Review

Cancer Research

Pleiotropic Roles for ZEB1 in Cancer

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Abstract

ZEB1 is a prime element of a network of transcription factors that controls epithelial-to-mesenchymal transition (EMT), a reversible embryonic transdifferentiation program that allows partial or complete transition from an epithelial to a mesenchymal state. Aberrant expression of ZEB1 has been reported in a variety of human cancers, where it is generally believed to foster migration, invasion, and metastasis. Over the past few years, *in vitro* and *in vivo* observations have highlighted unsuspected intrinsic oncogenic functions of ZEB1 that impact tumorigenesis from its earliest stages. Located downstream of regulatory processes that integrate microenvironmental signals and directly implicated in feedback loops controlled by miRNAs, ZEB1 appears to be a central switch that determines cell fate. Its expression fosters malignant transformation through the mitigation of critical oncosuppressive pathways and through the conferment of stemness properties. ZEB1 is also a key determinant of cell plasticity, endowing cells with the capacity to withstand an aberrant mitogenic activity, with a profound impact on the genetic history of tumorigenesis, and to adapt to the multiple constraints encountered over the course of tumor development. *Cancer Res; 78(1); 30–35. ©2017 AACR.*

Introduction

In multicellular organisms, interconversion between epithelial and mesenchymal phenotypes through the process of epithelial-to-mesenchymal transition (EMT) provides the flexibility and plasticity required during crucial steps of embryogenesis, such as mesoderm and neural crest formations (1). EMT is an evolutionarily conserved process, which is tightly regulated through the interplay between environmental signals from Wnt, TGFβ, FGF family members, and a complex web of intracellular signaling pathways that converge toward the activation of a network of EMT-inducing transcription factors (EMT-TF). This network involves zinc finger proteins (e.g., SNAI1, SNAI2), basic helix–loop–helix transcription factors (e.g., the TWIST family and E47) and zinc finger and homeodomain proteins (ZEB1 also named TCF8 or DeltaEF1, and ZEB2 also named SIP1). This reversible transdifferentiation process is characterized by a profound remodeling of the cytoskeleton, a switch from apical-basolateral polarity to front-back polarity and a loss of cell–cell adhesion. One of the earliest steps in EMT is the loss of E-cadherin function, a key determinant of adherens junctions, and the capacity of EMT-TFs to trigger EMT commitment relies on the direct repression of the *CDH1* promoter (which encodes E-cadherin). ZEB1 and ZEB2 proteins belong to the zfh family of transcription factors. They both have two flanking C2H2-type zinc finger clusters interacting with paired CACCT(G) E-box-like promoter elements and a central POU-like homeodomain deprived of DNA-binding activity (2). Several protein-binding domains are present in ZEB1 and ZEB2, including the Smad-, CtBP-, and p300-P/CAF-interaction domains, that are instrumental in the control of their transcriptional activity. As a consequence, although initially described as transcriptional repressors through their interaction with the CtBP corepressor, ZEB factors can also activate transcription, through their interaction with coactivators, such as p300 and P/CAF. This dual activity is of utmost importance in the control of EMT. Indeed, in contrast with other EMT-TFs, ZEB1/2 proteins may trigger the repression of epithelial genes encoding components of adherens and tight junctions, desmosomes and intermediate filaments while positively regulating mesenchymal factors, such as vimentin, fibronectin, N-cadherin and matrix metalloproteinases, facilitating the general dedifferentiation program. Importantly, although they exhibit a high structural homology, ZEB1 and ZEB2 display distinct expression domains, activities, and knockout mouse phenotypes. For instance, they exhibit antagonistic effects when controlling TGFβ/BMP signaling (3), as ZEB1 synergizes with Smad-mediated transcriptional activation and ZEB2 acts as a repressor by recruiting CtBP. Furthermore, *Zeb2* knockout causes embryonic arrest around stage E8.5, whereas *Zeb1*-null mice die perinatally, displaying respiratory failure, and major defects exclusively in skeletal elements and thymic T cells (4). Although a detailed *in situ* analysis of ZEB1 and ZEB2 has yet to be performed in human adult tissues, it is known that their mRNA expression levels vary significantly among tissues. Interestingly, ZEB1 and ZEB2 often show mirrored expression, as evidenced in the melanocyte lineage in which ZEB2 is expressed and represses ZEB1 expression, suggesting that they may have specific subfunctions, which are not redundant but complementary (4, 5).

ZEB1, Invasion, and Metastatic Dissemination

The significant parallels between cell plasticity in embryogenesis and tumor development led to the hypothesis that carcinoma cells rely on some elements of embryonic EMT during metastatic dissemination. Although it remains the subject of intensive debate (6), numerous studies support the notion that, in the course of

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tumor progression, extracellular molecules in the tumor microenvironment (TGFβ, FGF, EGF, HGF, Wnt, Notch, Hedgehog, etc.) and related pathways (MAPK, PI3K, NF-κB, Wnt/β-catenin, Notch, etc.) induce EMT, triggering the dissociation of malignant cells from primary tumors. Mesenchymal features and properties of EMT-committed carcinoma cells then facilitate migration and dissemination to distant sites. The acquisition of mesenchymal traits through EMT may thus occur in a minority of malignant cells, a notion that is supported by the observation of single cancer cells or small-cell clusters with reduced E-cadherin levels at the invasive front of carcinomas. Moreover, EMT-committed cancer cells may acquire a hybrid state, characterized by a dynamic combination of epithelial and mesenchymal traits, and such a partial and reversible activation of EMT has been shown to be critical for metastasis (1). The autocrine TGFβ/ZEB/miR-200 signaling regulatory network is believed to be a major driver of this cell plasticity (7). The TGFβ pathway is indeed a central activator of ZEB1 and ZEB2, while ZEB proteins and miR-200 family members (miR-200a, miR-200b, miR-200c, and miR-141) are involved in a double-negative feedback loop, which controls EMT both during development and tumorigenesis. The relevance of the miR-200/ZEB loop with regards to metastatic dissemination has been demonstrated *in vivo*. In a mouse model of lung adenocarcinoma, owing to the expression of mutant *K-Ras* and *p53*, miR-200 family members displayed the most prominent differential expression in metastasis-prone tumors relative to metastasis-incompetent tumors. Moreover, forced expression of the miR-200b cluster in metastasis-prone tumor cells abrogated their capacity to undergo EMT, invade, and metastasize in syngeneic mice (8). ZEB1 was shown to suppress the expression of cell polarity factors, repress basement membrane synthesis, and activate the expression of matrix metalloproteases, such as MMP-1, MMP-9, and MMP-14, thereby promoting the remodeling of the basement membrane and fostering invasion into surrounding tissues (7). In human colon, lung, and breast cancer cell lines, forced expression of ZEB1 increases invasive and migratory capacities *in vitro* and metastases *in vivo*. Using a mouse pancreatic cancer model driven by Pdx1-cre–mediated activation of mutant *K-ras* and mutant *p53*, the group of T Brabletz recently demonstrated that Zeb1 was a key factor for local invasion, colonization capacities, and distant metastasis (9). Of note, depletion of Snai1 or Twist1 EMT-TFs in the same model was not able to affect these processes, suggesting a prominent role for Zeb1 (10). Finally, the implication of EMT in the invasion–metastasis cascade highlights the dynamic nature of the process, the acquisition of mesenchymal features enhancing invasive and migratory capacities of malignant cells, while a mesenchymal–epithelial transition is required for metastatic colonization (1). This notion of epithelial–mesenchymal plasticity is supported by the findings of circulating tumor cells with a hybrid state in patients with advanced metastatic tumors. Cancer cell plasticity has recently also been observed in mouse mammary tumors using intravital microscopy, demonstrating that temporal acquisition of the mesenchymal state is important for migration (11).

ZEB1, Malignant Transformation, and Tumor Initiation

There is increasing evidence that functions of ZEB proteins are not limited to EMT regulation. Indeed, they might participate in a central switch that controls critical cellular functions and states, including differentiation, proliferation, response to DNA damage, and cell survival, with a dramatic impact on tumor development, from early steps of tumorigenesis to cancer progression (Fig. 1). In line with this notion, ZEB1 expression was observed in noninvasive neoplastic lesions, both in human samples and in animal models. For example, a significant fraction of human *in situ* pancreas adenocarcinoma shows high levels of the EMT-TF (12). In mice, *Zeb1* is overexpressed in noninvasive pancreatic lesions [pancreatic intraepithelial lesions (PanIN); ref. 10], and *Zeb1* depletion in mutant *K-ras* mice with a wild-type *p53* causes a reduction in both the number and grading of acinar ductal metaplasia and PanINs, suggesting that ZEB1 is a key driver of early steps of pancreatic tumorigenesis (9). Consistently, ZEB1 was shown to profoundly affect P53 and RB-dependent oncosuppressive pathways and to prevent both senescence and apoptosis, two critical barriers against tumor development. Mouse embryonic fibroblasts (MEF) from *Zeb1* knockout mice undergo early replicative senescence. Zeb1 is indeed able to repress the cyclin-dependent kinase inhibitors *CDKN1A* (best known as $p21^{waf1}$) and *INK4B* (best known as $p15^{ink4b}$), thereby allowing G_1–S cell-cycle progression (13). Of note, ZEB1 and ZEB2 might have opposing effects in controlling replicative senescence, as forced expression of ZEB2 in breast and hepatic cancer cell lines promotes senescence through transcription repression of h*TERT* expression (14). ZEB1 was also reported to overcome oncogene-induced senescence triggered by EGFR overexpression in human esophageal epithelial cells, via the repression of $p16^{ink4a}$ and $p15^{ink4b}$ expression (15). Its expression also increases drug resistance in pancreatic cancer cells (16), supporting data showing that EMT activators confer antiapoptotic properties to malignant cells (17–19). Underlying the role of ZEB1 in the control of cell proliferation and cell survival is the cross-talk between ZEB1 and the P53 family members (20). Indeed, ZEB1 is involved in the transcriptional regulation of *p63* and *p73* isoforms in MEFs as well as during myoblast and keratinocyte differentiation, while P53 controls ZEB1 levels through the transcriptional activation of miR-200c/141 and miR-200a/miR200b/429 clusters (21). Of note, other EMT-TFs, including SNAI1 and TWIST1 have been shown to control P53, highlighting the existence of a functional interaction between the network of EMT-TFs and the P53 signaling pathways with a critical role in the control of cell differentiation and oncosuppressive processes (19). As a consequence of its role as a molecular bypass of failsafe programs, ZEB1 cooperates with mitogenic oncoproteins for malignant transformation of epithelial cells. *In vitro*, ZEB1 expression in mammary epithelial cells, either ectopically induced or activated in response to permissive environmental conditions, reduces the number of genetic events necessary for malignant conversion (22). Moreover, ZEB1 causally promotes transformation of oncogenically manipulated human bronchial epithelial cells (23) and is required for tumor initiation in a mouse $KRas^{V12}$-mediated lung cancer model (12). In the latter model, Ras-induced Zeb1 expression directly represses the Pi3k pathway inhibitor Pten, providing a rational explanation for the low frequency of PTEN mutations in human tumors initiated by RAS mutants. Interestingly, this model also led to the demonstration that the role of ZEB1 in tumor initiation can be distinguished from that in EMT commitment and metastasis based on their requirements of different levels of the EMT-TF. Indeed, a low threshold of

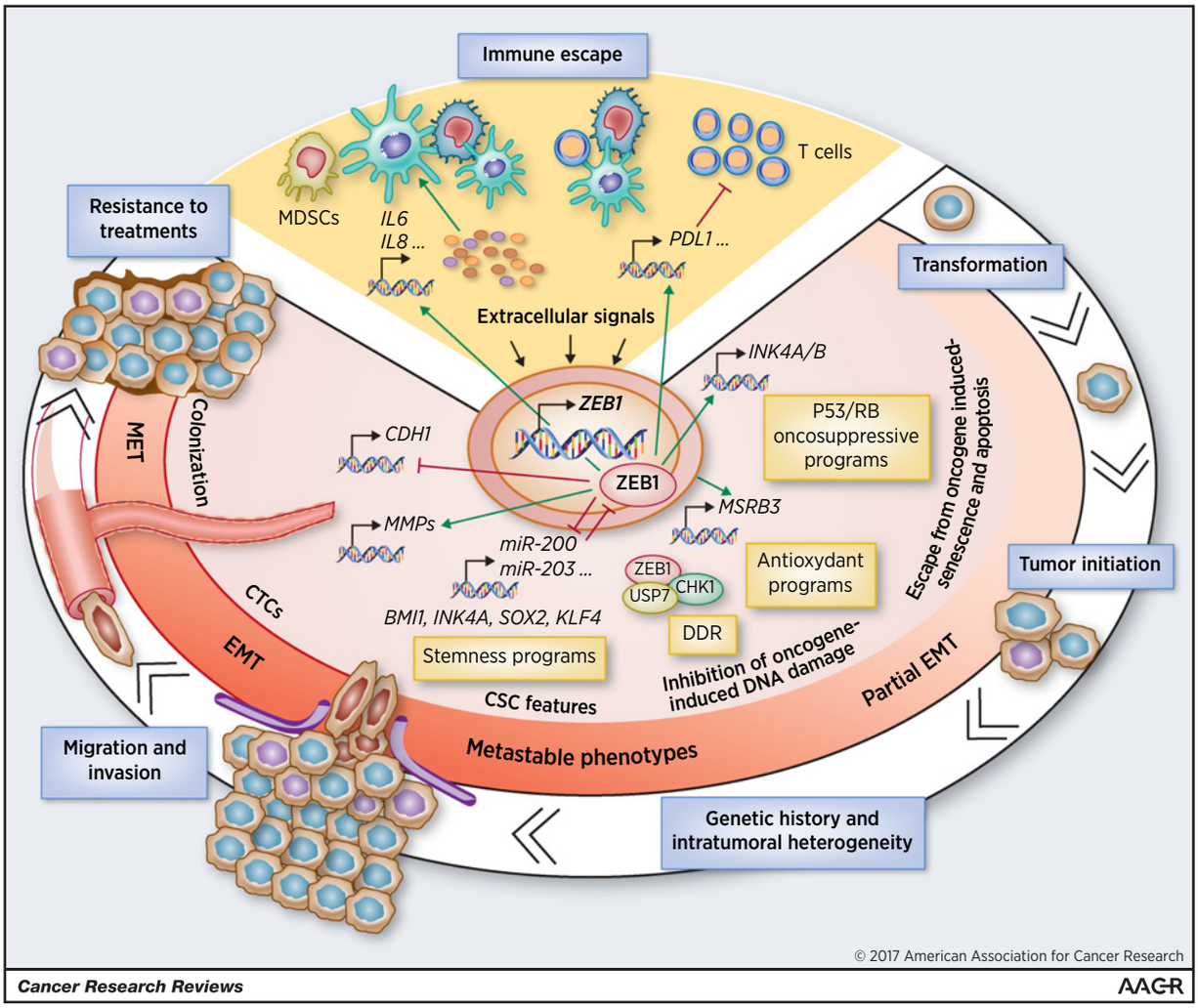

Figure 1.

Oncogenic functions of the EMT-inducing transcription factor ZEB1. The ZEB1 transcription factor is a central determinant of cell fate. It transcriptionally regulates factors involved in the control of cell differentiation, proliferation, survival, and motility. Its expression fosters the tumorigenic process from the earliest steps, including malignant transformation, and provides cancer cells with migratory and invasive capabilities. Over the course of tumor development, ZEB1 has a profound impact on cancer cell plasticity and intratumor genetic and epigenetic heterogeneity. ZEB1 also promotes an escape from the immune control in the context of an intricate cross-talk with immune cells and finally contributes to resistance to treatments. CTC, circulating tumor cells; MET, mesenchymal-to-epithelial transition; MDSC, myeloid-derived suppressor cells.

ZEB1 is sufficient for triggering tumor initiation, whereas further induction is necessary for promoting metastasis (12). Beyond malignant transformation, ZEB1 expression was also shown in lung and pancreatic cancer cells to be a determinant of K-RAS addiction, the epithelial differentiation state of K-RAS–mutant cells being associated with dependency on the mitogenic oncogene to maintain cell survival (24). Importantly, the oncogenic activities of ZEB1 are not restricted to epithelial cells. As an illustration, ZEB1 cooperates with $BRAF^{V600}$ in promoting transformation of immortalized melanocytes, and its depletion impairs or delays $BRAF^{V600}$-induced tumorigenesis in nude mice upon xenografting (5). Interestingly, ZEB2 displays an opposite pattern of expression in the course of melanocytic transformation and acts as tumor suppressor in these cells (5).

ZEB1, Cancer Stem Cells, and Genetic History of Tumorigenesis

The initial observation that, after EMT, transformed human mammary epithelial cells acquire stem-like features led us and others to propose that EMT commitment generates cancer stem cells (CSC; refs. 25, 26). CSCs are characterized by two major properties: the ability to self-renew and the capacity to regenerate the phenotypic heterogeneity of the parental tumor (27). These cells, that are believed to sustain primary tumor growth and to drive the seeding and establishment of metastases at distal sites, generally represent a minor fraction of the whole cancer cell population. Whereas CSCs were generally thought to reside at the apex of a unidirectional neoplastic cell hierarchy, the functional connection between EMT and stemness implies that CSCs

can be derived *de novo* from their non-CSC counterparts highlighting an unsuspected level of cancer cell plasticity within individual tumors. There is increasing evidence that ZEB1 plays a major role in the dynamic conversion between CSCs and non-CSCs. As an illustration, the group of RA Weinberg demonstrated in the model of basal-type breast cancers that in response to microenvironmental stimuli, such as TGFβ production, *ZEB1* increases the rate of transition from non-CSCs to CSCs (28). This dynamic process is epigenetically driven, the *ZEB1* promoter being maintained in a bivalent chromatin configuration, characterized by simultaneous repressive (H3K27me3) and activating (H3K4me3) histone modifications, resulting in an efficient inducibility. At the molecular level, ZEB1 regulates stemness by inhibiting the expression of stemness-repressing miRNAs, including miR-200, but also of miR-183 and miR-203, which together target BMI1 and possibly other stemness-associated factors, such as SOX2 and KLF4 (16). Importantly, although the induction of *ZEB1* expression through microenvironmental cues might be instrumental in promoting cancer cell plasticity, the EMT program has also been shown to be involved in the normal mammary epithelial stem cell state. Recently, we have shown that ZEB1 is expressed in normal human mammary stem cells and triggers an antioxidant program driven by the methionine sulfoxide reductase MSRB3 that protects stem cells against the oxidative stress normally induced by an aberrant mitogenic activation (29). This preemptive program, which declines as mammary cells differentiate, prevents the formation of oncogene-induced DNA damage. As a direct consequence, ZEB1 expression precludes the activation of the P53-dependent DNA damage response (DDR) and the subsequent induction of oncogene-induced apoptosis and premature senescence, two critical barriers against malignant transformation. Moreover, because double-strand breaks generated following an oncogenic activation are a major cause of genomic instability (30), endogenous ZEB1 expression ensures the maintenance of genomic stability over the course of tumorigenesis. Overall, these findings demonstrate that the differentiation status of the cell profoundly influences the early response to an oncogenic activation and is a key determinant of the onset of cancer chromosomal instability. They also provide a rational explanation for the existence of a subclass of aggressive breast neoplasms exhibiting high ZEB1 expression, a low frequency of P53 mutations and a subnormal genomic landscape. In addition, ZEB1 was recently shown to be phosphorylated and stabilized by ATM following ionizing radiation of breast cancer cells (31). ZEB1 upregulation triggers stabilization of CHK1 by activating the USP7 deubiquitylase, promoting radioresistance. Overall, these observations suggest that, during tumorigenesis, ZEB1 may both prevent the formation of oncogene-induced DNA damage by dampening the oxidative stress and increase the clearance of DNA breaks through the activation of the DDR.

ZEB1 and Resistance to Treatment

EMT commitment and stemness properties have been associated with resistance to standard radio- and chemotherapy, as well as with novel targeted therapies (32). Whether, in a given cell type and treatment condition, resistance to treatment is associated with mesenchymal features, or whether it is determined by specific functions of EMT-TFs remains unclear. Nevertheless, several lines of evidence suggest a specific role for ZEB1. First, as previously mentioned, the activation of the DDR by ZEB1 promotes radioresistance in breast cancer cells (31). Second, several miRNAs targeted by ZEB1 have been implicated in chemoresistance, namely miR-203, miR-429, and miR-200c. The negative feedback loop between ZEB1 and miR-429 has been involved in the development of resistance to cisplatin in epithelial ovarian carcinoma (33). As epithelial ovarian cancer cells exhibit higher resistance to cisplatin compared with those with a mesenchymal status (34), the mechanism of resistance is likely EMT independent in this context. In glioblastoma, miR-200c negatively regulates the *O*-6-methylguanine DNA methyltransferase (MGMT), via c-MYB, resulting in chemosensitivity (35). Expression of miR-203 also increases sensitivity of breast and pancreatic cancer cells to gemcitabine and paclitaxel (36). Interestingly, miR-203 levels increase upon exposure to the HDAC inhibitor mocetinostat, whereas the expression of ZEB1 reduces, paving the way for the use of epigenetic drugs to restore chemosensitivity through the reversion of the EMT/stemness phenotype. Third, ZEB1 has recently been reported to play an essential role in cellular lipid metabolism and in the synthesis, storage, and use of long-chain polyunsaturated fatty acids. These lipids are the substrates for lipid peroxidation, leading to the formation of toxic lipid peroxides that can ultimately trigger ferroptosis, a nonapoptotic form of cell death. In therapy-resistant mesenchymal cells, the phospholipid glutathione peroxidase GPX4 dissipates these reactive peroxides and thus protects them against cell death (37). Overall these findings suggest that GPX4 may be the Achilles' heel of resistant cancer cells, its targeting representing an innovative approach to deal with ZEB1-mediated resistance to treatment. Finally, several studies also suggest that ZEB1 expression promotes resistance to new anticancer therapies, including targeted therapies and immunotherapies (32). For example, a ZEB1-dependent EMT phenotype promotes resistance to erlotinib in EGFR-mutant non–small cell lung cancer (NSCLC) cell lines, with a major role for the receptor tyrosine kinase AXL (38). In $BRAF^{V600}$-mutated melanoma, high ZEB1 expression is associated with primary resistance to MAPK inhibitors (39). Experimentally, ZEB1 depletion sensitizes naïve melanoma cells to BRAF inhibitors (BRAFi) and decreases the viability of BRAFi-resistant melanoma cells, while forced expression of the EMT-TF in low ZEB1-expressing cells triggers a rapid drug-induced adaptation, induces a stem-like phenotype, and promotes resistance (39). In contrast, ZEB2 expression is associated with increased sensitivity to BRAFi, further highlighting the dual roles of ZEB1 and ZEB2 in melanomagenesis. The emergence of immune checkpoint inhibitors has revolutionized the therapy of several cancers, including melanoma, as the blocking of the interaction between the programmed cell death (PD)-1 protein and one of its ligands, PD-L1, promotes impressive antitumor responses. Nonetheless, the high degree of nonresponders, and in some cases the emergence of resistance in patients who initially respond, calls for the development of strategies aimed at overcoming primary and acquired resistance to these agents. Although the underlying mechanisms remain to be characterized, mesenchymal cancer cells appear to be primed to hijack immune defenses driven by natural killer cells and cytotoxic T lymphocytes. Interestingly, miR-200 has recently been shown to target PD-L1. ZEB1 expression relieves the miR-200 repression of PD-L1 on tumor cells, leading to $CD8^+$ T-cell immunosuppression (40). These findings are supported by robust correlations between the EMT score, miR-200 levels, and PD-L1 expression in NSCLC. Tumor cells are also able to recruit a protumoral immune microenvironment through the production

and release of inflammatory cytokines. In breast cancer cell lines, chromatin immunoprecipitation sequencing resulted in the identification of a ZEB1-regulated inflammatory phenotype (41). ZEB1 was demonstrated to transcriptionally activate the *IL6* and *IL8* genes in a direct manner, leading to the recruitment of myeloid-derived suppressive cells.

Conclusion and Future Directions

A growing body of evidence indicates that the role of ZEB1 in normal and cancer cells is not limited to the transition from an epithelial state toward a mesenchymal and motile phenotype. Indeed, it is a crucial regulator of fundamental intracellular decision-making processes, including stemness versus differentiation, cell proliferation versus senescence, and survival versus apoptosis. Owing to its strategic location downstream of regulatory processes integrating microenvironmental signals and to its direct implication in feedback loops controlled by miRNAs, ZEB1 has a pivotal role in cell fate determination. A key feature of ZEB1 is its implication in the regulation of cell plasticity. As a dynamic process that promotes the reversible conversion of tumor cells between metastable states, ZEB1-mediated plasticity is a prominent contributor to the capacity of premalignant cells and malignant cells to adapt to the multiple constraints encountered from the earliest steps of tumorigenesis to the invasion–metastasis cascade. The mechanistic links between EMT and epigenetics, including the characterization of the epigenetic profiles of CSCs versus non-CSCs and the identification of chromatin-modifying enzymes implicated in the transcriptional regulation of EMT-TFs, are important areas for future investigation. A better understanding of the intrinsic mechanisms underlying ZEB1-mediated cancer cell plasticity and of the role of the tumor microenvironment in shaping this plasticity is also needed to control the emergence of resistance to treatment and to ensure more effective cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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