Role of Par-4 in EMT

Mir Mohd Faheem, Archana Katoch, and Anindya Goswami

Abstract

The importance of Par-4 in apoptosis has been deciphered in depth. Interestingly, a paradigm shift is emerging with respect to the non-canonical roles of Par-4. The intricacy between Par-4 and EMT is significantly gaining traction, which is the main focus of this chapter. The chapter commences as we first delineate EMT's transitory and dynamic nature as opposed to the conventional view that portrays EMT as unidirectional and irreversible. We have emphasized EMT's culpability in the genesis of the metastatic program and how EMT-associated transcription factors (EMT-TFs) manipulate the cancer cells to acquire a motile phenotype suitable for intravasation, migration, and secondary metastasis. We as well discuss the molecular signaling pathways regulating EMT and the challenges rendered by the acquisition of EMT in cancer therapeutics. In the later sections, we have diligently highlighted the emergence of Par-4 as a prospective EMT nullifying candidate and therapeutic opportunities thus evolving around it. Particular emphasis is attributed to novel burgeoning role of Par-4-mediated negative regulation of the following anti-metastatic cascades; for example, modulation of β-catenin pathway, cytoskeletal rearrangements, and extracellular (ECM) remodeling and of course the anti-metastatic microRNAs. Lastly, we put forth innovative insights that link Par-4- and TGF-β-mediated lethal EMT.

Keywords

Epithelial to mesenchymal transition (EMT) · Cancer stem cells (CSCs) · Mesenchymal to epithelial transition (MET) · Extracellular matrix (ECM) degradation · Matrix metalloproteinases (MMPs) · E-cadherins · β-Catenin · Cytoskeletal rearrangements · Par-4 · SAC domain · Secretagogue · miR-200c · Vimentin · EMT-associated transcription factors (EMT-TFs) · Twist-1 · Zeb-1 · TGF-β · Lethal EMT · Chemoresistance

1 Introduction

1.1 Epithelial to Mesenchymal Transition (EMT)

For the first time in 1908, Frank Rattray Lillie described the interconversion between epithelial cells and mesenchymal cells [1]. However, much later, in a seminal finding, Greenburg and Hay, on their studies in the primitive streak of chick embryos, unveiled that EMT is an evolutionarily conserved distinct cellular process involving epithelial to mesenchymal phenotype changes [2]. EMT is a distinct physiological roadmap illustrating a trans-differentiation process that allows an epithelial cell to attain a mesenchymal phenotype as illustrated by the following features: (1) massive transcriptional reprogramming (2) loss of cell adhesions and the apical-basal polarity (3) extracellular matrix (ECM) remodeling (4) transitions in cellular morphology (from a cobblestone epithelial morphology to a spindle-shaped mesenchymal one) (5) alteration in the signaling pathways controlling cell shape as well as motility (6) reprogramming of the gene expression [3]. Owing to this chain of transforma-

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tion, EMT generates a mesenchymal cell with increased motility and invasiveness compared to its epithelial counterpart. Although this dynamic process was initially coined as epithelial to mesenchymal transformation, it is in this day and age precisely known as epithelial-mesenchymal transition (EMT) to highlight its transient nature. Pertinently, the morphological changes that a cell endures all through the EMT phenomena are neither single-step/unidirectional alterations (from epithelial to mesenchymal) nor a *fait accompli*. Rather, cells undergoing EMT are distinguished by multiple quasi-mesenchymal states of intermediary nature (Fig. 1). The capability of cells to transition between the epithelial and mesenchymal states, partly or completely, demonstrates the inherent plasticity of epithelial cells. EMT is a reversible process, and mesenchymal cells can experience reverse transition by a process known as mesenchymal to epithelial transition (MET), which is of utmost therapeutic significance.

The epithelial-mesenchymal transition (EMT) is a biological phenomenon in the course of physiological processes, for example, embryonic development, induction of pluripotency, embryonic stem cell differentiation, tissue repair, and wound healing, respectively [4]. Surprisingly, unlike true epithelial features, the mesenchymal cells are truly aggressive in terms of their massive invasive as well as migratory properties exercise through the extracellular matrix (ECM). Therefore, the extremely rigorous differentiation potential of mesenchymal cells is considered as a vital cog for normal embryonic development in various organisms. As a result, EMT not only succeeds in orchestrate cellular rearrangements but, at the same time, it also facilitates the organization of highly specialized tissues and organ systems [5]. This could explain why the essential molecular pathways regulating EMT, including TGF-β, Twist, Slug/Snail, Cripto, Six1, and Wnt/β-catenin, are highly conserved among mammalian systems [5]. Contextually, the convergence of all these pathways has empowered EMT in several pathophysiological conditions such as tissue/organ fibrosis, tumorigenesis, and metastasis as well as influencing the cancer stem cell behavior [6]. While the role of EMT during embryonic developmental stages, wound healing, and tissue remodeling processes is cumulatively beneficial for normal physiological events, contrast, the activation of EMT in cancer rather predispose the malignant cells more aggressive with acquired abilities of invasion, migration, stemness, and drug resistance. Albeit, these new postulates have driven the researchers to define EMT more explicitly so that we can more accurately distinguish between the physiological and pathological EMT processes and rationally discriminate the relationship between the two.

Although there is a substantial advancement in our understanding of the involvement of EMT in the invasion, migration, and metastasis of the tumor over the past decade, there

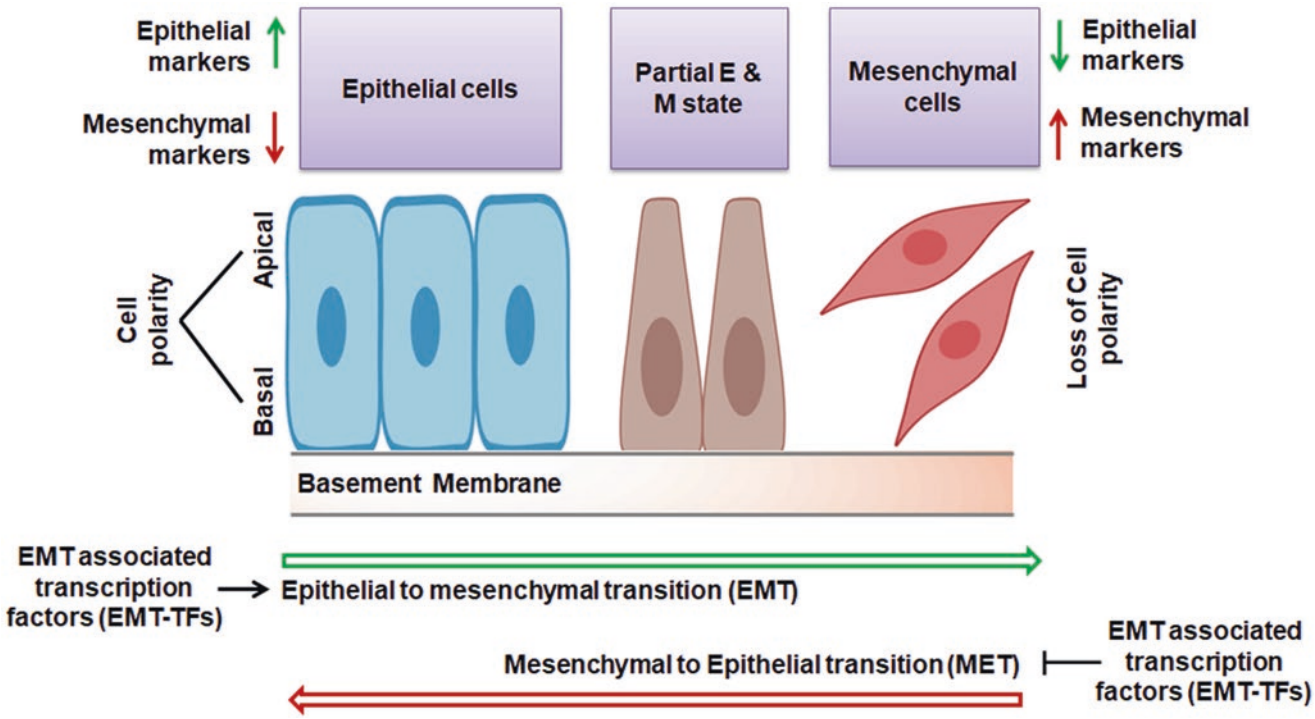

Fig. 1 Epithelial-mesenchymal transition (EMT). EMT encompasses a series of molecular events that result in the transition of a polarized epithelial cell into a mesenchymal cell. This transition is accompanied by a gradual loss of epithelial markers and a simultaneous gain of mesenchymal markers. The reverse process is known as mesenchymal-epithelial transition (MET)

are still large voids concerning the prognosis during the activated EMT state of cancer patients. Robert Weinberg and his colleagues have successfully defined "metastases" as one of the eight hallmarks of cancer. Metastasis truly empowers the terminally differentiated cancer cells to pierce surrounding matrix and distant sites, conferring the cascade as the most challenging aspect of cancer in regard to cancer therapeutics and clinical prognosis. Nonetheless, it is a well-established fact that EMT lies at the core of the metastatic cascade's genesis and, in this context, is of utmost therapeutic significance. As such, the successful treatment of many cancers may be considerably improved given our ability to prevent or even reverse the process of metastasis.

1.2 Regulators of EMT

In a devastating pathophysiology of cancer, a range of diverse players, including transcription factors, signaling intermediates, cytoskeleton proteins, are grossly involved in paving the smooth function of EMT cells. Indeed, activation of epithelial-mesenchymal transition (EMT) is not only responsible for the integration of signaling crosstalk engaged in proliferative pathways, but it also triggers cancer cells survival in the unfavorable catastrophic milieu. A diverse range of signaling pathways have been implicated in the modulation of EMT; among them, the most intensely studied being the TGF-β, Notch, and Wnt signaling pathways. Since the loss of adheren junction proteins, e.g., E-cadherin and Claudins, is a landmark event during EMT, most EMT-inducing signaling pathways are involved in the regulation of the repressors of E-cadherin and other adheren junction proteins. Of note, Zeb family, Snail family, and the Twist-1 are some of the critical EMT effectors which impede the E-cadherin expression [7–10]. Accordingly, in the following paragraphs, we recapitulate the handpick of vital effectors and regulators of EMT.

1.2.1 TGF-β Signaling Pathway

The overwhelming role of TGF-β signaling has been largely implicated in the context of dual purposes—physiological development as well as in promoting malignancy [3, 11, 12]. Not only it acts as a multifunctional and ubiquitously expressed cytokine, apart from EMT, TGF-β also regulates various cellular activities, including cell growth and tissue fibrosis [13, 14].. This multifaceted function of TGF-β primarily concedes its dual nature, i.e., a tumor-suppressive role in the early phases of tumor development but promoting metastasis in the later stages [15, 16]. Albeit, among all the TGF-βeta subtypes, TGF-β1 strongly adheres to the induction of EMT in tumor cells [17]. TGF-β regulates EMT through canonical Smad-dependent and Smad-independent manner. In the Smad-dependent signaling, binding of TGF-β to the TGF-β type II (TβRII) receptor trans-phosphorylates the TGF-β type I (TβRI) receptor which, in turn, activates the Receptor-Smads (R-Smads), Smad2, and Smad3. Following receptor activation, the R-Smads can regulate the gene expression by binding to the Common Smad (Co-Smad) - Smad4 and translocating into the nucleus [18–22]. Eventually, Zeb-1, Slug, Snail1, and Twist-1 transcription factors are examples of vital downstream targets of activated Smads, which predominantly alter the TME by triggering EMT-cascade [23, 24]. For generating a proof of concept, in an elegant experimental setup, using various mutant R-Smad constructs, Valcourt et al. have shown that a dominant-negative mutant of either Smad2 or Smad3 significantly abrogates the EMT induction in response to TGF-β [25]. Even though Smad3 is considered fundamental for EMT induction, the consequences of Smad2 on EMT induction are distinctly proven controversial [26–28].On the other hand, the Smad-independent signaling pathways are equally detrimental since they, too, elicit diverse cellular responses, including EMT induction [25, 29–31]. Intriguingly, TGF-β facilitates EMT, independent of Smads, by regulating the Ras, Rho-like GTPases, p38, Erk, and PI3K/Akt pathways and via extensive modulation of Notch, Wnt, and integrin signaling pathways [32].

1.2.2 Wnt Signaling

The canonical Wnt signaling pathway during embryonic development as well as tumorigenesis in the context of EMT induction has been well-documented in the literature [33–35]. β-catenin plays a significant role in the deregulated Wnt signaling pathway in a vast range of cancers. However, as far as its stability is concerned, β-catenin is phosphorylated by GSK-3β and degraded via the ubiquitin-dependent pathway in the absence of activated Wnt signaling, thereby maintaining lower cytoplasmic β-catenin levels. As soon as Wnt signaling pathway is activated, cytoplasmic β-catenin translocates to the nucleus, where it facilitates to the formation of a complex with TCF/LEF transcription factors and stimulates the expression of EMT-inducing target genes. Furthermore, β-catenin, together with TCF augments the expression of one of the central EMT-effector molecules, Slug [36] and prevents the degradation of Snail [37]. As mentioned above, Wnt signaling indirectly aids EMT by collaborating with the TGF-β and PI3K/Akt signaling pathways. In that context, one of the elegant examples is the stabilization of the β-catenin by PI3K/Akt signaling through Wnt ligands via the blockage of GSK-3β activation leading to promote spontaneous tumor formation [38]. In another classical experimental set up with palate medial-edge epithelial cells, Nawshad et al. have demonstrated a consistent suppression of E-cadherin protein levels due to the formation of stable complex, engaging LEF, Smad2, and Smad4 proteins [39]. Apart from its prominent role to curtail the cytoplasmic

E-cadherin levels, LEF, on the other hand, in alliance with Smad4 drastically augments the mesenchymal markers Vimentin, and fibronectin and thus facilitates cellular motility [39].

1.2.3 Notch Signaling

Notch signaling is a vital signaling arm that controls cell fate through regulating essential cellular functions, including cell proliferation and apoptosis [40]. However, the enigma of Notch signaling in EMT has been extensively studied in relevance to cancer progression [41, 42]. The promising outcome of some classical researches unearths that constitutive activation of Notch signaling governs the binding of Notch ligands to the transmembrane receptors, Jagged or Delta Like Ligands (DLL) of adjacent cells (DLL) [40], leading to the activation of the Notch pathway and subsequent cleavage of Notch to release its intracellular domain. As a consequence, Notch intracellular domain translocates to the nucleus and sequesters CSL (CBF-1-Suppressor of Hairless/Lag1), resulting in the transcription of Notch-target proteins, Hey1, Snail, Cyclin D, and c-Myc [43–45]. Apart from triggering EMT and therapeutic resistance, aberrant Notch signaling is widely prevalent in many cancers [46, 47]. However, crosstalk between the Notch and TGF-β signaling pathways are considered imperative for the TGF-β-induced EMT and migration [40, 44].

1.2.4 HIF-1α Signaling

A low oxygen level, also known as hypoxia, is a frequently observed phenomenon in primary tumors. What are the vital consequences when cancer cells undergo prolonged hypoxic stress? Hypoxic stress diligently fuels up in the accumulation of hypoxia-inducible factors (HIFs), which are known to induce EMT via Twist and Snail [48–50]. While HIFs are the major effectors of hypoxia, ERK, PI3K/AKT/mTOR, and NF-κB are vital pathways found to be extensively regulated by hypoxia-induced EMT [48, 51–53]. Burgeoning pieces of evidence show that loss of E-cadherin and augmented expression of Vimentin, N-cadherin, CXCR4, and SMA are prominent hallmarks of hypoxia-induced EMT [54]. While considering a major basic helix-loop-helix (bHLH) transcription factor Twist-1 in EMT activation, HIF-1α directly regulates Twist-1 by binding to the HRE elements in the Twist-1 promoter. Moreover, from a mechanism of action perspective, Twist-1 is found to be indispensable for HIF-1α-mediated EMT and metastasis [50, 55]. Despite its versatile role in the regulation of EMT, hypoxia indirectly potentiates the TGF-β-induced EMT via steadily augmenting Slug and Snail expression with concurrent inhibition E-cadherin [54]. Additionally, in pancreatic cancer cells, the Hedgehog signaling also regulates hypoxia-induced EMT and invasion [56].

1.2.5 Integrin Signaling

Integrins, a family of transmembrane receptors comprising α and β subunit, are extensively corroborated in the process of building an intracellular network through cell attachment between the neighboring cells or ECM. Notably, this meshwork is an essential component pertinent to cell proliferation, differentiation, adhesion, and migration [57, 58]. However, perturbed integrin signaling is deeply associated as a core mechanism in EMT / chemoresistance [57]. The mechanism which would explain the role of integrins in ECM destruction underscores the deliberate involvement of receptor tyrosine kinases (RTKs) to amplify pro-survival signals via ERK and PI3K/AKT axis [59]. As a part of this comprehensive mechanism, integrin signaling, possibly via integrin αv, could augment the TGF-β1- mediated downregulation of E-Cadherin, facilitating the EMT cascade [60]. Although cancer cells are adequately equipped with their own intrinsic survival and proliferation signals compared to non-cancer cells, specific integrins family members, even on top of that, further exaggerate the tumorigenesis. In contrast, some other integrins may either inhibit or confer negligible impetus on tumor promotion [58]. Nevertheless, the tumor-promoting integrins, αvβ3 and α6β4, seemingly work together with other RTKs for ECM degradation [61, 62]. Therefore, integrin signaling represents a potential target that may yield better clinical outcomes in anti-metastatic therapeutic development.

1.2.6 microRNAs

microRNAs (miRNAs) are small non-coding RNAs, approximately 20 to 22 nucleotides in length, overtly facilitate transcriptional and post-transcriptional gene regulation. miRNAs bind to the '3'untranslated region (UTR) of their target mRNA resulting in gene silencing via target degradation or translational repression. Interestingly, miRNAs are severely implicated in the pathogenesis of cancer, especially in the EMT process. While few miRNAs positively regulate EMT, others are yet to be explored. On the basis of their oncogenic activities, miRNAs are categorized into oncogenic miRNAs (oncomirs) or tumor-suppressor miRNAs. Table 1 lists some of the miRNAs that are altered during EMT and tumorigenesis.

miR-21 is a well-characterized oncomir known to target a major tumor-suppressor protein, PTEN (phosphatase and tensin homolog), to induce EMT [73]. However, miR-21 inhibition causes the restoration of the PTEN levels via inactivation of one of the indispensable arms of AKT/ERK1/2 signaling, which ultimately reverses EMT [74]. Alternatively, a more detailed study of molecular signatures of miR-21 also targets another tumor-suppressor, Leucine zipper transcription factor-like 1 (LZTFL1), for the restoration of EMT [75]. In a concerted effort to gear up advanced carcinogenesis, miR-10b, an oncogenic miRNA, is largely concerned

Table 1 EMT-regulating microRNAs

miRNA	Effect on invasion/metastasis	Target	Reference
miR-1	Suppression	Twist	[63]
Let-7 family	Suppression	NANOG, BCL2L1, Twist-1, Twist-2, c-MYC	[63, 64]
miR-10b	Promotion	HOXD10, Vimentin, KLF4, Apaf-1	[65]
miR-16	Suppression	Twist-2, CDK1, CDK2	[63, 66]
miR-22	Suppression	CDK6, SIRT1, SP1	[67]
miR-23a	Suppression	Twist-2	[63]
miR-30a/b	Suppression	Snail1	[68]
miR-33b	Suppression	Twist-2, Zeb-2	[63]
miR-34a	Suppression	Slug, Twist-1, Zeb-1, Snail1, and Notch	[69]
miR-141	Suppression	Zeb-1/Zeb-2	[63]
miR-200	Suppression	Zeb-1/Zeb-2	[70]
miR-300	Suppression	Twist-2	[63]
miR-337	Suppression	Twist-2	[71]
miR-373	Promotion	CD44	[72]
miR-506	Suppression	Snail2	[63]

because its regulation is controlled by Twist-1, and down the way, hyperactivated miR-10b targets the homeobox D10 (HOXD 10). On the other hand, recent research unveils that Twist-1 instigates the expression of miR-10b-mediated HOXD 10 suppression convincingly confers activation of pro-metastatic protein Ras homolog family member C (RHOC) [76]. In order to promote migration, another oncomir miR-9 directly targets CDH1 causing cell motility and invasion [77]. Downregulation of miR-9-mediated E-cadherin expression induces β-catenin signaling, contributing to the upregulation of VEGF tendering tumor angiogenesis. Clinically, miR-9 overexpression found in tumors is correlated with aggressive phenotypes and poor prognosis [78]. High levels of miR-103/107 are also associated with metastasis and poor outcome [79]. miR-103/107 functions to inhibit the expression of Dicer, causing global miRNA downregulation.

In contrast to the oncomirs, the tumor-suppressive miRNAs are attributed to stall malignant transformation. The cumulative miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) as prospective candidates to halt tumorigenesis is vividly characterized and known as an epithelial phenotype's guardians in breast cancer [80]. Predictably, loss of miRNA-200a is frequently observed in breast cancer, but this loss does not predict tumor recurrence or patient survival [81]. The miR-200 family activates the Sec23a-mediated tumor cell secretome, which leads to the secretion of metastasis-suppressive proteins [82]. miR-200 family members are encoded from two clusters and directly target the messenger RNAs of the E-cadherin transcriptional repressors Zeb-1 and Zeb-2. Notably, Burk et al. and other studies have shown that both promoter regions are repressed in mesenchymal cells by Zeb-1 and Zeb-2 through binding to the E-box elements [80, 83]. A double-negative feedback loop controlling Zeb-1-Zeb-2 and miR-200 family expression is vital for regulating the plasticity of the cancer cells. Another miRNA, miR-375, targets short stature homeobox 2 (SHOX2) to suppress EMT [84]. A novel miRNA, miR-506, significantly suppresses the expression of mesenchymal markers in the MDA-MB-231 human breast cancer cell line. In addition to restraining the transforming growth factor (TGF)-β-induced EMT, miR-506 also plays a vital role in the post-translational control of EMT-related genes [85]. miR-203 represses endogenous Snail, forming a double-negative miR-203/Snail feedback loop [86]. Additionally, miR-203 also targets Slug while TGF-β-induced Slug promotes EMT by repressing the miR-203 promoter to inhibit its transcription [87].miR-34 is one of the most studied tumor-suppressor miRNAs. It is implicated in the inhibition of EMT mediated by p53. It has been reported that activation of p53 downregulates the EMT induced by the transcription factor Snail via induction of the miR-34 gene. Suppression of miR-34 attributes the upregulation of Snail and endorses cell migration/invasion. Moreover, miR-34a prevents TGF-β-induced EMT, and the repression of the miR-34 gene by Snail is known to be a part of the EMT program [88].

1.3 EMT Markers

A variety of markers, including proteins as well as miRNAs, have been explored in pre-clinical settings to assess the extent of EMT. These markers have been categorized as (i) epithelial markers that are concerned with the maintenance of the epithelial state and (ii) mesenchymal markers that sustain the mesenchymal phenotype. Since EMT is characterized by the transition of an epithelial cell to a mesenchymal state, the attenuation of epithelial markers with simultaneous acquisition of mesenchymal markers lies at EMT's core. Table 2 highlights some of the EMT markers that are studied to assess EMT. Here, we summarize some of these well-accepted EMT markers that are analyzed to assess the EMT phenomenon.

Table 2 Markers of EMT and their role in tumorigenesis

Category	Marker protein	Role in EMT/ tumorigenesis	Reference
Epithelial markers	E-cadherin	Functional and expressional loss of E-cadherin during EMT and cancer. Downregulation increases cellular motility	[89]
	Claudins	Integral membrane proteins localized at tight junctions and maintain the epithelial cell polarity. Repressed during EMT to promote cancer cells invasion and migration	[90]
	Zonula occludins	Component of tight junctions and adherens junctions; controls cell migration; downregulated during EMT	[91]
Mesenchymal markers	N-cadherin	E-cadherin to N-cadherin switching during EMT. High expression in mesenchymal cells. Promotes cancer cell survival, invasion, and migration. High levels depict poor prognosis	[92]
	Vimentin	Established mesenchymal marker. Regulates cell shape as well as cell motility	[93]
	Fibronectin	Component of the tumor matrisome. Regulates the integrin signaling to facilitate EMT, invasion, and metastasis	[94]
	Snail1/2	Key repressor of E-cadherin and highly expressed in cancers. Promotes EMT and metastasis; predicts poor prognosis	[95]
	Twist-1	E-cadherin repprossor, promotes EMT, metastasis, and formation of cancer stem cells	[96]
	Zeb-1/2	Strong repressor of E-cadherin and aids EMT	[97]
	EPCAM	Highly expressed in circulating tumor cells	[98]

1.3.1 Epithelial Markers

Intact adheren junctions are a hallmark of epithelial morphology, which keep the cells tethered to each other. For maintaining cellular integrity, E-cadherin, encoded by the *CDH1* gene, is an important component protein identified at the adheren junctions, known to regulate the epithelial phenotype [99]. However, during malignant transformation, switching of E-cadherin to N-cadherin is prevalent and regulated by diverse signaling pathways [92]. Notably, loss of E-cadherin and subsequent EMT activation imparts overwhelming migration capability leading to metastatic dissemination. By analyzing clinical data, we can clarify how functional loss of E-cadherin, via chromosomal deletions, mutations, epigenetic silencing, or proteolytic cleavage, has been implicated in the development of pancreatic, breast, gastric, and skin cancers [100, 101]. On the other hand, the CDH1(E-cadherin) gene promoter's hypermethylation is extensively observed in malignant cells associated with EMT initiation [102].

Apart from E-cadherin, few other epithelial markers, including claudin family members, are involved in maintaining cell polarity and permeability. Claudins serve as a vital component of the tight junctions (TJs) [103]. Claudins comprise a large family of tetraspan membrane proteins, which are expressed in a tissue-specific manner. Similar to E-cadherin expression, a wide range of clinical samples display an altered expression of claudins, with claudin-1, -3, -4, and -7 being the most recurrently affected among the claudin family [104]. Strikingly, loss of claudin-3 (CLDN3) and claudin-4 (CLDN4) not only triggers robust morphological changes but adequately enhances growth, migration, and invasion processes. A deficit of CLDN3 and CLDN4 significantly boosts the E-cadherin protein levels with simultaneous N-cadherin downregulation [103]. Notwithstanding their EMT modulatory functions, some members of the claudin family are consistently downregulated during tumorigenesis, which is fairly constant with their function as a tight junction protein; however, claudin overexpression has also been reported in some cancers [104].

1.3.2 Mesenchymal Markers

In cancer, the role of EMT has been grossly corroborated into the severity of the disease and thus providing a mechanism for cancer cells to dislodge from their primary site and colonize at distant secondary sites. Rationally, therefore, a successful accomplishment of EMT warrants the activation of mesenchymal markers. In that context, major extensively studied mesenchymal markers include N-cadherin, Vimentin, and Epithelial cellular adhesion molecule (EpCAM) [105]. During malignant transformation, the induction of N-cadherin protein levels is a well-studied event. This E-cadherin to N-cadherin switch, also known as the cadherin switch, is a hallmark of EMT and is a designated biomarker for the evaluation of circulating tumor cells (CTCs). Based on substantial evidence, it can be assumed that elevated N-cadherin levels are significantly associated with increased tumor invasion, metastatic dissemination, and poor patient prognosis [106]. Surprisingly, N-cadherin also modulates the Wnt signaling because forced N-cadherin expression leads

to the elevated localization of β-catenin at the plasma membrane [107]. In order to promote β-catenin translocation, studies unveil that N-cadherin modulates the TCF/LEF-mediated gene transcription, which could be the causal root of excessive cell motility [108]. Additionally, the highly integrated cooperation between the FGFR-Akt with N-cadherin signaling in the perspective of EMT/ stemness induction has been extensively examined as well [109]. Sequentially, the next vital metastatic marker-Vimentin, which is a type III IF (intermediate filament) expressed during embryonic development as well as tumorigenesis. Out of the six major IFs, Vimentin is considered the most important facilitator for mesenchymal cellular stiffness. Vimentin is stimulated in epithelial cells as soon as EMT is activated; otherwise, these cells express keratin solely as a major IF. In order to analyze the coherent connection between Vimentin and Keratin, Polioudaki et al. [110] confirmed a significantly low Vimentin to Keratin ratio (Vim/K) in an epithelial phenotype, whereas a mesenchymal phenotype is associated with a high Vim/K ratio in CTCs in breast cancer patients. Although Vimentin overexpression is often ubiquitous in a diverse range of cancers, its aberrantly high expression is positively correlated with tumor progression, metastatic dissemination, invasiveness, and chemoresistance [111, 112].

Epithelial-cellular adhesion molecule (EpCAM), also known as CD326, is a transmembrane glycoprotein known to be associated as a vital cell adhesion protein in epithelial cells; however, its role in epithelial malignancies has been consistently emerging [113]. Contextually, a recent study unveils a high expression of EpCAM in triple-negative breast cancer (TNBC) cells [114]. Moreover, the metastasis incidence in TNBC is also directly correlated with EpCAM expression [115]. Importantly, EpCAM possesses a critical role in maintaining the pluripotency of cancer stem cells (CSCs), conferring it as a classical CSC marker [116].

1.4 EMT Paves the Way for Tumor Metastasis

Metastasis is a scientific terminology referring to the spreading and colonization of the primary tumor cells to distant secondary organs. It is responsible for the majority of cancer-related deaths. Although the lion's share of primary tumors can be treated with surgery and adjuvant therapy, the systemic nature of the metastatic disease renders it mostly incurable. Furthermore, the disseminated tumor cells are highly resistant to the existing anti-cancer therapeutic agents and often cause recurring disease [117]. The lethality due to metastasis is now well-recognized and remarkable efforts have been made to uncover the cellular and molecular basis of this systemic phenomenon. A series of cell-biological events, collectively termed as the invasion-metastasis cascade, are executed on the onset of the successful establishment of secondary metastases at an anatomically distant organ site. The invasion-metastasis cascade involves (1) local invasion of the cancer cells into the basement membrane and the surrounding extracellular matrix (ECM) and stromal cell layers, (2) intravasation into the endothelial lamina of blood vessels and entry into the systemic circulation, (3) surviving through the rigors of systemic transport (4) arrest at distant organ sites and extravasation into the parenchyma of distant tissues, (5) enduring the foreign microenvironments to form micrometastases and re-initiate their proliferative programs at metastatic sites, thereby generating macroscopic, clinically detectable neoplastic growths [12]. We have illustrated the various steps involved in the metastatic cascade in Fig. 2. While the majority of these events are controlled by the molecular mechanisms (genetic and epigenetic) functioning within the cancer cells, the non-neoplastic stromal cells also exert overwhelming resistance influencing the invasion-metastasis cascade [118]. In the next sections, we will critically analyze the inherent *modus operandi* of tumor metastasis.

1.4.1 EMT and Malignant Transformation

Post-EMT malignant transformation of TME is an intricately synchronized process driven by intrinsic genetic changes, alterations in the local microenvironment, or environmental factors. The initial escape from the primary site is an essential prerequisite for tumor cells to adopt motile phenotype and thus degrade the underlying basement membrane/ECM to initiate an invasion, which can only be accomplished upon EMT induction. Although EMT is typically considered a delayed event during overall tumorigenesis, metastasis commences with EMT induction in a tumor cell subset. The crucial role that EMT- associated transcription factors dictate the initial malignant conversion cannot be undermined. From the repository of emerging recent literatures, aberrant Twist-1 mRNA levels have been detected in the early stages of primary tumor development [119, 120]. Such information supports the critical role of Twist-1-mediated E-cadherin suppression during malignant conversion. One of the other mechanisms by which Twist-1 aids malignant transformation is binding to the tumor-suppressor protein, p53 leading to its degradation. Twist-1-mediated p53 degradation nullifies the oncogene-induced senescence and apoptosis executed by p53 [121]. Of note, the Twist-1-mediated p53 degradation, on the other hand, contributes to the Her-2 and H-Ras-driven malignant transformation [122]. Similarly, Wnt, Notch, and other signaling pathways that regulate EMT are also implicated in malignant transformation of TME, primarily through the activation of EMT-TFs [123, 124].

Post-activation of EMT-TFs, degradation of the underlying basement membrane is indispensable for invasion and is executed through the upregulation of various matrix degrad-

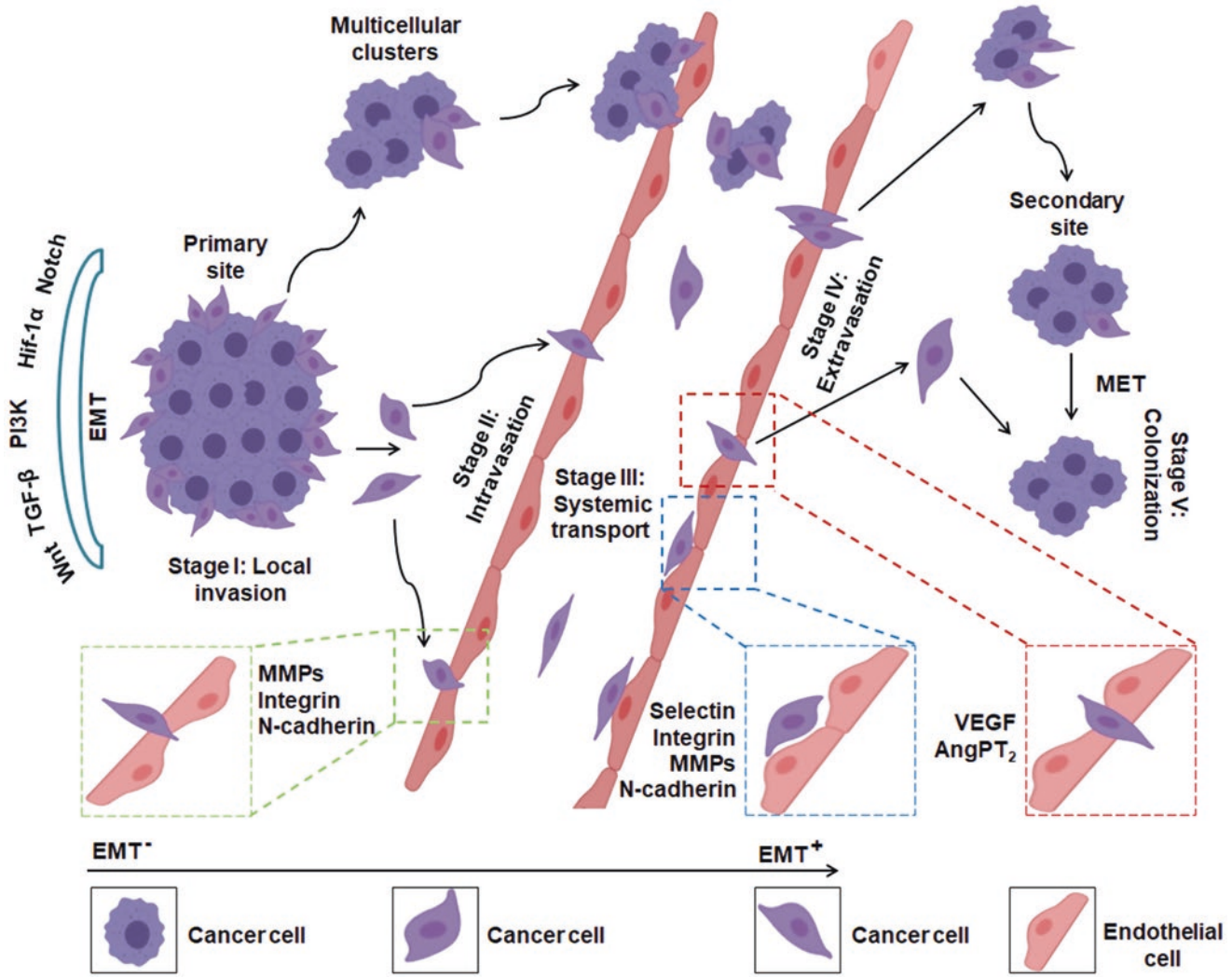

Fig. 2 EMT and tumor metastasis. The figure depicts the various stages of tumor metastasis. The tumor cells undergo EMT at the primary tumor site, travel to distant locations, and finally undergo MET to establish successful secondary metastases

ing enzymes. Interestingly, EMT-TFs orchestrate the formation of invadopodia to degrade ECM [125]. Notably, invadopodia facilitates the ECM degradation by means of involving diverse proteases, including matrix metalloproteases (MMPs), membrane-tethered proteases (MT-MMPs), and ADAMs (a disintegrin and metalloproteases) to the cell-matrix contact points [126]. Remarkably, EMT TF Twist-1 promotes invadopodia formation through the activation of PDGFRa/Src signaling. On the other hand, TGF-β equally contributes towards invadopodia formation by augmenting the expression of Twist-1 and the focal adhesion protein Hic-5 [127]. In a robust integrative approach, Zeppo1, another metastatic promoter found to impede E-cadherin expression along with the stimulation of invadopodia-like structures [128]. In a similar manner, Snail1 is known to assist the expression of MT1-MMP, MT2-MMP, and MMP9 as well as promotes the basement membrane's degradation [129]. Furthermore, EMT-TF Snail2 as well regulates tumor metastasis through induction of MT4-MMP and MMP2 [3]. Hence, cumulatively, all the above evidences imply that post-EMT preparatory phase is indispensable for tumor cells to ensure dissociate from tight gap junctions, attain migratory phenotype, and as a consequence, degrade the ECM to initiate the metastatic cascade.

1.4.2 Intravasation

Intravasation, the second step of metastasis, ensures the tumor cells invade the endothelial lamina, infiltrating into the lymphatic or blood vessels, and shelter into the vasculature accordingly. Following entering into the circulation, tumor cells either migrate directionally in response to chemokine or growth factor gradients, else get carried away passively by the stream of blood flow. Nevertheless, growing evidences suggest that cells in transition desperately need various

ligand-receptor molecules for stable adhesion. Besides, cytokines and growth factors that augment vascular permeability to allow transmigration through the vascular wall also adequately serve as an impediment [126]. Strikingly, during this initial phase of the journey, expression of N-cadherin spikes robustly; although present at the adherens junctions, N-cadherin is either negligibly expressed or absent in the epithelial cells. Notably, this newly synthesized pool of N-cadherin-mediated adhesion between the cancer cells and the endothelial cells governs the entire intravasation process. Eventually, following N-cadherin-mediated adhesion, downstream activation of Src kinase / β-catenin further potentiates the trans-endothelial migration [127].

What is the role of integrins in the intravasation cascade? Integrins are vividly implied in the metastatic intravasation process [128]. For example, melanoma cells expressing the integrin VLA-4 are known to stimulate adhesion and trans-endothelial migration via binding to VCA-1 localize on endothelial cells. Despite strong host-immunogenic resistance, aberrant expression of several selectins, viz. E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L) are known to facilitate binding and rolling of cancer cells on endothelium [129]. Contextually, EMT-transcription factors, for example, Zeb-1 and Snail1, also regulate the migration of the cancer cells through the endothelial barrier [130]. Of note, Snail1 overexpression specifically activates the membrane-bound MMPs (like MT1-MMP and MT2-MMP) but not secreted MMPs, suggesting physical contact of MMPs with the endothelium is a prerequisite for intravasation [131]. In the next phase, the EMT cells gradually degrade the surrounding matrix in order to pave the way for invasion and intravasation, while the non-EMT cells follow the course to infiltrate into the vasculature [132]. Paradoxically, non-EMT cells are believed to be more competent than the EMT cells in reestablishing colonies in the secondary sites due to their superior adhesive properties that allow them successful extravasation into the secondary site [133].

1.4.3 Systemic Transport

Once the tumor cells detach from each other and enter the vasculature, they must override immunological resistances, shear forces, and anoikis. Anoikis is a form of programmed cell death that is instigated when anchorage-dependent cells detach from the surrounding ECM [134]. Consequently, one such survival mechanism is initiated when the integrins on tumor cells interact with ECM, activating focal adhesion kinase (FAK), which phosphorylates its downstream effector molecules leading to Akt activation. Paradoxically, loss of contact between integrins and ECM impedes the survival signals and initiates cell death by triggering the expression of pro-apoptotic proteins [135]. EMT, on the contrary, supports the cancer cells to overcome anoikis by E-cadherin to N-cadherin switching, which is a decisive factor in promoting invasion. The importance of EMT can be evaluated from the landmark studies underscoring the presence of EMT markers in CTCs [136]. A handful of these relevant studies have demonstrated that the mesenchymal phenotype is adequately prevalent among the CTCs and solely accompanied by Zeb-2 overexpression [137]. Furthermore, in the squamous cell carcinoma-mouse tumor model, Twist-1 induction triggers a dramatic boost in the mesenchymal CTCs as indicated by low E-cadherin and high Vimentin levels [136]. Notably, the CTCs trigger tumor cell-induced platelet aggregation (TCIPA), which tether to the surface of CTCs via GPIIb-IIIa-fibrinogen bridge [138]. Platelets also secrete TGF-β that aids CTCs in maintaining the EMT state [139]. Moreover, platelet-derived TGF-β efficiently reduces the expression of the immunoreceptor-NKG2D, thus inhibiting Natural Killer (NK) cell activity [140]. On the other hand, mushrooming evidences elicit that platelets may shield the CTCs against immune assault by NK cells [141]. Furthermore, the transfer of the major histocompatibility complex (MHC) from activated platelets to CTCs favors the escaping of the immune surveillance [142]. The platelet-derived VEGF, at the same time, provides synergic impetus to CTCs by stalling the maturation of primary antigen-presenting cells/dendritic cells [143]. Further down the course, CTCs also take part in the construction of micro tentacles, which are believed to be microtubule-based membrane protrusions, probably aiding in CTC aggregation and tethering [144]. In that direction, burgeoning evidence implies that major EMT TFs, Twist-1/Snail1 play a pivotal role in promoting micro-tentacle formation, suggesting that CTC survival via micro-tentacle-based attachment of CTCs to platelets and endothelium could be potentiated by EMT [145].

1.4.4 Tumor Cell Extravasation and EMT

Most of the tumor cells trespassing into the bloodstream hardly confront the rigors of the circulation, including the hemodynamic shear forces as well as attacks of the immune system and anoikis due to the loss of adhesion to the ECM. Only a few surviving cells may arrest in the vascular lumen and manage to extravasate through the capillary endothelium into the parenchyma of distant organs and thus orchestrate micrometastasis [146]. However, the evidence for the involvement of extravasation in various pathogenic processes is mounting. For example, a multifunctional non-kinase receptor for semaphorins family, neuropilin-2 (NRP-2), is identified on the surface of renal carcinoma and pancreatic cancer cells. This receptor aids vascular adhesion and extravasation by interacting with endothelial α5 integrin [147]. Notably, in prostate cancer metastasis to bones, adhesion of E-selectin ligands as well as β1 and αVβ3 integrins, are sequentially required for extravasation. Furthermore,

while validating the role of integrins in adhesion and extravasation, a handful of emerging evidences uncover that integrins αvβ3, αvβ5, α5β1, α6β4 expressed on tumor cells correlating with metastatic progression in melanoma, breast carcinoma, pancreatic, lung, and prostate cancer [61]. Consequently, extravasation is orchestrated via active collaboration with prometastatic genes, Twist-1, Integrin beta-1 (ITGB1), and VEGFA [148]. In order to continue steady migration in this phase of metastasis, cancer cells deliberately recruit versatile motile structures. For example, filopodium-like protrusions (FLPs) by the tumor cells containing integrin-β1 constitutively interact with the ECM of the distant tissue parenchyma to alter the TME [148]. Similarly, Snail1 can induce the formation of FLPs, and most strikingly, the mesenchymal states of some breast cancer cells are closely associated with their ability to generate FLPs. Together, the above studies underscore the EMT program confers a significant role in promoting extravasation and dissemination of tumor cells to distant organs.

1.4.5 Metastatic Colonization and MET

Of the total fraction of tumor cells that metastasize from the primary site, only a minuscule subset of cells proceed to establish micrometastases under the catastrophic resistances by the unmet stromal environment [12]. As discussed above, the metastatic cascade till the extravasation stage is majorly driven by the EMT, as is evident by the EMT signatures noted in the primary carcinomas and CTCs. However, it is surprising that the macrometastases are largely epithelial, in contrast to the proposed mesenchymal nature, suggesting that EMT involvement during metastasis is likely to be functionally dynamic. In that context, Bonnomet et al. noted a heterogeneous expression pattern of Vimentin in the primary MDA-MB-468 tumor xenografts and the resulting lung metastases, while high levels of Snail1, Snail2, and Vimentin prevail in CTCs. This finding implies that the Vimentin-negative macrometastases might originate following MET in the Vimentin-positive CTCs, highlighting the epithelial-mesenchymal plasticity [149]. Similarly, another study rationally pointed out that EMT activation aids the metastasis's initial phases, including local invasion, intravasation, and extravasation. However, EMT inhibition is equally essential for tumor cell proliferation and macrometastasis formation at the distant site [136]. A novel EMT inducer, Prrx1, cooperates with Twist-1 to promote a more invasive phenotype in human breast cancer cells. On the basis of the evidence, downregulation of Prrx1 is an essential prerequisite to revert EMT and for lung metastasis colonization [150]. In an exactly similar fashion, EMT activation by Zeb-2/Snail1 leads to the inhibition of Cyclin D activity, thereby suppressing cell division [151]. All these studies together assert that EMT reversal could be essential to restart proliferation at the secondary site for metastasis colonization although these highly coordinated mechanisms warrant detailed investigation.

Another new perspective in this context is the emerging players of miRNA families, such as the miR-200 family (including miR-200a, miR-200b, and miR-200c). These miRs maintain the cells' epithelial nature by negatively regulating the EMT inducer Zeb-1 and vice versa (as we have discussed earlier). Interestingly, the Sec23a-mediated secretion of metastasis-suppressive proteins are by and large prevented upon the re-expression of miR-200 family members, which ultimately trigger colonization, possibly by repressing Igfbp4 and Tinagl1 [152]. These studies indicate that both the loss of EMT-inducing signals and the induction of MET-promoting cues may be required simultaneously to actively promote micrometastases. Given that micrometastasis outgrowth is a critical stage in the invasion-metastasis cascade, more studies on MET's molecular regulators could shed light on therapeutic approaches to inhibit tumor colonization.

1.5 EMT Acquisition by Mesenchymal Cells—A Real Challenge in the Development of Cancer Therapeutics

The association between major EMT-associated transcriptions factors and the development of novel therapeutic strategies on that basis is of great interest to the scientific community. The underlying molecular mechanisms involved in EMT acquisition are primarily governed by the EMT-associated transcription factors (EMT-TFs). EMT-TFs include transcription factors belonging to the basic helix loop helix family (Twist-1 and 2), zinc finger family proteins (Zeb-1/2, Snail, and Slug) and β-catenin. Acting in isolation or conjunction, EMT-TFs regulate certain EMT-associated marker proteins. Epithelial markers such as E-cadherin, Claudins, Occludins, and Cytokeratin are transcriptionally repressed by EMT-TFs with concomitant upregulation of mesenchymal markers like Vimentin, N-cadherin, Fibronectin, and matrix-metalloproteases (MMPs). Activation of EMT-TFs and subsequent EMT induction in cancer cells is invariably considered as the building blocks of acquired chemoresistance, leading to enhanced stemness/plasticity of the malignant cells. Stemness refers to the core properties exhibited by stem cells, for example, self-renewal and production of differentiated progeny. These properties related to stemness are physiologically displayed by embryonic stem cells and adult stem cells during development and tissue homeostasis as well as regeneration. Extensive studies of the tumor tissue have pointed towards the presence of stem-like cells, termed as the cancer stem cells (CSCs), within tumors [153]. The CSCs behave in an equivalent malignant manner to normal stem cells in terms of stemness

[154]. A mesenchymal phenotype is a commonality between the CSCs and the normal stem cells that allows them to retain the stemness as well as the migratory properties [155–157]. CSCs have been linked to EMT phenotypes by epigenetic programming in many types of cancer. The EMT process enables cancer cells to disseminate and to self-renew during tumor metastasis. For example, non-transformed immortalized human mammary epithelial cells undergo an EMT process upon Snail1, Twist-1 expression, or the presence of TGF-β1. The subpopulation of $CD44_{high}/CD24_{low}$ immortalized human mammary epithelial cells that possess stem-like properties increases with concomitant induction of EMT phenotype [158]. Contrariwise, CSCs confer prodigious carcinogenic potential and plasticity in comparison to the non-CSCs subset of cancer cells. This finding indicates that an EMT process generates cells with similar properties commonly observed in self-renewing stem cells. In this regard, it appears that the EMT process that enables cancer cells to disseminate from a primary tumor (i.e., metastasis) also promotes cancer cell self-renewal.

The potential application of the identification of interplay between CSCs and EMT has just begun to unveil. For instance, loss of the tumor suppressor p53 in mammary epithelial cells has been shown to induce EMT and enrich CSCs through repression of miR200c, suggesting that the p53–miR200c pathway can be activated to suppress EMT-associated CSCs to treat cancer [159]. Furthermore, EMT harboring CSCs are resistant to platinum-based conventional chemotherapies (oxaliplatin, cisplatin) (Fig. 3) due to the modulation of genes involved in cell survival or evasion of apoptosis. CSCs and EMT seem to be an axis of evil in cancer, a better understanding of which may contribute to establishing novel therapeutic platforms. Rationally, EMT acquisition in cancer cells puts forth a two-fold challenge, i.e., drug resistance and stemness, both of which are implicated in the progression of the metastatic cascade. Hence, the true challenges in anti-cancer therapy development must confront the EMT accretion of cancer cells.

2 Par-4 Emerges out as a Prospective EMT Modulatory Protein

Cellular fate between apoptosis or survival depends upon the balance between both survival (EMT) and pro-apoptotic cascades (Program cell death); this equilibrium stage is adequately explained in pre-clinical settings where therapeutic administration could modulate tumor suppressor's function to eradicate tumor burdens. Until recently, Par-4 as a tumor suppressor protein is well-established owing to its cancer-specific expression and apoptosis-inducing ability, but Par-4 research has attained a new height by illustrating EMT stalling properties of Par-4. Several research groups have indeed dissected the signaling mechanisms involved in Par-4 activation to augment apoptotic cascades [160–163]. However, new developments in Par-4 research have not only widened its therapeutic potential but dominantly proclaim its imperative role in modulating autophagy, senescence, and other therapeutically relevant avenues. One of such daunting task is the halt in EMT induction and prevention of metastasis by Par-4. In this section, we have extensively envisaged the prospective role of Par-4 with reference to EMT, the molecular signaling involved, and therapeutic implications. This section has also been summed up in Fig. 4.

2.1 Structural Aspects of Par-4(SAC Domain) that Link it with EMT

Par-4 is a leucine-zipper protein that has distinct nuclear localization and entry sequences. It comprises of two nuclear localization sequences (NLS1 and NLS2) at the N-terminal region, a nuclear export sequence (NES), a "selective for apoptosis of cancer cells" domain (SAC) unique to the Par-4 protein, and a leucine zipper domain (LZ) at the carboxyl-terminal region (Fig. 5) [164].

In an elegant study by Zhao et al., bone marrow from SAC transgenic mice transplanted into SAC-non-transgenic irradiated littermates serves as a pool for SAC-expressing cells that are resistant to tumor growth. In the tail vein mice metastatic model, recombinant Par-4 (TRX-Par-4) and SAC (TRX-SAC) proteins are competent in inhibiting the formation of metastatic lung nodules [165]. One of the principal mechanisms for Par-4 functionality is its inhibition of NF-κB. Par-4 that has a defective or lacking NLS2 (amino acid residues 137–153) domain is retained in the cytoplasm and is unable to block NF-κB activity [166], downstream targets of which include major EMT-related genes, for example, Twist-1, Snail1, and β-catenin. Of note, the NLS2 domain is encompassed by the larger SAC domain in totality. Rationally, the relevance of the SAC domain to the anti-EMT potential of Par-4 is beyond question. The question that begs to be asked is whether or not additional structural domains in Par-4 are unequivocally responsible for its anti-EMT activity. The leucine zipper (LZ) motif of Par-4 is essential for its interaction with other proteins and binding to DNA sequences to carry out its co-transcriptional activity. The interactions mediated by the LZ motif can be perceived as an alternate mechanism by which Par-4 can either interact with EMT markers (Vimentin) or bind to regulatory DNA sequences of EMT-TFs (Twist-1, Snail1, Zeb-1). However, unlike the SAC domain-mediated inhibition of NF-κB, the LZ motif's role in the abrogation of EMT needs in-depth exploration as there is a substantial dearth of evidence.

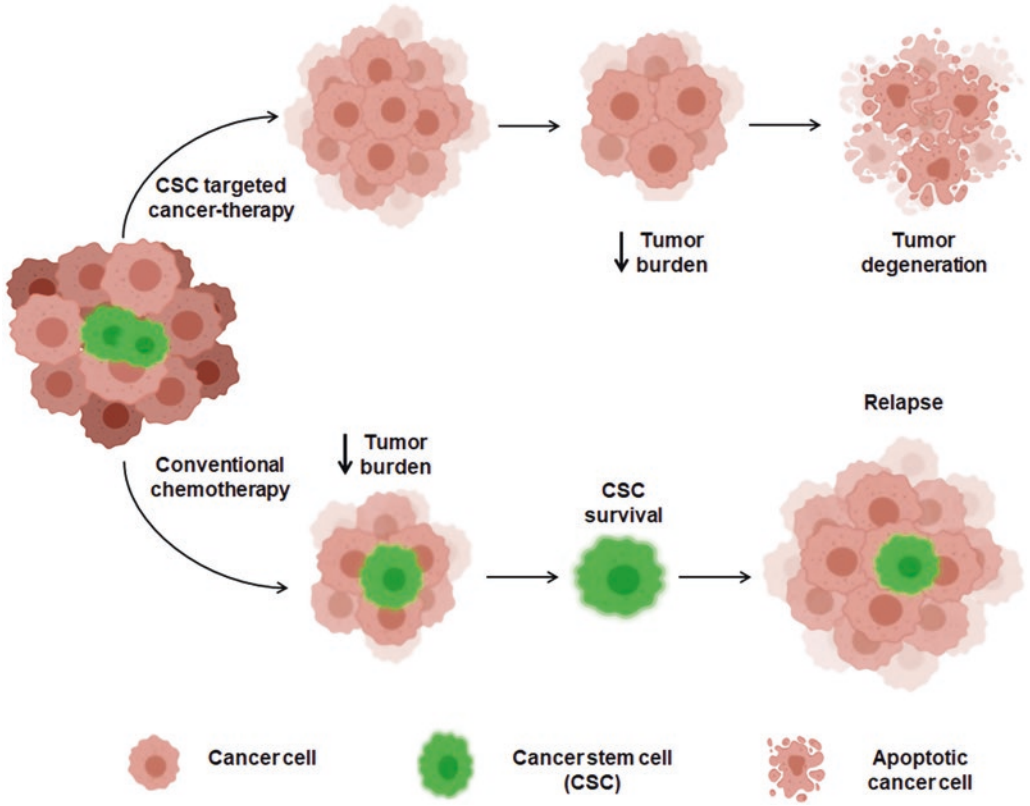

Fig. 3 Cancer stem cells (CSCs) and chemoresistance. The figure depicts the outcomes of conventional cancer therapies versus stem cell-specific therapies. The CSCs constitute a small subset within the tumor cells that drive chemoresistance as well as tumor recurrence following conventional chemotherapy

2.2 Regulation of NF-κB Activity by Par-4

EMT has been perceived as a deliberate ploy employed by cancer cells to evade cytotoxic threats and accomplish survival. Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB, is a master transcription regulator that is essential for cell survival and is critically relied upon by cancer cells to ensure their survival. Notably, translocation of the NF-κB into the nucleus drives the expression of genes regulating diverse biological processes [167]. However, the majority of the cancer types are prone to altered levels of NF-κB that are positively correlated with tumor growth, invasion, metastasis, and chemoresistance. Interestingly, the NF-κB and Par-4 proteins are antagonistic to each other [168]. The NLS2 sequence found within Par-4 is essential for its nuclear translocation (as detailed earlier) and subsequent suppression of the NF-κB-dependent transcription activity, binding to Par-4 partner proteins WT1, ZIPK/DAXX, and THAP; and thus induction of apoptosis [169]. Apart from the direct inhibition of the NF-κB activity, Par-4 may also indirectly stall NF-κB via stabilizing AKT. AKT is a serine/threonine-protein kinase that regulates a variety of cellular processes, including proliferation, survival, and protein translation. However, AKT overexpression is a catastrophic event reported in almost all cancers, rendering it a very important therapeutic target. Of note, AKT activation not only stimulates NF-κB activity to instigate survival of cancer cells but at the same time, AKT blocks the pro-apoptotic transcription factor, FOXO3a [170]. However, an elaborate study by Joshi et al. has demonstrated that Par-4 autonomously inhibits AKT via PKCζ that phosphorylates AKT at Ser124 [171]. AKT phosphorylation at Ser124 impacts the phosphorylation status of the two most important residues, Ser473 and Thr308, that are critical for AKT activity [172]. Another detailed contextual study by Choudhry et al. reveals Par-4 to be one of the downstream targets of TGF-β signaling involved in the EMT induction. The TGF-β-mediated induction of Par-4 expression, as well as its nuclear localization, was revealed to be executed via the Smad4 and NF-κB pathways. Moreover, the study also reveals that the interaction of Par-4 with Smad4 results in the abrogation of the NF-κB and XIAP protein levels, culminating in an EMT halt [173]. Further, NF-κB also hinders the apoptosis process by enhancing the transcription of the anti-apoptotic protein, $Bcl_{xl,}$ and X-linked IAPs (XIAP) [174]. Par-4 counteracts the pro-survival effects of NF-κB by initiating the assembly of the death-inducing signaling complex (DISC) by augmenting the interaction of FAS receptor and

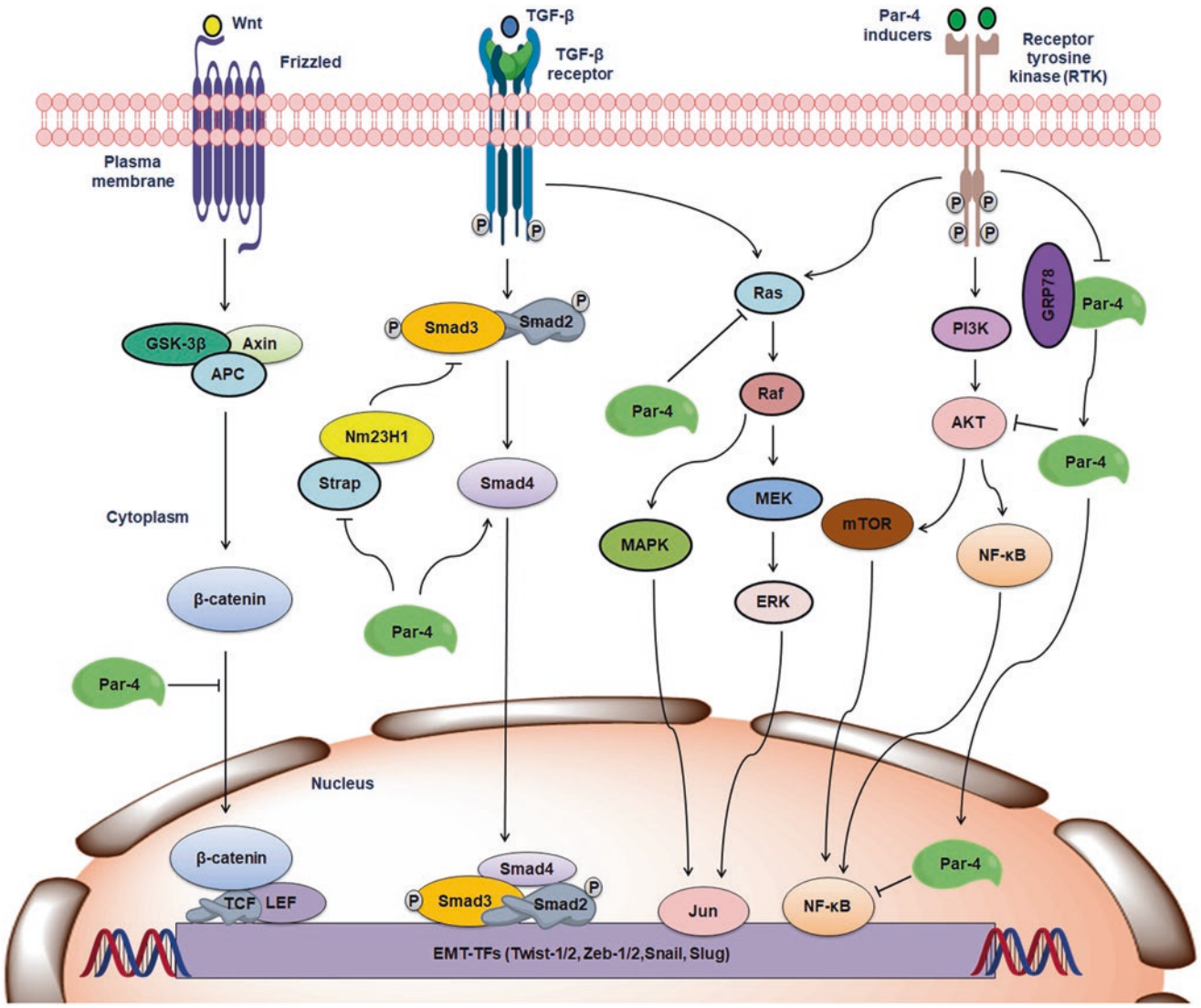

Fig. 4 Signaling involved in Par-4-mediated abrogation of EMT. The figure depicts various signaling pathways that initiate and/or facilitate EMT as well as the axes that are targeted by Par-4 to execute its anti-EMT and anti-metastatic function

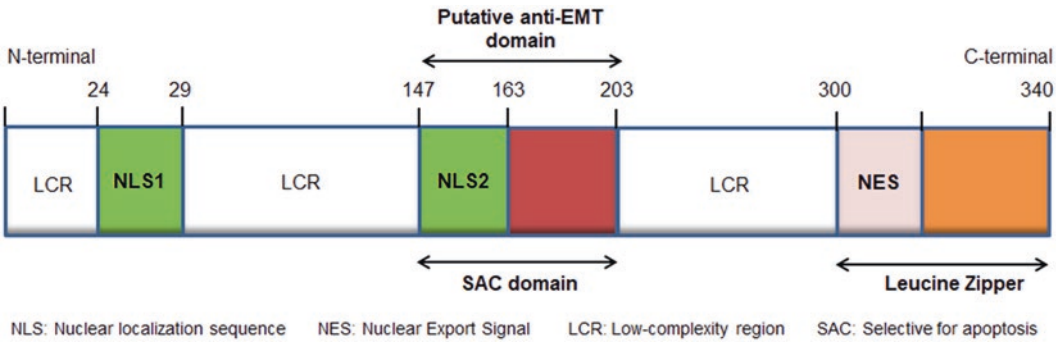

Fig. 5 Structural aspects that link Par-4 and EMT. Functional domains of Par-4 include two nuclear localization signals (NLS1 and NLS2) at the N-terminal, an SAC (selective for apoptosis in cancer cells) domain in the middle, and a leucine zipper (LZ) domain at the C-terminal

FAS Ligand with FADD and inducing apoptosis in a hormone-independent manner [175]. MMPs, the active players in ECM components degradation, are well-accepted for their involvement in cancer progression and metastasis. Importantly, MMPs can also confer apoptosis resistance to the cancer cells by negatively regulating the Fas-FADD-mediated death signaling [176]. However, the extracellular Par-4 can rescue the anti-apoptotic signaling associated with cancer cells by diminishing MMP-2 [177], activating downstream caspase-3 as well as nullifying the pro-metastatic effects of c-FLIP to exert an extrinsic apoptotic effect [178, 179]. Thus, the apoptotic induction, as well as abrogation of invasion, could be controlled independently by secretory Par-4 in diverse cellular background. Since Par-4 negatively regulates NF-κB protein which double-edged function is grossly equipped with the regulation of cancer cell survival through modulation of EMT-TFs. By and large, NF-κB induces the transcription of EMT-TF genes Twist-1, Slug, and SIP1 by directly binding to their promoter regions, ultimately attributing the EMT process to promote an aggressive phenotype [180]. Hence, all these above studies in this subsection authenticate the direct contribution of NF-κB in EMT promotion and as well provide compelling evidences that Par-4-mediated anti-EMT effects could majorly be attributed to the inhibition of NF-κB. Although Par-4 presents a foolproof theoretical approach to tackle the NF-κB-mediated tumorigenesis and chemoresistance, the feasibility of NF-κB-targeting therapies has to be carefully evaluated.

2.3 Regulation of EMT-Associated Transcription Factors by Par-4

In the horizon of Par-4 research, we have witnessed emerging evidences unleashing its novel functions. One of such fascinating functions emphasizes the anti-EMT role of Par-4. Multiple studies have recently revealed the anti-EMT role of Par-4 [177, 181]. Importantly, exogenous Par-4 is well-documented to positively correlate with E-cadherin expression and down-modulation of various EMT-TFs, including Twist-1, Snail, Slug, Zeb-1, and Zeb-2. As a consequence of diminished EMT-TF transcriptional activity, mesenchymal markers, viz. Vimentin, N-cadherin, MMPs, and fibronectin are consistently found to be repressed. However, whether or not EMT-TFs are directly regulated by Par-4 remains to be thoroughly examined. Although evolutionarily, Twist-1 transcription factors are attributed to embryonic development, their expression is limited post-embryogenesis in most of the cell types [182]. Elevated expression of Twist-1 is often associated with tumor progression, metastasis, and poor patient prognosis [183]. Twist-1-mediated E-cadherin suppression is critical for the induction of EMT that ultimately converges into metastatic dissemination [184]. Moreover, Twist-1 also positively modulates the expression of the mesenchymal markers Vimentin, Fibronectin, and N-cadherin to promote cellular motility. Recent studies elegantly postulate the relevance of Par-4-mediated Twist-1 inhibition in cancer cells concomitant with E-cadherin upregulation although the exact mechanism remains obscure [181, 185, 186]. One of the plausible mechanisms by which Par-4 may impede Twist-1 could be via the regulation of AKT1. AKT1, on the contrary, phosphorylates Twist-1 at the Serine-42 residue resulting in incremental Twist-1 transcriptional activity to mediate E-cadherin suppression [187]. Besides, we have discussed in the above section, Par-4, via the recruitment of PKCζ inhibits AKT activation to exert anti-tumorigenic effects [171]. These studies subtly point out the possibility of Twist-1 inhibition by Par-4, which requires more scientific validation.

Alternatively, Twist-1 is also an evolutionarily conserved target of NF-κB [188]. Since Par-4 is a well-known repressor of NF-κB, it may also possibly abrogate the NF-κB-Twist-1 upregulation. TNF-α is a pro-inflammatory cytokine that is deeply corroborated into EMT activation, cancer stemness as well as angiogenesis [189]. Both IKK-b and NF-κB p65 are required for TNF-α-induced expression of Twist-1, suggesting the involvement of canonical NF-κB signaling. Moreover, activation of NF-κB, as well as Twist-1, blocks programmed cell death (PCD). The protective activity of NF-κB is also crucial for oncogenesis as well as aids cancer chemoresistance. Together, these findings indirectly suggest that Par-4-mediated NF-κB inhibition may contribute to the Twist-1 suppression observed upon the ectopic expression of Par-4 in cancer cells. Although these could be the proposed mechanism of Twist-1 inhibition via the Par-4, more studies are warranted to validate the Twist-1-suppressing effects of Par-4 as well as its consequences on tumor progression and metastasis.

2.4 Role of Par-4 in Regulation of Cytoskeletal and ECM Remodeling

Basal levels of Par-4 secreted by cancer cells are generally inadequate to cause substantial apoptosis; secretagogues that augment the release of Par-4 represent an alternate approach to repurpose our objectives in Par-4-dependent therapeutic development. Notwithstanding, the implications of apoptosis-instigating mechanisms in relation to radiation or chemotherapy may provide clues to better explain the selection of proper targets in cancer. In a classical approach, Burikhanov et al. have utilized a unique chemical-genetic entity to underscore the ability of secretagogue-Arylquin to enhance Par-4 function. This secretagogue-Arylquin -mediated functional enhancement of Par-4 was executed by facilitating Par-4 secretion via the classical secretory pathway as

well as by aborting the interaction of Par-4 with Vimentin [190]. While the sequestration of Par-4 by Vimentin in cancer cells not only attributes an important role in the induction of EMT and maintenance of mesenchymal state, but it may also corroborate to drug resistance mechanisms and EMT, particularly in the advanced stage of cancer. Notably, in order to achieve a robust anti-tumor efficacy, such disruption of the Par-4–Vimentin interaction leads to the release of Par-4 to execute its pro-apoptotic function. Therefore, Par-4 rescue may not only sensitize the cancer cell to apoptosis but may abrogate the induction of EMT. This axis of Vimentin-mediated Par-4 regulation in cancer cells portraits a distinct post-translational therapeutic window to target Par-4, Vimentin, or both. Since our goal is to discover the novel function of Par-4, interestingly, our group has unfolded a potential MMP-2 inhibition by extracellular Par-4 [177]. Although secreted by a classical BFA-sensitive pathway, conditional media (CM) containing Par-4, in this research, found to abrogate ex vivo tumor growth in matrigel plug assay. Of note, MMP-2 is a highly proficient metalloproteinase that degrades the extracellular matrix and facilitates the invasion and migration capabilities of cancer cells. In this study, Rah et al. demonstrate that the MMP-2 expression and activity were simultaneously abolished by the secretory Par-4. These results were confirmed by the Par-4 knockdown studies where MMP-2 expression was restored along with a steady increase in invasion potential of cancer cells upon silencing of Par-4. Thus, the strategic use of small molecule inducers of Par-4 for the regulation of intracellular Par-4 could be an effective tool to control the cancer cell metastasis. These reports together put forward a novel paradigm of controlling deregulated malignant signaling by regulating Par-4 (Fig. 6), hence, revealing a new dimension of Par-4 extrapolation for advancement in metastatic cancer therapeutics.

2.5 Par-4 and Destabilization of β-Catenin Pathway

β-catenin signaling pathway is considered one of the critical axes concerning cancer metastasis and drug resistance issues. Deregulation of this pathway by activating mutations in the upstream components converges upon the nuclear accumulation of β-catenin, thereby driving the expression of genes implicated in cancer cell survival, proliferation, and EMT-TFs [191]. While intact cadherin–catenin complex is a critical prerequisite for the maintenance of the cellular homeostasis, however, the lack of cadherins regulating cell adhesion (primarily the E-cadherin) and/or altered subcellular distribution of β-catenin disrupts the cadherin–catenin complex, leading to increased invasiveness, migration, and poor clinical outcome.

Notably, constitutive activation of the phosphatidylinositol 3-kinase (PI3K) signaling triggers the dephosphorylation of β-catenin and finally its accumulation and translocation into the nucleus that culminates in the inactivation of glycogen synthase kinase 3-beta (GSK-3β) [192]. Contextually, in a breakthrough research, Amin et al. have elucidated that small-molecule inducer of Par-4 abrogates EMT and invasion by modulating β-catenin localization and its transcriptional activity in aggressive prostate and breast cancer cells [181]. This study revealed that 3-AWA (a withaferin-based potent Par-4 inducer) sequestered nuclear β-catenin and augmented its cytoplasmic pool as evidenced by diminished β-catenin transcriptional activity. Moreover, exogenous Par-4 attenuated AKT activity and rescue phospho-GSK-3β to promote β-catenin destabilization. Furthermore, Par-4-induced E-cadherin expression along with sharp downregulation of c-Myc and cyclin D1 proteins. The results from the Par-4 knockdown studies, as is performed using siRNA, validates that the 3-AWA-mediated inhibition of nuclear β-catenin is Par-4 dependent. Therefore, Par-4 and β-catenin proteins are mutually regulated and inversely correlated in normal as well as cancer contexts, and strategic modulation of intracellular Par-4 could be an effective tool to control and EMT and cancer cell metastasis.

3 New Insights Linking Par-4 and EMT

3.1 Lethal EMT: TGF-β Signaling and Par-4

Deregulation of transforming growth factor-beta (TGF-β) signaling is well-accepted to be one of the major deregulations observed in the pathophysiology of diverse cancer types. Through different stages of cancer initiation and progression, TGF-β plays a multifaceted and paradoxical role. TGF-β signaling can be pro-tumorigenic or tumor-suppressive. The particular cases where the duality of TGF-β role is observed are well-studied in pancreatic ductal adenocarcinomas (PDACs). TGF-β mediator-Smad4 is frequently found inactivated in PDACs, along with other gastrointestinal cancers. Typically, TGF-β-induced EMT program is considered to be a pro-tumorigenic phenomenon. But in TGF-β-sensitive PDAC cells, on the conversion of TGF-β-induced Sox4, from an enforcer of tumorigenesis into a promoter of apoptosis, the tumor-promoting EMT switches to lethal EMT [193].

Along with the already available therapeutic approaches to mitigate pro-survival/anti-apoptotic factors, the novel lethal EMT approach is a robust example of EMT-linked cellular transcription factor landscape remodeling, including the repression of Klf5, the gastrointestinal lineage master regulator. For the successful progression of cancer, vivid cooperation between Klf5 and Sox4 is crucial, and this asso-

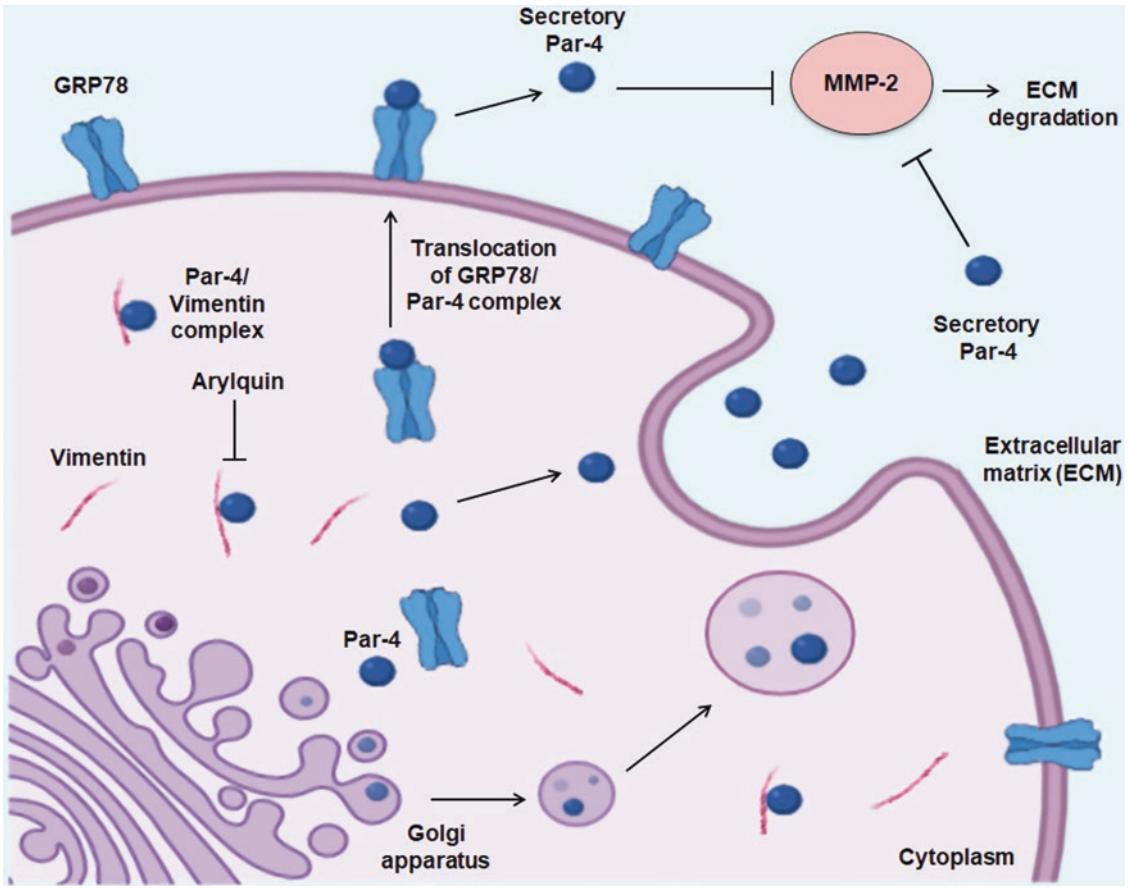

Fig. 6 Secretory Par-4 and ECM degradation**.** During ER stress conditions, Par-4 and GRP78 bind to each other, and the paired proteins relocate to the plasma membrane. Par-4 is then released as secretory Par-4, leaving GRP78 at the plasma membrane. Secretagogues like Arylquins disrupt the Par-4/Vimentin complex; as a result Par-4 is free to be secreted out. The secretory Par-4 then abrogates the ECM degradation, mainly through inhibition of MMP-2 activity

ciation impedes Sox4-induced apoptosis. Smad4 (also called DPC4) is a component of paramount importance in this axis. It is an established tumor repressor that is frequently lost/mutated in pancreatic cancer. However, it is noteworthy to mention that tumor growth of colon carcinoma cells is obstructed by the presence of Smad4 protein, which constitutively reactivates E-cadherin and therefore abrogates neo-angiogenesis. While Smad4 is indispensable for EMT, it is not an absolute prerequisite for Sox4 induction by TGF-β. On the one hand, TGF-β-induced Sox4 is spontaneously available to support progenitor identity. Simultaneously, an essential partner of Sox4 in oncogenesis is stripped away by Smad-dependent EMT. For achieving a viable therapeutic intervention, the Smad4-dependent EMT is grossly activated by induction of TGF-β in the PDAC cells. Intriguingly, the activation of Smad-dependent EMT successively acts as a whistleblower for apoptosis. However, to achieve the desired result, the pro-tumorigenic function of Sox4 needs to be switched to pro-apoptotic mode. This transition is obtained by Snail-mediated suppression of Klf5, a crucial master regulator of endodermal progenitors. These results, collectively, illustrate a paradigm shift in which TGF-β tumor-suppressive action revolves around an EMT-associated disruption of a pro-tumorigenic transcriptional network.

As mentioned above, a dual role is perceived by TGF-β in the successful accomplishment of tumor growth and invasion. The apoptosis promoting potential of TGF-β along with the termination of epithelial cell cycle progression leads to tumor suppression in the early stages of cancer. Contrariwise, in the later stages, it promotes tumor growth owing to interference with a chain of factors such as modulating genomic instability, cell motility, immune evasion, neo-angiogenesis, and metastasis. In recent studies, Par-4 has been emerging as a vital constituent to influence TGF-β-induced EMT. Most strikingly, the anti-metastatic function of Par-4 has been implicated as a crucial downstream target of the TGF-β signaling pathway [173]. Echoing this, Faheem et al. also demonstrate that Par-4 plays an essential role in regulating the TGF-β/Smad4 pathway in pancreatic ductal adenocarcinoma (PDAC) models [194]. In a breakthrough finding, authors

proclaim that overexpression/induction of Par-4 convincingly results in apoptosis in conjunction with TGF-β by positively regulating Smad4. Interestingly, Par-$4^{+/+}$ cells show far more significant Smad4 induction in comparison to Par-$4^{-/-}$ cells in the presence of TGF-β. Faheem et al. have diligently found that Smad4 expression is robustly spiked by ectopic Par-4 through the restoration of the TGF-β/Smad4 axis. Furthermore, Par-4 drags the PDAC cells to G1 arrest in the presence of TGF-β by boosting the p21 and p27 levels and attenuating Cyclin A and E to trigger lethal EMT via caspase 3 cleavage augmentation. Interestingly, in this report, the authors hypothesize that Par-4 dependent and TGF-β-mediated lethal EMT is embarked in these cells following restoration of Smad4 in the Smad4 null BxPC3 cell line. However, mechanistically this research work underscores that disruption of Nm23H1-Strap interaction is the cornerstone of Par-4-mediated Smad4 activation. Nm23H1 is a nucleoside diphosphate (NDP) kinase and a putative metastatic suppressor. Nm23H1–Strap interaction is not only essential for simultaneous p53-mediated apoptotic functions as well as regulating TGF-β-mediated biological activity. In addition to this, this interaction controls intrinsic Nm23H1 activity [195, 196]. Nm23H1/Strap interaction obstructs the downstream signaling of TGF-β as an intact Nm23H1/Strap complex acts in tandem with the inhibitory Smads (Smad7 particularly), which results in a lowered capacity of receptor Smads (Smad2 and 3) to couple with Smad4 [197]. Given that Par-4 positively modulates the TGF-β/Smad4 pathway in PDAC cells and favors the tumor-suppressive role of TGF-β. Hence, Par-4 is a crucial element that helps to restore the apoptotic functions of the TGF-β pathway.

3.2 BMP and ALK Signaling

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and constitute a diverse, evolutionarily conserved family of secreted signaling molecules critical for various developmental processes [198]. BMP7 is known to counteract TGF-β-induced EMT in developmental stages [199]. ALK2, on the other hand, also termed ACTRI, is an activin type I receptor that mediates responses for BMP7 [200]. ALk2 phosphorylates Smad1/5/8 and, as a result, triggers its association with Smad4 incurring MET phenotypes. Apart from its EMT alleviating role, Par-4 has been reported to induce MET in highly aggressive cancers [201]. Recently, Katoch et al. have conceived a dual mechanism of Par-4-mediated inhibition of EMT and concomitant alleviation of MET in metastatic pancreatic cancer cells [186]. Authors demonstrate that induction of Par-4, either ectopically or by NGD16 (a small molecule derivative of diindolylmethane), strongly impede invasion, migration, and metastatic index of these cells. In the same experimental setup, authors have found a robust amplification of epithelial marker E-cadherin concomitant with downregulation of canonical mesenchymal marker Vimentin. However, siRNA-mediated silencing of either endogenous Par-4 or Smad4 resulted in the reversal of MET phenotypes with diminished E-cadherin levels underscoring the appearance of MET phenotypes were due to the augmentation of ALK2/ Smad4 signaling in a Par-4-dependent manner. These findings are in concordance with the emerging role of BMP7 in MET induction, possibly by ALK2, phosphorylation of Smad 1, 5, and 8; and inhibition of EMT-TFs, viz. Slug, Twist-1, and Snail. Therefore, ALK2 induction can be perceived as a plausible mechanism of Par-4-mediated abrogation of EMT and induction of MET in PDAC cells.

3.3 Anti-Metastatic miRNAs and Par-4

microRNAs (miRNAs) are non-coding single-stranded RNAs that negatively control post-transcriptional gene expression to degrade multiple target mRNAs and execute translational suppression [202]. miRNA dysregulation has been implicated in the etiology, pathogenesis, diagnosis, and treatment of cancer [203]. In the myocardium, miR-17-3p-mediated Par-4 abrogation was demonstrated to attenuate cardiac aging [204]. This event leads to the upregulation of its downstream proteins, including CEBPB, FAK, N-cadherin, Vimentin, Oct4, and Sca-1 (stem cell antigen-1), and downregulates E-cadherin. Thus, repression of Par-4 by miR-17-3p augments the transcription of CEBPB and FAK. This, in turn, results in EMT acquisition and self-renewal, culminating in cellular senescence and apoptosis resistance. A growing number of studies have demonstrated altered levels of the miRNA-200 family members in the cells undergoing EMT [205, 206]. miR-200c is a positive regulator of E-cadherin and represses the expression of E-cadherin repressor, Zeb-1, to maintain the epithelial phenotype in the cells, thus attenuating EMT [207]. Consequently, extensive investigation has unveiled the role of miR-200c in cell proliferation, apoptosis, EMT, invasion, therapy-induced resistance, and metastasis in diverse cancer types [208]. However, miR-200c and Zeb-1 possess an inverse relationship in the context of the EMT phenomenon as well as their regulation *vis a vis;* miR-200c directly targets and impedes Zeb-1 and Zeb-2 expression. Albeit, the aberrant miR-200c loss with a simultaneous increase in Zeb-1 expression has been correlated to orchestrate EMT by downregulating E-cadherin [209]. From that standpoint, our group recently demonstrated that the consequences of Par-4 upregulation in the amelioration of Zeb-1-mediated EMT by enhancing the miR-200c levels [185]. Of note, the global proteome changes in Panc-1

cells upon ectopic restoration of miR-200c / Par-4 identify overlapping protein targets in the miR-200c and Par-4 axis. Intriguingly, reverse phase protein (RPPA) analysis for the whole proteome of miR-200c and GFP-Par-4-transfected Panc-1 cells identify 82 proteins which consistently overlap in both the sample sets. Cumulatively, these proteins include phospho-p44/42 MAPK; Bcl-xl; Bim; phospho-Rb (Ser807, Ser811); phospho-Akt (Ser473); phosphor-Smad1/5 (Ser463/Ser465); and Zyxin. The expressional changes in these distinct proteins might be exerted independently by different arms of the miR-200c and Par-4 signaling pathways. This work by Katoch et al. has unveiled a novel role of Par-4 as a positive regulator of miR-200c expression that results in halt in EMT progression.

4 Conclusion, Limitations, and Future Perspectives

Over the years, Par-4 research has been largely focused on unveiling its pro-apoptotic role. Mounting evidences, however, suggest towards the beneficial role of Par-4 in the abrogation of EMT and subsequent metastasis in various cancers. Therefore, exploration of Par-4 in EMT progression warrants detailed investigation. Small-molecule inducers of Par-4 or recombinant Par-4 are ideal for examining the effects of Par-4 on EMT associated markers (epithelial/mesenchymal) both in in vitro and in in vivo contexts. Since Par-4 modulates major metastasis-related proteins like Vimentin and MMPs, targeting bystander effects of Par-4 would be an attractive strategy to control EMT in aggressive cancers. Furthermore, induction of programmed cell death by Par-4 is independent of its novel β-catenin signaling modulatory role; however, future studies need to divulge into the mechanisms by which Par-4 deters Wnt/ β-catenin signaling. Whether the pro-apoptotic role of Par-4 is mutually exclusive to its anti-metastatic role or these roles are concomitantly intertwined with each other needs deciphering. In this context, the integration of the SAC domain with respect to the anti-metastatic potential of Par-4 is of significant relevance and yet to be comprehended. Pertinently, a dearth of evidence so far links the effects of Par-4 on EMT-associated markers (epithelial/mesenchymal) and *vis-à-vis* subsidiary signaling nodes like NF-κB, β-catenin, etc. There is a shortfall of evidences underscoring the regulation of EMT-TFs by Par-4. The need of the hour is to decipher whether or not Par-4 directly interacts with any of the EMT-TFs. Albeit, with the identification of novel signaling intersections between Par-4 and EMT programs, the opportunity to examine this axis holds a promising field in future study. Further, identification, development, and exploration of novel Par-4 inducing small molecules that impede EMT cascades represent significant progress in the right direction. All this relevant information should facilitate the development of Par-4 targeted novel anti-metastatic therapeutic regimens in the future.

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