abilities to disseminate, migrate and invade the surrounding tissue to allow for metastasis to occur. Thus, a reactivation of developmental pathways resulting in an EMT-like program is one possible mechanism by which cells acquire these capabilities and are able to form distal metastasis. Intriguingly, many similarities between developmental and oncogenic EMT have been identified and has led to our understanding of common signaling pathways (including TGF-beta, Ras and Wnt), transcriptional regulators (including the Snail, Zeb and Twist families) and microRNAs (including let-7 and miR-200 families) which regulate EMT. Aberrant regulation of these pathways and factors is associated with increased metastatic potential *in vitro* and in animal models and correlate with poor clinical outcomes. This chapter focuses on the EMT program in cancer, its regulation, its parallels to developmental EMT and its significance to the progression to metastatic disease.

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2.1 Defining EMT and Its Relevance in Cancer

Epithelial-mesenchymal transition (EMT) describes the progression of cellular phenotype from an epithelial to mesenchymal state. Within differentiated tissues, epithelial cells are rigid and perform surface-barrier and secretory functions, whereas mesenchymal cells are highly migratory and perform scaffolding, anchoring and maintenance functions. Normal epithelial cells are constrained in a two dimensional cobblestone sheet and are connected by specialized structures including tight junctions, adherence junctions, desmosomes and gap junctions. Within this sheet, epithelial cells exhibit an organized polarity that is maintained by these junctions in which the cells have a free apical (luminal) surface and an adhesive basal-lateral surface which anchors to the basement membrane (see Fig. 2.1). The basement membrane serves as a key extracellular partition made of extracellular matrix proteins that separates and impedes the movement of epithelial cells into the surrounding stroma. In contrast, mesenchymal cells exhibit a spindled shaped, bi-polar

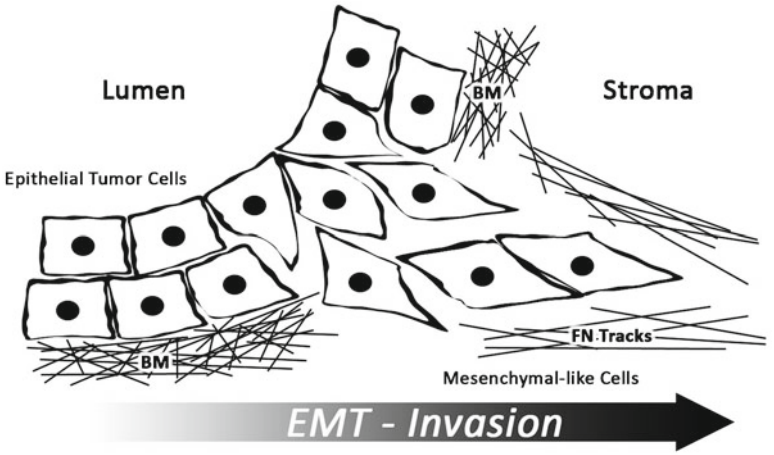

Fig. 2.1 Reactivation of the developmental Epithelial-Mesenchymal Transition (*EMT*) program promotes progression towards metastatic disease in cancer. During EMT cells lose the rigid 'cell-cell' junctions characteristic of epithelial tissues, including E-cadherin based adherence junctions, which allows for cells to disseminate from the primary tumor mass. Cells also change the composition of their cytoskeleton, losing epithelial cytokeratins and gaining vimentin and FSP-1 expression, changing their morphology and promoting cell motility. Furthermore, cells begin to express mesenchymal proteases, such as MMP-2 and MMP-9, and lay down extracellular matrix proteins, like fibronectin (*FN*), which help degrade the basement membrane (*BM*) surrounding epithelial tissues and creates tracks in the surrounding stroma to promote local invasion. Such changes allow cells access to the lymphatic and circulatory systems to advance the formation of distant metastases. EMT can be induced by genetic changes (Ras hyperactivity) and factors (such as TGF-beta) present in the microenvironment from tumor cells, stromal fibroblasts and tumor associated inflammatory cells and is regulated by a variety of oncogenic and developmental transcription factors and miRNAs

morphology and lack the apical-basal polarity of epithelial cells. They also migrate individually and do not create rigid contacts with neighboring cells. This results in mesenchymal cells forming loosely organized, fibrotic tissues. The prototypical mesenchymal cell is the fibroblast, which is a highly motile cell responsible for maintaining connective tissue and the stroma surrounding epithelial tissues. Because each of these cell types represents a distinct lineage, each with a unique gene expression profile, this transition represents a considerable change in cellular physiology and biochemistry.

EMT events for the most part have been described as part of germ layer reorganization and tissue remodeling during embryonic development. Specifically, these EMT events are critical for mesoderm formation during gastrulation, neural crest maturation, organ morphogenesis as well as wound healing and tissue repair in the adult [1–4]. Cellular and embryonic studies of EMT have resulted in three observational changes in phenotype which have since defined this phenomenon [5, 6]. First, EMT is associated with morphological changes in which epithelial cells no longer exhibit the cobblestone network and apical-basal polarity of normal epithelia. Instead, cells become more dispersed and fibroblast-like in their morphology. Second, EMT is associated with changes in protein expression wherein cells down regulate the expression of cell-cell junction proteins like E-cadherin and epithelial cytokeratin filaments and begin to express mesenchymal associated proteins such as fibronectin, vimentin and N-cadherin. Last, cells change their physiology from a rigid stationary cell to a more motile phenotype and begin to express matrix proteins and proteases that aid in their migration and invasion through tissues.

Most solid human tumors (>90%) are carcinomas which arise from epithelial glands. Surgery to remove the primary tumors is an effective treatment for many cancers and patients treated prior to metastases are often cured. Unfortunately, treatment of advanced cancer is often complicated by metastatic disease where cancer cells have migrated to distant sites. Thus, the majority of cancer deaths are caused by the ability of cancer cells to become detached from the neoplastic epithelia and form metastases where surgery becomes impossible. It has become increasingly apparent that in order for cancer cells to accomplish this, an EMT-like program must be activated to prime nodular epithelial cells for the dissemination, movement and invasion required for metastatic spread [7]. The concept that EMT events are involved in the formation of metastatic cancer are primarily based on mechanistic studies done *in vitro* and in mouse models and observations that loss of epithelial characteristics and acquisition of mesenchymal markers within tumors is often associated with advanced disease. It must be noted, however, that EMT changes do not appear to occur in consistent patterns in cancers nor is the commitment to a mesenchymal phenotype always permanent and the reverse process, mesenchymal-epithelial transition (MET), is also observed [8].

Although many similarities in the major concepts of developmental and pathological EMTs exists, Kalluri and Weinberg have classified EMT events into three separate categories to help clarify key differences in the functional consequences and regulation of these events [9]. Accordingly, EMT events associated with early

embryo and organ development are considered type-1 EMT. This type of EMT is tightly regulated, does not induce fibrosis or systemic, uncontrolled invasion and results from cells which have not fully matured. Type-2 EMT events are associated with wound healing, tissue regeneration and fibrosis. This type of EMT is less controlled and occurs in adult tissues in response to inflammation. Type-3 EMT occurs in carcinoma cells in which changes in oncogenes and tumor suppressor genes in conjunction with tumor associated inflammation utilize the EMT machinery to induce a migratory phenotype and invasion. While there are functional differences between tumor progression and normal embryonic development it also has become quite clear that many similarities also exist [10]. Thus, it is limiting to assert that type-1 and type-3 EMT events are distinctly different and it should be considered that a type-3 EMT event is a reactivation of developmental pathways observed in type-1 EMT resulting from the cellular and environmental changes in cancer. This reactivation in the context of cancer consequently enhances epithelial cell plasticity and promotes aberrant invasive and migratory activities of the cancer cell.

Unfortunately, and unlike type-1 and type-2 EMT events, it has been difficult for pathologists to conclusively document type-3 EMT events associated with tumor progression and metastasis in humans [11]. EMT has, however, been directly observed at the leading edge of a spontaneously driven breast cancer model *in vivo* in mice utilizing stromal and epithelial cell specific *cre*-transgene markers [12]. Although this study definitively showed cells of epithelial origin becoming mesenchymal during tumor progression and being associated with increased invasion and metastasis, not all tumors that gave rise to metastases exhibited robust EMT. Therefore, it could be concluded that EMT is not the only mechanism by which tumors become invasive and metastatic. Invasive carcinomas may invade surrounding tissues as multicellular epithelial sheets which maintain cell-cell junctions and polarity in a process known as collective migration [13]. However, it has recently been demonstrated that cells restricted to collective invasion were only capable of lymphatic invasion and metastasis to adjacent organs, but not entry into blood vessels or dissemination to distant organ sites [14]. Furthermore, while in many tumors the presence or absence of local lymph node metastasis is a strong predictor of distant metastatic disease, in others, as many as 30% of patients free of lymph-node metastasis still develop disease at distant sites [15]. In agreement with these observations, cancers which display clear forms of collective migration, such as squamous cell carcinomas, rarely form distant metastases [16]. Collectively migrating cells also appear to always follow the tracks of a leading stromal cell but may also follow tracks or signals of migrating epithelial cells that have undergone EMT, suggesting that a cooperative interaction by cells with each other and the surrounding stroma is required for collective invasion [17]. The overall picture emerging from such observations is that collective migration may not be a sufficient for the formation of distant metastases. Regardless, it is clear that changes in the local extracellular matrix and adaptation of a motile phenotype are required for metastasis and such changes can occur as a result of EMT.

We suggest that type-3 EMT events play a significant role in the formation of distant metastasis. These events may represent transient processes of EMT-MET conversions whereby invasive cells which undergo EMT regularly revert back to a normal morphology following specific interactions with the surrounding stroma or to a new environment. Cells which exhibit the plasticity to undergo EMT and MET conversions are likely to be highly adaptable, metastatic and capable of forming distant metastases. While proof that such events occur during metastasis is not conclusive, given the discrete nature in which EMT might occur, standard pathological examination is not conducive to observing EMT or associating EMT with advanced and metastatic cancer. This is primarily because type-3 EMTs occur to different extents and exhibit different phenotypes, with some cells retaining more epithelial traits then others. Interestingly, many cells undergoing EMT adopt a cancer stem cell-like phenotype and are able to produce metastases in mice at very low titer [18, 19]. These results suggest that cells undergoing EMT might not have to en mass in order to promote the formation of metastasis. Accordingly, such events might go unobserved during pathological observation of tumors and the frequency of cells displaying EMT markers might not correlate with metastatic spread. So, while it is apparent in animal and *in vitro* models that different type-3 EMT events are related to metastatic potential, it is unclear whether the extent which a cell loses epithelial characteristics or gains mesenchymal traits or whether the quantity of cells undergoing EMT has any correlation to metastasis in humans. Thus, evidence in support of EMT will require real-time Imaging since the dynamic nature of such EMTs are difficult to capture in fixed tissues where only a small number of tumor cells might exhibit EMT markers at any one time. Furthermore, standard histological examination of human tumors is observational and not mechanistic in nature and therefore limited in its ability to understand the contribution EMT might have to metastatic progression. Until experimental techniques are available which resolve these issues in humans, the importance of EMT to human cancer metastasis will remain controversial.

2.2 EMT Biomarkers and Their Functional Significance

In order to generate cells with specific functions cells during development must exhibit a level of plasticity that allows them to give rise to or morph into other phenotypes. As this process proceeds, cells must change the repertoire of proteins they express in order to function in their new role. Identification of such changes in protein expression can be used as biomarkers to identify cells associated with a specific purpose. A variety of biomarkers specific to cells of epithelial and mesenchymal origin have been identified to demonstrate the three subtypes of EMT. Indeed, the changes in these biomarkers, including changes in ‘cell-cell’ adhesion, cytoskeleton dynamics and matrix remodeling, have functional consequences and clinical significance as they all play important roles in metastatic progression.

2.2.1 Disruption of Epithelial Cell-Cell Junctions

E-cadherin is an adherence junction protein that is expressed at 'cell-cell' junctions in nearly all epithelial cells but is absent in mesenchymal cells [20]. Furthermore, E-cadherin expression is critical for proper organization and differentiation of epithelial cells during development, as well as for maintaining the apical-basal polarity that is a hallmark of epithelial tissues [21, 22]. Decreases in E-cadherin expression are nearly always observed during EMT associated with development, tissue fibrosis, wound healing and cancer progression and thus its loss is the major biomarker for an EMT event [23]. The most convincing evidence for EMT being associated with invasive and metastatic disease is that loss of E-cadherin-based adherence junctions is consistently associated with progression to invasive carcinoma and poor prognosis in most human epithelial cancers, including carcinomas of the breast, colon, prostate, stomach, liver, esophagus, skin, bladder, kidney and lung [24]. The loss of E-cadherin at 'cell-cell' junctions promotes metastasis by enabling cells to detach in response to the shear forces found in lymphatic vessels, venules and arterioles, facilitating their dispersion from the tumor mass [25].

Impairment of E-cadherin-mediated cell adhesion during tumor progression has been shown to occur by deletion, mutation, chromatin rearrangement and hypermethylation. In addition, loss of E-cadherin promoter activity has been found to occur in many metastatic malignancies [24, 26]. In fact, knockdown of E-cadherin alone can induce wide-ranging transcriptional and functional changes which manifest in EMT and contributes to metastatic dissemination [27]. Conversely, several groups have demonstrated that forced re-expression of E-cadherin in malignant cells results in a reversion of the EMT phenotype and inhibits invasion and metastasis [28–31]. Additionally, in an *in vivo* model for spontaneous pancreatic cancer, maintenance of E-cadherin expression during beta-cell tumorigenesis inhibited invasion and arrested tumor development at the adenoma stage [30]. The implication being that loss of the E-cadherin adherence junction complex is one of the rate limiting step in EMT and progression to invasive cancer *in vivo* and thus serves as the gatekeeper of the epithelial phenotype [32].

Critical for maintaining E-cadherin based adherence junctions is its interaction with p120 CAS and alpha-, beta-, and gamma-catenin, which link E-cadherin to the actin cytoskeleton. Disruption of the intracellular E-cadherin–catenin complex alone is sufficient for a loss of 'cell-cell' adhesions and the tissue rigidity characteristics of epithelial tissues which occurs in tandem with tumor invasion [33]. In particular, beta-catenin is released from E-cadherin complexes into the cytoplasm when these 'cell-cell' junctions are disrupted and can act as a signaling molecule. The signaling activity of beta-catenin regulates cellular plasticity and type-1 EMT during heart cushion development [34]. Accordingly, beta-catenin localization has been used as a biomarker of EMT in both fibrosis and cancer [35, 36]. In normal epithelium, cytoplasmic beta-catenin is degraded by the ubiquitin-proteasome pathway through a multiprotein destruction complex containing APC and GSK-3beta. Upon activation of Wnt signaling pathways within the cell (which regulate

proliferation, morphology, motility, and fate during embryonic development) the activity of GSK-3beta is inhibited. This results in the accumulation of beta-catenin in the cytoplasm which can translocate into the nucleus where it can regulate gene expression. Thus, cytoplasmic or nuclear localization of beta-catenin is indicative of and a biomarker for EMT and an invasive phenotype [36]. For example, in advanced colorectal cancers the central region of the tumor exhibits membrane localized beta-catenin associated with E-cadherin at cell junctions. In contrast, at the invasive front membrane staining is lost and beta-catenin is localized in the nucleus [36, 37]. Active Wnt/beta-catenin signaling and nuclear beta-catenin accumulation also correlates with EMT, invasion and a poor prognosis in breast cancers [38, 39].

During EMT loss of the E-cadherin cell junction complex is often accompanied by a concomitant up-regulation of mesenchymal cadherins, such as N-cadherin and cadherin-11 [40]. This process is described as "cadherin switching" and readily occurs during type-1 EMT when cells separate from the epiblast layer to ingress the primitive streak and when epithelial cardiomyocytes migrate toward the endocardium during heart morphogenesis [41, 42]. N-Cadherin is typically expressed by mesenchymal cells, fibroblasts and neuronal cells, however, aberrant expression of N-cadherin is also observed in invasive breast, prostate and melanoma cancer cells [43–45]. During type-3 EMT events, N-cadherin expression promotes loss of epithelial cell polarity and increased cell motility, invasion and metastasis, having the opposite effect as E-cadherin expression [46, 47]. Like N-cadherin, cadherin-11 is not expressed by normal epithelial cells but is induced during EMT events associated with development [48]. In addition, cadherin-11, is also expressed in aggressive melanoma, breast and prostate cancer cell lines and appears to coincide with greater cellular invasiveness and a poor clinical prognosis in patients [43, 49, 50]. Expression of these mesenchymal cadherins promotes tumor cell dissemination and invasion independent of E-cadherin down-regulation, highlighting their potent tumor promoting activities [51]. It appears that mesenchymal cadherins promote local invasion by allowing dynamic interactions with the endothelial and stromal components surrounding epithelial tumors [44]. In sum, loss of E-cadherin, nuclear beta-catenin localization and gain of mesenchymal cadherin expression are all currently recognized as key biomarkers for EMT and metastatic potential.

2.2.2 Changes in Cytoskeleton Dynamics

Studies of EMT have also revealed that the expression of vimentin and Fibroblast Specific Protein-1 (FSP1), also known as S100A4, are commonly associated with EMT during embryogenesis and highly invasive tumor cells. Vimentin is a mesenchymal intermediate filament which is known to play a role in maintaining cell integrity in response to mechanical stress [52]. Vimentin also plays a key functional role in the migration and contractibility of cells, as vimentin null mice have fibroblasts with a decreased ability to migrate and exhibit impaired wound healing [53]. During embryogenesis vimentin is expressed in mesodermal cells which exhibit a

highly motile and invasive phenotype [54, 55]. Specifically, vimentin is turned on at the onset of when cells first detach and migrate from the epithelium to form the mesoderm, and continues to be expressed in all mesenchymal cells making it an excellent biomarker for the mesenchymal phenotype [56]. Carcinoma progression to an invasive phenotype is also often accompanied by increased expression of vimentin in a wide range of cancers [57–61]. Conversely, knocking down the expression of vimentin in highly malignant colon, prostate and breast cancer cells inhibits their ability to migrate and become invasive [62, 63]. Vimentin still remains a controversial type-3 EMT marker, however, as pathologists do not always observe significant increases in vimentin expression in cancer and it is sometimes associated with benign tissue [64]. However, the limitations in histological examination of human tumor sections appear to be part of the problem. Advances in analysis of circulating tumor cells have recently associated vimentin expression with metastatic disease, thus vimentin positive circulating tumor cells might be indicative of tumor associated EMT [65, 66]. In addition to up-regulation of vimentin, rigid epithelial cytokeratins are also down-regulated during EMT and actin filaments are organized into stress fibers [67]. The consequence of this dramatic remodeling of the cytoskeleton is facilitating pseudopod formation at the leading edge of the cell to promote invasion and migration.

Fibroblast Specific Protien-1 (FSP-1) appears to be another biomarker for EMT that is important for the transformation of epithelial cells to a metastatic phenotype. FSP-1 is a member of the S100 family of cytoplasmic proteins and is homologous to S100A4. Members of this family are calcium binding, low molecular weight proteins that function as both homo and heterodimers and are implicated in cytoskeletal-membrane interactions, calcium signal transduction, and cellular growth and differentiation [68]. FSP-1 expression was first observed in cells of mesenchymal origin during mesoderm formation and during inflammation induced fibrogenesis [69]. FSP-1 is absent in normal epithelial cells but is prevalent in mesenchymal cells including fibroblasts, monocytes, macrophages and lymphocytes, all of which exhibit a migratory phenotype [69–71]. Transfection studies have shown that FSP-1 is involved in the stimulation of cellular motility and it has been shown to colocalize with myosin IIA and actin filaments at the leading edge of migrating cells [72].

FSP-1 expression is commonly observed in cultured epithelial cells undergoing growth factor induced EMT as well as in EMT during renal fibrosis in transgenic mice [73, 74]. The FSP1 gene is a direct target of nuclear beta-catenin and is associated with promoting the progression of invasive and metastatic cancer [75]. Overexpression of FSP-1 in non-metastatic breast cancer and bladder cancer cells has been shown to promote local tumor invasion and metastasis to lungs and lymph nodes [76, 77]. Conversely, knock down of FSP-1 reduces cell motility, invasiveness and metastatic potential *in vivo* [78]. In addition, FSP-1 is expressed by invasive tumor cells undergoing EMT in PyV-mT induced mammary tumors in mice and mice over-expressing FSP1 crossed into tumorigenic backgrounds have offspring which exhibit increased frequency of metastasis even tough primary tumor incidence and size do not change [7, 79]. Finally, human patient studies have further revealed that in a panel of 349 breast cancer patients, FPS-1 expression was the